MASTER'S THESIS IN MEDICAL MICROBIOLOGY (BIO-3930)

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Prevalence of plasmid-mediated quinolone resistance in Norwegian and Swedish clinical isolates of *Escherchia coli* and *Klebsiella* spp.

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Dedication

I dedicate this work:

- To *my mother and father* for their unlimited love and for all of their commitment to being there for me throughout my life.

- To three persons I exceptionally love *Khalil, Mirella, and Jowel*.

- To *Hani,* my dear lovely sweet huge young brother.

- To treasured *Ritta*.

- And to *my relatives, friends, and colleagues*.

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Abbreviations

bp	Base pair/base pairs
BLAST	Basic Local Alignment Search Tool
BSAC	British Society for Antimicrobial Chemotherapy
3`-CS	3`-Conserved sequence
5`-CS	5`-Conserved sequence
CR	Common region
CFU	Colony forming unit
ddH2O	Distillate deionised water
EtBr	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum-ß-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
F	Forward
IS	Insertion sequence
IR	Inverted repeat
K-res	Reference Centre for Detection of Antimicrobial Resistance
MIC	Minimum inhibitory concentration
NIST	National Institute of Standards and Technology
NORM	The Norwegian Surveillance Programme for Antimicrobial Resistance
NWGA	Norwegian Working Group on Antibiotics
ORF	Open reading frame
PCR	Polymerase chain reaction
R	Reverse
res	Recombination site
SRGA	Swedish Reference Group for Antibiotics
UNN	University Hospital of North Norway

Abstract

Three plasmid-mediated quinolone resistance (PMQR) mechanisms have recently been described; the first mechanism is related to Qnr proteins of the pentapeptide repeat family that protect type II topoisomerases from inhibition by quinolones, the second mechanism involves the AAC(6')-Ib-cr protein representing a new variant of the common aminoglycoside acetyltransferase which is capable of acetylating and thus reducing the activity of norfloxacin and ciprofloxacin, and the third and most lately described mechanism involves the quinolone efflux pump protein QepA.

The aim of this study was to examine the presence of plasmid-mediated *qnr* and *aac(6')-Ib-cr* resistance genes among *Escherichia coli* and *Klebsiella* spp. clinical isolates obtained between 2003 and 2005 from Norway and Sweden. The isolates were selected based on resistance to nalidixic acid and/or reduced susceptibility or resistance to ciprofloxacin.

The prevalence of *qnr* genes was low (1.6 %) with *qnr* genes being more common among *Klebsiella* spp. (7.7 %) than *E. coli* (0.7). *qnrS1* was detected in six isolates and was the most prevalent, *qnrB* was detected in two isolates (one was *qnrB1* and the other was *qnrB7*), while no *qnrA* was detected. The *aac(6')-Ib* gene was detected in 15.4 % of the isolates, 69 (92%) of these were of the *aac(6')-Ib-cr* variant. Transfer of the *qnr* genes was successfully achieved in five isolates, three by transconjugation and two by transformation. The *qnrS1* genes were located on plasmids ranging in size from 140 to ≥200 kb. However, one *qnrS1* gene could be chromosome-located. IS*Ecl2* (*orfB-ΔorfA*) was detected downstream and *Δres*, *orf259*, and *orf213* were detected upstream to the *qnrS1* gene in all the 6 *qnrS1*-positive isolates. In addition, the *pbp3* and bla_{LAP1} genes were detected further downstream to the *qnrS1* gene in three isolates. The presence of IS*Ecl2* close to *qnrS1* could play a role in the dissemination of this gene while the repeated association between *qnrS1* and bla_{LAP-1} could be a factor in the growing spread of ciprofloxacin- and β-lactam-resistant clinical isolates of *Enterobacteriaceae*.

1. Introduction

Antimicrobial agents

Antimicrobial agents represent a main therapeutic tool to control and treat a variety of bacterial infectious diseases. The first antimicrobial compounds used in modern medicine were produced and isolated from living organisms such as the penicillins from fungi of the genus *Penicillium*, or streptomycin produced by bacteria of the genus *Streptomyces*. With the advent of organic chemistry many antimicrobial agents are now obtained by chemical synthesis, such as the sulfa drugs and the quinolones. At the highest level, antibmicrobial agents can be classified as either bactericidal or bacteriostatic (Hancock. 2005). Bactericidals kill bacteria directly while bacteriostatics prevent them from dividing. However, in practice, both of these are capable of ending a bacterial infection.

Classification of antimicrobials can also be done according to their mechanism of action (Tenover. 2006). Mechanisms include interference with cell wall synthesis (e.g., β-lactams), inhibition of protein synthesis (macrolides), interference with nucleic acid synthesis (quinolones), inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole), and disruption of bacterial membrane structure (polymyxins) (table 1).

Table 1. Mechanisms of action of antimicrobial agents (Tenover. 2006).

Mechanism	Antimicrobial(s)
1- Interference with cell wall synthesis	β-lactams: penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: vancomycin, teicoplanin
2- Protein synthesis inhibition	
• Bind to 50S ribosomal subunit	Macrolides, chloramphenicol, clindamycin.
• Bind to 30S ribosomal subunit	Aminoglycosides, tetracyclines.
3- Interference with nucleic acid synthesis	
• Inhibit DNA synthesis	Quinolones.
• Inhibit RNA synthesis	Rifampin
4- Inhibition of metabolic pathway	Sulfonamides, folic acid analogues
5-Disruption of bacterial membrane structure	Polymyxins, daptomycin.

Bacterial antimicrobial resistance

During the last five decades, the use and sometimes misuse of antimicrobials in both human and veterinary medicine has resulted in the emergence of strains of bacteria that no longer respond to antimicrobial therapy (McDermott *et al*. 2003). Anti microbial resistance develops through a number of different mechanisms: (1) changes in the permeability of the bacterial cell wall/membrane, restricting the access of anti microbials to target sites; (2) active efflux of the anti microbials out the cell; (3) mutation in the target site; (4) enzymatic degradation or modification of the anti microbial agent; and (5) acquisition of alternative metabolic pathways to those inhibited by the anti microbial agent (table 2).

Table 2. Mechanisms of antimicrobial resistance (McDermott *et al*. 2003).

Mechanisms of resistance	Antimicrobial(s) affected
1. Modification of the antimicrobial agent	Aminoglycosides, chloramphenicol, and ß-lactams.
2. Alteration or protection of the target site	Aminoglycosides, ß-lactams, macrolides, quinolones, rifampicin, trimethoprim, and tetracycline.
3. Decreased antibiotic accumulation	
• Decreased uptake	Many antibiotics (quinolones).
• Increased efflux	Tetracycline, macrolides, quinolones, and chloramphenicol.
4. Alteration of the metabolic pathway	Sulfonamides, trimethoprim.

Bacteria may be intrinsically resistant to one class or more of antimicrobial agents, or resistance can be acquired by de novo mutation or by the acquisition of resistance genes from other organisms (Tenover. 2006). Intrinsic resistance can be described as a natural phenomenon when it is displayed by all members of a species and is a function of the physiological or biochemical structure of that species (Harbottle *et al*. 2006). For example, *Enterococci* are intrinsically resistant to cephalosporins due to a decreased binding affinity to the penicillin-binding proteins. On the other hand, acquired resistance can result from the acquisition of a mutation in the regulatory or structural genes and/or the acquisition of a foreign resistance gene (Harbottle *et al*. 2006). Acquired resistance is not present in the entire species but within only a certain lineage of bacteria derived from a susceptible parent.

Horizontal transfer of antimicrobial resistance

Most bacterial genomes that have been sequenced contain DNA segments that have been acquired from other sources (Normark & Normark. 2002). It was found that this horizontally acquired DNA usually encodes functions that are of selective advantage to the organism such as antibiotic resistance, virulence and biodegradation pathways.

The three common mechanisms for horizontal gene transfer are: (1) Transformation, a process by which bacteria take up free DNA directly from their environment, (2) Transduction, through which bacterial DNA is moved from one bacterium to another by a bacteriophage, and (3) Conjugation, a process by which a living bacterial cell transfers genetic material through cell-to-cell contact. A number of different DNA elements have played a main role in the development of resistance in bacteria (Normark & Normark. 2002). Such genetic elements include plasmids, transposons, genomic islands, phage, integrons and gene cassettes.

Plasmids

Plasmids are extra-chromosomal DNA molecules that replicate independently of the chromosome (Baron *et al*. 1996). They are typically supercoiled, circular, and double-stranded DNA molecules. However, linear plasmids have also been discovered in *Borrelia* and *Streptomyces* (Baron *et al*. 1996). There may be one copy, generally for large plasmids, to hundreds of copies of the plasmid in a single cell, and cells can harbor more than one type of plasmids. Plasmids vary in size from about five to more than several hundred kilobase pairs and they generally do not encode functions essential to bacterial growth, such as RNA polymerase, ribosomal subunits, or enzymes of the tricarboxylic acid cycle (Baron *et al*. 1996). Instead, plasmids often carry genes that confer a selective advantage to the bacterium harboring them, such as genes conferring antibiotic resistance. Other functions carried by plasmids include: synthesis of antibiotics, synthesis of toxins and proteins for bacterial pathogenesis, synthesis of enzymes for the utilization of unusual carbon sources, and resistance to heavy metals.

Some plasmids are transferable (figure 1); they contain genes (the *tra* genes) that encode all the functions they need to transfer themselves and other DNA elements among cells (Snyder & Champness. 2007). Transconjugation is most commonly mediated by such plasmids. Whereas, other plasmids are only mobilizable (figure 2); they encode some but not all of the proteins required for transfer and consequently

need the help of the transferable plasmids to move from one bacteriuom to another (Snyder & Champness. 2007).
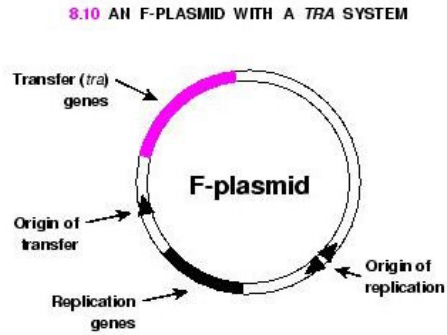
Figure 1. An F-plasmid, an example of a transferable plasmid
(www.science.siu.edu/microbiology/micr302)

Plasmids that carry resistance genes are called R plasmids (Harbottle *et al*. 2000) (figure 2). These plasmids, originally called R factors, were first discovered from *Shigella flexneri* strains in the 1950s (Watanabe & Fukasawa. 1961). Since then, they have been increasingly associated with both Gram-positive and Gram-negative bacterial pathogens and commensal organisms. Plasmid-associated resistance genes have been detected for almost all the clinically available antimicrobials, including most recently the quinolones, and it is not uncommon for a single plasmid to simultaneously mediate resistance to multiple antimicrobials and to be shared among different bacterial genera (Harbottle *et al*. 2000). In addition to carrying resistance genes, plasmids can serve as vehicles for other genetic elements important in antimicrobial resistance, such as transposons and integrons.
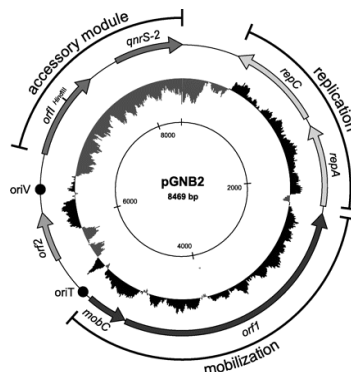
Figure 2. Plasmid pGNB2, an example of a mobilizable R-plasmid containing the *qnrS2* gene
that confers low-level quinolone resistance (Bönemann *et al*. 2006)

Transposable elements

Transposons are mobile genetic DNA elements that encode a site-specific transposase allowing site-specific insertions and excisions (Normark & Normark. 2002). There are three types of transposable elements: Insertion sequence elements, composite transposons, and non-composite transposons (figure 3).

IS element

| IR | Transposase | IR |

Composite transposon

←——— IS element ———→   Different determinants   ←——— IS element ———→

| IR | Transposase | IR | (antibiotic resistance) | IR | Transposase | IR |

Noncomposite (complex) transposon

Different determinants

| IR | Transposase | Resolvase/*res* | (antibiotic resistance) | IR |
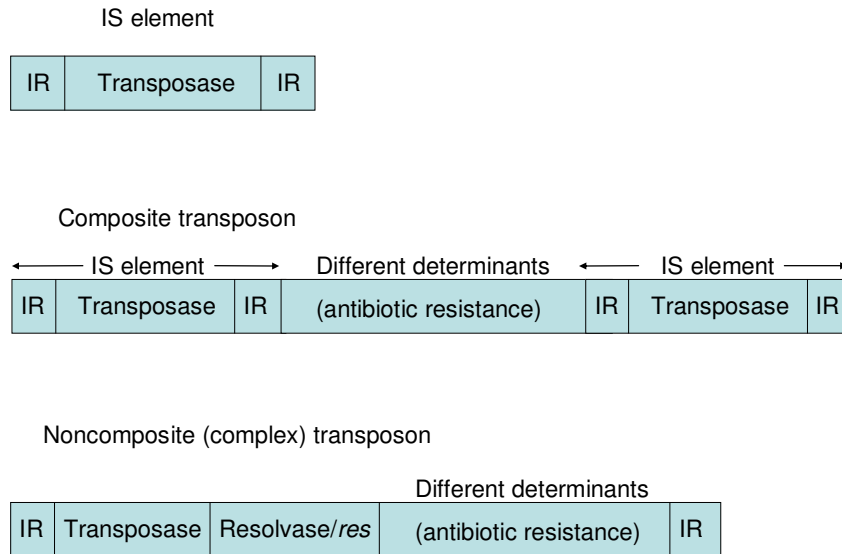
Figure 3. Transposable elements

Insertion sequence (IS) elements are usually 750 to 2000 bp long and consist of a gene encoding the transposase enzyme that promotes their transposition and two inverted repeats at the ends (Snyder & Champness. 2007). Thus far, thousends of different IS elements have been identified in bacteria. Plasmids also often contain IS elements. Composite transposons are made of two IS elements of the same type (two copies of the same IS element) bracketing other genes (Snyder & Champness. 2007). Different resistance determinants have been detected inside composite transposons in many bacterial species. Noncomposite transposons do not have complete copies of IS elements at their ends, still they contain short inverted repeats at their ends (Snyder & Champness. 2007). They contain *tnpA* (the transposase gene), *tnpR* (the resolvase gene), and *res* (the site at which resolvase acts). Noncomposite transposons may also contain resistance genes as an integrated part of the transposon.Transposons may

move into various genera of bacteria during transfer of promiscuous plasmids or via transducing phage. Furthermore, some transposons are themselves conjugative or can be induced to form phage (Harbottle *et al.* 2006). Conjugative transposons are able to excise from the genome of the donor cell, promote their conjugation to a neighboring bacterium and, following conjugation, integrate into the recipient chromosome or plasmid.

Gene cassettes and integrons

Gene cassettes are discrete genetic elements that exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another (Bennett. 1999). Gene cassettes are part of the integron only when they are integrated. They normally contain only a single gene and a short sequence, called a 59 base element, which functions as a specific recombination site, called *attC* standing for *att*achment Cassette. The cassettes are small, normally of the order of 500–1000 bp and the genes carried on gene cassettes usually lack promoters and are expressed from a promoter on the integron. Integrons are genetic elements capable of capturing and mobilizing gene cassettes, but they themselves are not mobile (Bennett. 1999) (Fluit & Schmitz. 2003). An integron is defined as a genetic element that (1) encodes an enzyme called integrase and mediates site-specific recombination events; the integrase excises and integrates the gene cassettes from and into the integron, and (2) possesses a site called *attI* standing for *att*achment Integron and at which additional DNA, in the form of gene cassettes, can be integrated by these site-specific recombination. The promoters for genes carried on the cassettes are mostly found within the *intI* gene.

Integrons can be divided into two main groups: the resistance integrons which carry mostly gene cassettes that encode resistance to antibiotics and can be located either on the chromosome or on plasmids, and the super-integrons which are chromosomally-located and contain gene cassettes with a varity of functions (Fluit & Schmitz. 2003). At least three distinct classes of resistance integrons have been discovered based on the integrase gene sequence. Class 1 are the most prevalent integrons among clinical isolates to date. The structure of class1 integrons consist of two conserved regions,

called 5'-CS region and 3'-CS region, flanking a variable region of gene cassettes (Bennett. 1999). The 5'-CS region contains the integrase gene, *intI*, the site for insertion of gene cassettes, *attI*, and a promoter region, which is located within *intI*. While the 3'-CS region encodes resistance to sulphonamides, mediated by the *sul1*gene, and has a truncated version of the detergent resistance gene *qacEΔ1*, and two open reading frames, *orf5* and *orf6*, encoding proteins of unknown functions (figure 4).
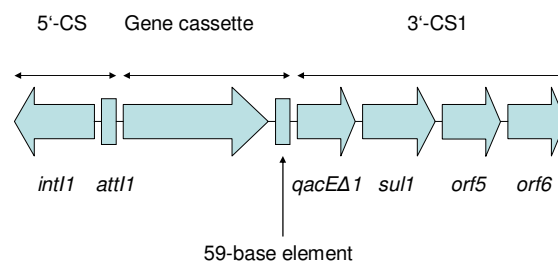
Figure 4. Class 1 integron structure (adapted from Bennett, 1999).

Both class 2 and class 3 integrons show the distinctive features of an integron (the integrase gene *intI* and its associated *attI* site). Nevertheless, it is important to know that the *intI2* integrase is not functional due to an internal stop codon while the properties of the IntI3 integrase are comparable to those of the IntI1 integrase. The first super-integrons was detected in *V. cholerae* and was considered as a class 4 integron (Fluit & Schmitz. 2003). Although super-integrons can be very large and may contain hundreds of ORFs, the super-integron of *Shewanella oneidensis* contains only three gene cassettes. Many scientists consider these super-integrons as the reservoir of gene cassettes for resistance integrons.

The quinolones

While some of the first antibiotics discovered during the past century were isolated from living organisms, the quinolone class of antimicrobial agents was synthesized by chemists (Andriole. 2005). Nalidixic acid was accidentally discovered in 1962 as a by-product of the synthesis of the antimalarial compound chloroquine. This discovery led to the development of a set of quinolone compounds widely described in clinical use at the present time (figure 5).
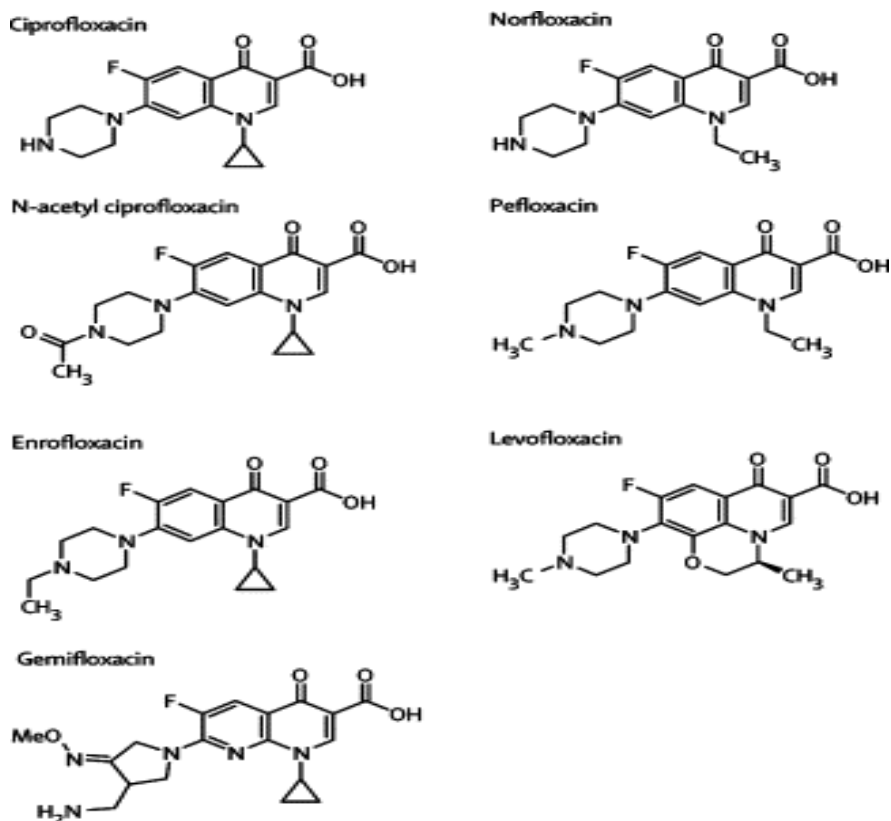
Figure 5. Chemical structures of fluoroquinolones (Andriole. 2005).

The antimicrobial activity of the early quinolones (the first-generation quinolones), such as nalidixic acid and cinoxacin, were excellent against aerobic Gram-negative bacteria but not very active against aerobic Gram-positive bacteria or anaerobic bacteria (Andriole. 2005). The second-generation quinolones, including norfloxacin,

ciprofloxacin, ofloxacin, levofloxacin, lomefloxacin, and pefloxacin, have antimicrobial activity against both aerobic Gram-positive and Gram-negative bacteria, but they still lacked activity against anaerobic bacteria. The addition of fluorine at the C-6 position resulted in the innovation of the "fluoroquinolones" with norfloxacin being the first fluoroquinolone to be discovered. Newer fluoroquinolones (the third-generation fluoroquinolones), including grepafloxacin, gatifloxacin, sparfloxacin, and temafloxacin, had greater potency against Gram-positive bacteria, particularly pneumococci; they also had good activity against anaerobic bacteria. The last group of quinolone compounds (trovafloxacin, clinafloxacin, sitafloxacin, moxifloxacin, and gemifloxacin) was termed "fourth-generation fluoroquinolones," and had effective activity against anaerobes and improved activity against pneumococci (table 3).

Table 3. Classification of quinolone antimicrobials (adopted from Andriole. 2005).

Generation	Selected examples
First generation	Nalidixic acid
Second generation	Norfloxacin, Ciprofloxacin*, Ofloxacin, Levofloxacin
Third generation^	Sparfloxacin, Gatifloxacin, Grepafloxacin
Fourth generation˝	Trovafloxacin, Moxifloxacin, Gemifloxacin

* Most potent agent against *Pseudomonas aeruginosa*.

^ More potent against *Streptococcus pneumoniae* and anaerobes.

˝ Most potent against *Streptococcus pneumoniae* and anaerobes.

Molecular mechanisms of action of quinolones

Each bacterium contains a chromosome which is 1300 mm long, but the average bacterium is only 2 mm long and 1 mm wide so bacteria have to deal with this topological problem (Nordmann & Poirel. 2005). Therefore, the chromosome is subdivided into ~65 regions, which are called "domains," each of which is ~20 mm long, and the size of each domain is reduced by negative supertwisting (i.e., supertwisting that occurs against the normal direction of the helical state of DNA in its linear form). Several DNA topoisomerases have been identified in bacteria, all of them are able to nick one or both of the strands of a double-stranded circular DNA,

introduce or remove negative supercoils, and then seal the nicked DNA (Nordmann & Poirel. 2005). Introducing and removing such supercoils are vital in DNA replication, transcription, recombination, and repair.

Quinolones act by inhibiting the action of topoisomerases II (DNA gyrase) and topoisomerase IV. For Gram-negative bacteria the prime target of quinolones is the DNA gyrase, whereas in the Gram-positives it is the topoisomerase IV (Andriole. 2005). Quinolones act by binding to gyrase/topoisomerase IV–DNA complex. Formation of quinolone-gyrase/topoisomerase IV-DNA complex is responsible for the inhibition of DNA replication and the bacteriostatic action of the quinolones while their lethal action is thought to be a separate event from complex formation, and to arise from the relapse of free DNA ends from quinolone–gyrase–DNA complexes (Nordmann & Poirel. 2005).

Resistance to quinolones

Successful treatment outcomes of bacterial infections with quinolones have led to increasing use and the extensive use of fluoroquinolones, in turn, has led to mounting resistance to these antimicrobials (Hooper. 1999). High rates of resistance to quinolones have been reported from different parts of the world. In China, for example, more than 50% of the clinical strains of *E. coli* isolated during 1997-1999 were resistant to ciprofloxacin (Wang *et al*. 2001). In Norway, rates of resistance to quinolones are somewhat lower (NORM/NORM-VET report, 2006); the prevalence of resistance to nalidixic acid for urinary tract and bloodstream *E. coli* clinical isolates was 5.4% and 9.3 %, respectively, while it was 13.1 % for bloodstream *Klebsiella* spp. clinical isolates.

Quinolone resistance generally results from stepwise chromosomal mutations (Ruiz. 2003). Three mechanisms of chromosomal-mediated quinolone resistance have been established to date: alterations in the targets of quinolones and decreased accumulation of quinolones due to impermeability of the membrane or due to an over expression of efflux pump systems. Target alterations are predominantly in the

quinolone-resistance determining region (QRDR), a portion of the DNA-binding surface of the topoisomerase at which amino acid substitutions can diminish quinolone binding and subsequently cause resistance to quinolones (Ruiz. 2003). Recently, plasmid-mediated quinolone resistance (PMQR) has been discovered. This resistance comprises the production of Qnr proteins protecting the targets against the effects of quinolones (Robicsek *et al.* 2006), the enzymatic inactivation of certain quinolones (Robicsek *et al.* 2006), and most recently an efflux pump encoded by the *qepA* gene (Yamane *et al.* 2007).

The Qnr proteins

Three Qnr determinants have been discovered so far, namely QnrA, QnrS, and QnrB. The first plasmid-mediated quinolone resistance gene, named *qnrA1*, was detected in a *K. pneumoniae* clinical isolate from the USA in 1998 (Martinez-Martinez *et al.* 1998). Later on, the *qnrS1* and the *qnrB1* genes were first discovered from a clinical *Shigella flexeneri* 2b isolate in Japan (Hata *et al.* 2005) and from a *K. pneumoniae* strain isolated in South India (Jacoby *et al.* 2006), respectively.

Although QnrB and QnrS share only about 40% and 60% amino acid identity with QnrA, respectively, the three determinants belong to the pentapeptide repeat family and they all protect the DNA gyrase and topoisomerase IV from the action of quinolone agents, including the fluoroquinolones (Robicsek *et al.* 2006). By making a search in the GenBank® sequence database (http://www.ncbi.nlm.nih.gov/Genbank/) provided by the National Center for Biotechnology Information (NCBI), 6 variants of QnrA, 2 variants of QnrS and 17 variants of QnrB have been identified so far (appendix A. table 1).

Distribution and origin of Qnr determinants

Qnr determinants have then been identified worldwide in remotely related areas from America, Europe, Asia, and Africa (Nordmann & Poirel. 2005 and Touati *et al*. 2008). They have been detected in a series of enterobacterial species, including *E. coli*, *Citrobacter freudii*, *Enterobacter spp*, *Klebsiella* spp, *Providencia stuartii*, *Salmonella* spp, *Proteus mirabilis*, and *Serratia marcescens* (Jeong *et al*. 2005, Mammeri *et al*. 2005, Nazic *et al*. 2005, Poirel *et al*. 2006, Rodriguez-Martinez *et al*. 2006, Wang *et al*. 2003, Cambau *et al*. 2006, and Park *et al*. 2007). In addition, QnrA has recently been discovered in *Acinetobacter baumannii*, a non-*Enterobacterial* Gram-negative (Touati *et al*. 2008). So far, none of these determinants have been identified in *Pseudomonas aeruginosa*, a clinically important non-*enterobacterial* Gram-negative bacterium, though this could be due to modest screening (Robicsek *et al*. 2006). The wide geographical distribution of *qnr* genes in different enterobacterial species means that these genes have been in flow, though hidden, for some time.

While the *qnrA* gene is thought to have originated in *Shewanella algae,* an environmental species from marine and fresh water (Poirel *et al*. 2005), Qnr-like proteins have been detected from water-borne *Vibrionaceae* bacterial isolates and also proposed to be a possible origin of the clinically more important QnrA, QnrS, and QnrB determinants (Poirel *et al*. 2005). These Qnr-like proteins shared 40-67% identity with the clinically important Qnr-proteins and once cloned in *E. coli* they conferred reduced susceptibility to quinolones. Moreover, two new genes named *qnrVS1* and *qnrVS2* were identified from *Vibro splendidus* and another *Vibro* spp. environmental isolates, respectively, and considered as a natural reservoir of *qnrS* genes (Cattoir *et al*. 2007). All these findings underlines that Gram-negative bacterial species of the aquatic environment such as *Shewanellaceae* and *Vibrionaceae* maybe the source and the reservoir of the *qnr* genes.

Resistance levels mediated by Qnr determinants

The *qnrA* determinant increased MICs of nalidixic acid and fluoroquinolones when was cloned in *E. coli* J53 from 4 μg/ml to 32 μg/ml and from 0.008 μg/ml to 0.25 μg/ml, respectively (Tran & Jacoby. 2002). By comparing the results from several reports, it can be found that the increase in quinolone resistance due to *qnrA*, *qnrS*, or *qnrB* gene is quite comparable. Also, it was noticed that *qnr* proteins reduce the activity of nalidixic acid less than that of ciprofloxacin. The *qnrA*-mediated resistance to nalidixic acid was increased 2 folds (from 4 μg/ml to 8 μg/ml) in some studies to 12 folds (from 4 μg/ml to 48 μg/ml) in other studies while *qnrA*-mediated ciprofloxacin resistance was increased between 12.5 folds (from 0.008 μg/ml to 0.1 μg/ml) and 250 folds (from 0.008 μg/ml to 2 μg/ml) (Wang *et al*. 2004, Corkill *et al*. 2005, Nordmann & Poirel. 2005, and Robicsek *et al*. 2006). The *qnrS* gene increased the MIC of nalidixic acid 3 - 8 folds and of ciprofloxacin between 16 - 62.5 folds (Chen *et al*. 2006, Gay *et al*. 2006, Kehrenberg *et al*. 2006, Poirel *et al*. 2006, and Hu *et al*. 2008). Transfer of *qnrB* resulted in 4 - 8 folds increase in the MIC of nalidixic acid and 8 - 62.5 folds in the MIC of ciprofloxacin (Jakoby *et al*. 2006, Gay *et al*. 2006, Pai *et al*. 2007, Kehrenberg *et al*. 2007).

The donor bacteria, which are generally clinical isolates, most often exhibited higher levels of quinolone resistance than the transconjugants. This can be explained by the co-presence of additional chromosome-encoded mechanism of quinolone resistance, particularly mutations in the QRDR of *gyrA* and *parC* genes (Nordmann & Poirel. 2005). In some cases, the differences in resistance levels among transconjugants can be due to the transfer of other PMQR mechanisms, such as the AAC(6`)-Ib-cr enzyme (Robicsek *et al*. 2005).

Genetic environment of *qnrA*

The *qnrA* variants are typically located on plasmids (Nordmann & Poirel. 2005). These plasmids frequently carry other antibiotic resistance genes and although they were generally transferable, in some cases they were not (Nordmann & Poirel. 2005).

However, *qnrA* was recently detected in an *E. coli* clinical isolate from Denmark where, interestingly, hybridization with the *qnrA* probe was observed to the chromosomal DNA (Cavaco *et al*. 2007).

qnrA genes were often embedded in complex *sul1*-type integrons (Nordmann & Poirel. 2005). These genetic structures possess one 5`-conserved segment (5`-CS) which contains *intI1* gene encoding for the integrase enzyme and duplicated 3`-conserved segments (3`-CS) each of them contains *qacEΔ1* and *sul1* genes. The two 3`-CS surround a common region (CR) which contains the IS*CR1* (*orf513*) and a unique region (Nordmann & Poirel. 2005). The Orf513 protein may act as a recombinase for mobilization of downstream-located antibiotic resistance genes. The *qnrA* gene was not associated with a 59-be element as a form of a gene cassette (Nordmann & Poirel. 2005) (figure 6).
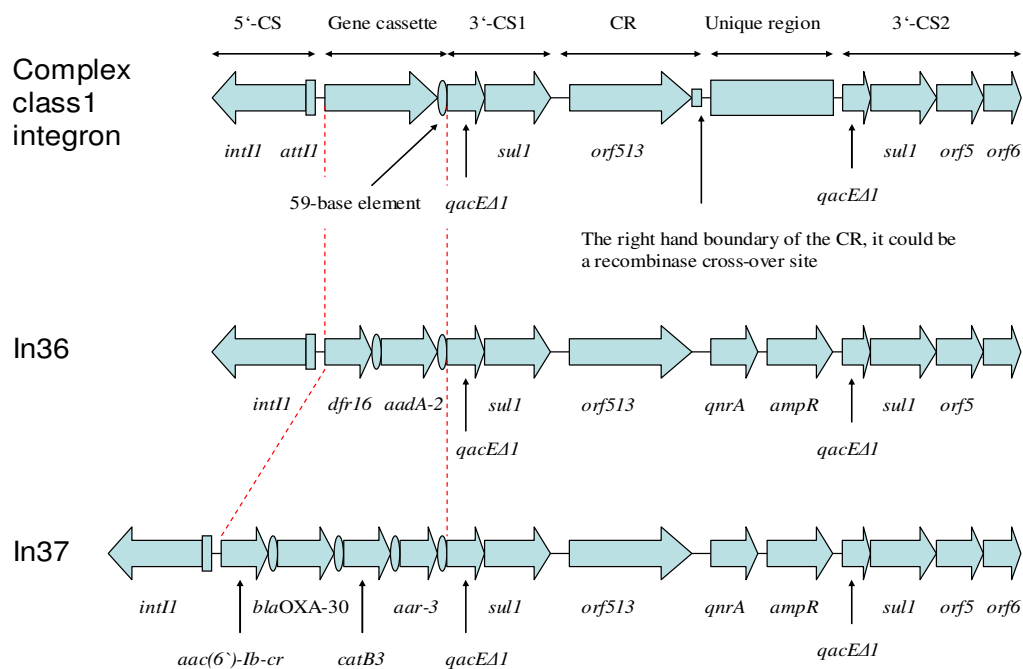
Figure 6. Genetic environment of *qnrA* located in complex class1 integrons 36 and 37

Genetic environment of *qnrS*

The *qnrS* genes are also typically located on plasmids but, in contrast to *qnrA*, they are not part of a *sul1*-type class 1 integron structure (Hata *et al*. 2005). Association between *qnrS1* and a Tn*3-like bla*$_{TEM-1}$-containing transposon has frequently been detected; on a conjugative 47 kb plasmid, pAH0376, from a *Sh. Flexneri* isolate from Jaban (Hata *et al*. 2005) (figure 7), on a conjugative 58 kb plasmid, pINF53, from a *Salmonella enterica* serovar Infantis isolate from Germany (Kehrenberg *et al*. 2006) (figure 7), and in three *Salmonella enterica* serovar Virchow isolated from Turkish foods of avian origin (Avsaroglu *et al*. 2007).

A novel Ambler classA ß-lactamase gene, now named *bla*$_{LAP-1,}$ instead of the Tn*3* element, has also been repeatedly detected upstream to *qnrS*; on a non-conjugative 98-kb plasmid, pK245, from a clinical *K. pneumoniae* strain isolated in Taiwan (Chen *et al*. 2006), in five *E. cloacae* isolates from France as well as in one *E. cloacae* isolate from Vietnam (Poirel *et al*. 2007), and on a conjugative plasmid isolated from a clinical *K. pneumoniae* strain from Spain (Lavilla *et al*. 2007). The region containing *qnrS* and *bla* in pK245 appears to be a composite transposon bounded by two flanking IS*26* elements in a head-to-tail arrangement which may be responsible for the mobility of *qnrS* (Chen *et al*. 2006) (figure 7).
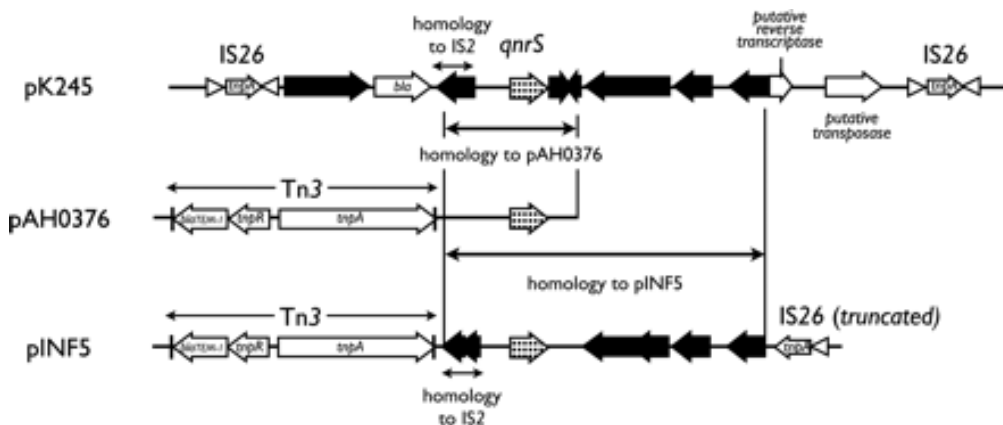
Figure 7. Schematic diagram comparing the *qnrS*-containing region of pK245, pAH0376 from *S. flexneri*, and pINF5 from *S. enterica* serovar Infantis (Chen *et al*. 2006)

In addition, the two resistance genes were always separated by 1,597 bp that included the insertion sequence IS*Ecl2* (Poirel *et al*. 2007). In silico analysis showed that this IS*Ecl2* element was also present in the *qnrS1*-positive pINF5 plasmid of *S. enterica* serotype Infantis and in the *qnrS1*-positive pAH0376 plasmid of *Sh. flexeneri* 2b (Poirel *et al*. 2007) (figure 8). A truncated resolvase protein and a segment showing significant homology with the *E. coli* CS12 fimbrial gene cluster have been identified downstream to the *qnrS1* gene (Kehrenberg *et al*. 2006 and Poirel *et al*. 2007) (figure 8). However, this segment was not detected in two *E. cloacae* isolates from France (Poirel *et al*. 2007) (figure 8). Furthermore, it was in the opposite orientation in three other *E. cloacae* isolates from France (Poirel *et al*. 2007) (figure 8) and in one *Salmonella typhimurium* isolate from the UK (Kehrenberg *et al*. 2007).
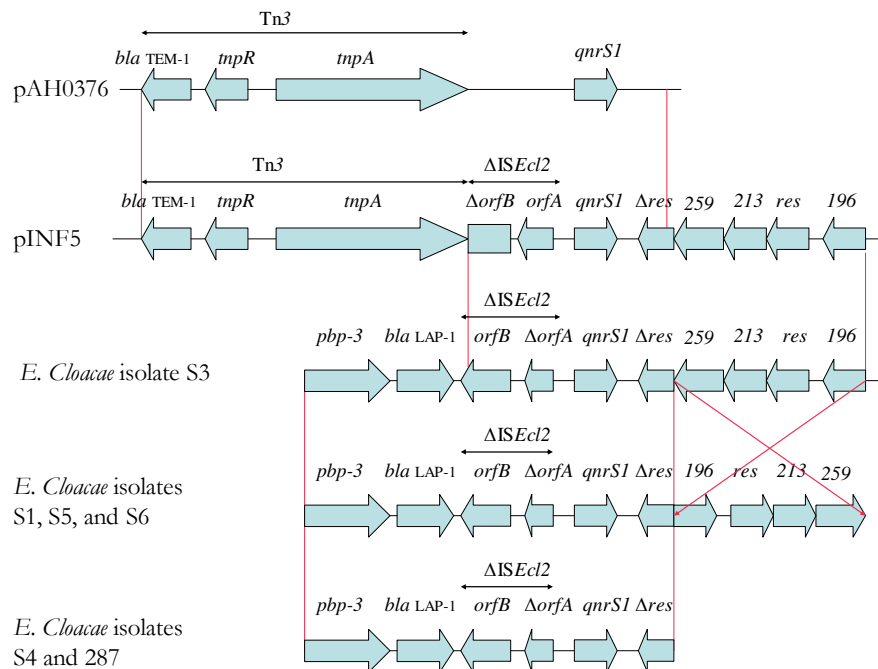
Figure 8. Schematic diagram comparing the *qnrS*-containing region of pAH0376 from *S. flexneri* and pINF5 from *S. enterica* serovar Infantis with that detected in *E. cloacae* isolates (adapted from Poirel *et al*. 2007).

Another variant, QnrS2, having 92% amino acid identity to QnrS1 was later on detected in a highly mobile broad-host-range 8.5 kb plasmid, pGNB2, isolated from

an activated sludge bacterial community of a wastewater treatment plant in Germany (Bonemann *et al*. 2006). This *qnrS2* gene was not part of an integron but was linked to *orf1, a* remnant of a Tn*1721*-like transposon. Interestingly, the G+C content of the *orfI-qnrS2* gene region differs considerably from the rest of the plasmid, suggesting that pGNB2 acquired *qnrS2* together with Tn*1721 orfI* (Bonemann *et al*. 2006).

Genetic environment of *qnrB*

Although *qnrB* variants are generally plasmid-carried, a chromosomal location of *qnrB12* gene appeared most likely since repeated plasmid transformation-conjugation experiments yielded negative results, and Southern blot hybridization studies with plasmid profiles and with I-CeuI-digested genomic DNA gave a signal only with the largest fragment, approximately 800 kb, in each strain (Kehrenberg *et al*. 2008).

The *qnrB1* gene was reported to be associated with the *orf1005* gene, encoding a putative transposase (Jacoby *et al*. 2006). Between *orf1005* and *qnrB1*, there was an open reading frame, *orf1*, which resembles a truncated *pspF* gene coding for the transcriptional activator of the stress-inducible *psp* operon (figure 9). *orf1* was again found upstream *qnrB2* while downstream it there were other two open reading frames; *orf2*, which resembles genes encoding hypothetical proteins of several Gram-negative species, and *orf3*, which has 83% identity to the *sapA* gene encoding a peptide transport periplasmic protein (Jacoby *et al*. 2006). *orf1005* was not present in any of the strains containing *qnrB2* (figure 9).
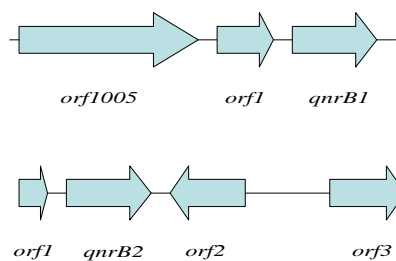
Figure 9. Genetic environment of *qnrB1 and qnrB2* genes. *orf1* resembles *pspF*, *orf2* is related to genes of unknown function in various Gram-negative bacteria, and *orf3* resembles *sapA* (adapted from Jacoby *et al*. 2006).

The *qnrB2* determinant was also reported to be located in a complex *sul1*-type integron (Garnier *et al*. 2006). The genetic structure contained two class 1 integrons surrounding two common regions (CRs) separated by a partial 3'-conserved segment. The *qnrB2* gene was adjacent to the first CR and in the opposite orientation (figure 10). Once more, the *qnrB2* determinant and the *qnrB6* determinant in *Citrobacter* spp. clinical isolates from Spain were all carried in complex *sul*1-type integrons (Sanchez-Cespedes *et al*. 2007).
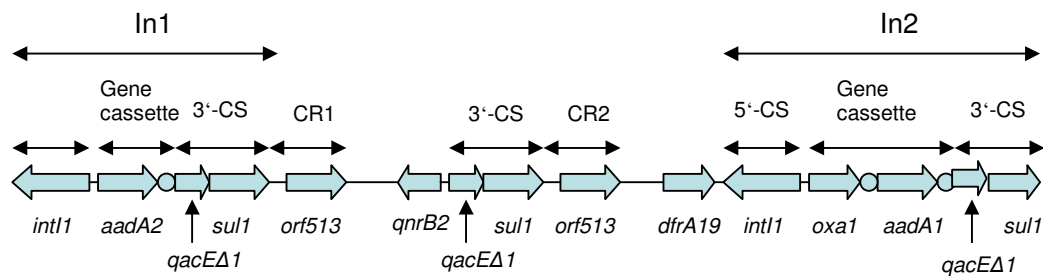
Figure 10. Genetic environment of the *qnrB2* gene (adapted from Garnier *et al*. 2006).

The genetic environment of the *qnrB4* gene was found to be bracketed at its 5' extremity by a *psp* operon coding for putative phage shock proteins and at its 3' extremity by a *sap* operon coding for a putative peptide transport system permease (Cattoir *et al*. 2007). However, the *qnrB4* gene carried on plasmid pHS7 from a clinical isolate of *K. pnemoniae* from China was located in a *sul1*-type integron with *orf1*, *bla*DHA-1*, *ampR*, *psp* operon, and partial *qacEΔ1* upstream and *aphA1*, IS26, and *sapA* and partial *sapB* genes downstream the *qnrB4* gene (Hu *et al*. 2008) (figure 11). The structure adjacent to *qnrB4* was similar to that in plasmids, pRBDHA and pMPDHA with GenBank accession numbers AJ971343 and AJ971344, respectively (Hu *et al*. 2008).
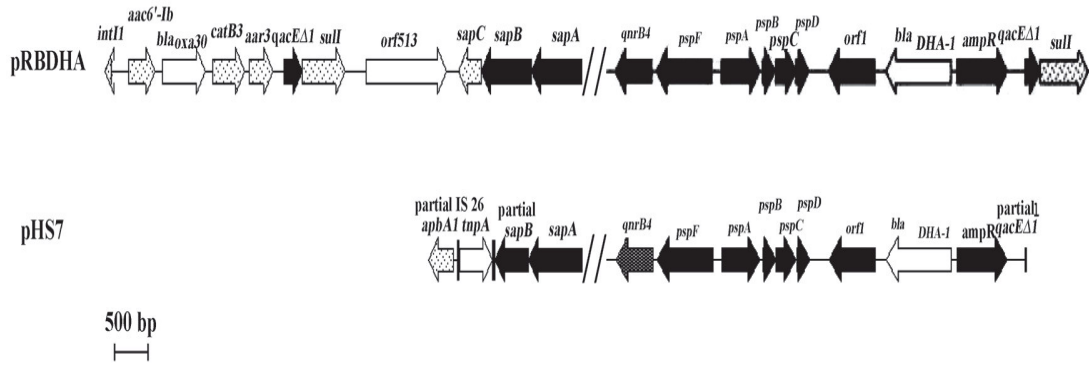
Figure 11. Genetic environment of the *qnrB4* gene (Hu *et al*. 2008).

qnrB10 was located in the unique region of three different complex class 1 integrons downstream of IS*CR1* (*orf513*), one of them also containing *aac*(*6'*)-*Ib-cr* within its the variable region (Quiroga *et al*. 2007) (figure 12). While, the *qnrB12* gene was located downstream of four open reading frames, *pspF*, *pspA*, *pspB*, and *pspC*, encoding phage shock proteins (Kehrenberg *et al*. 2008).
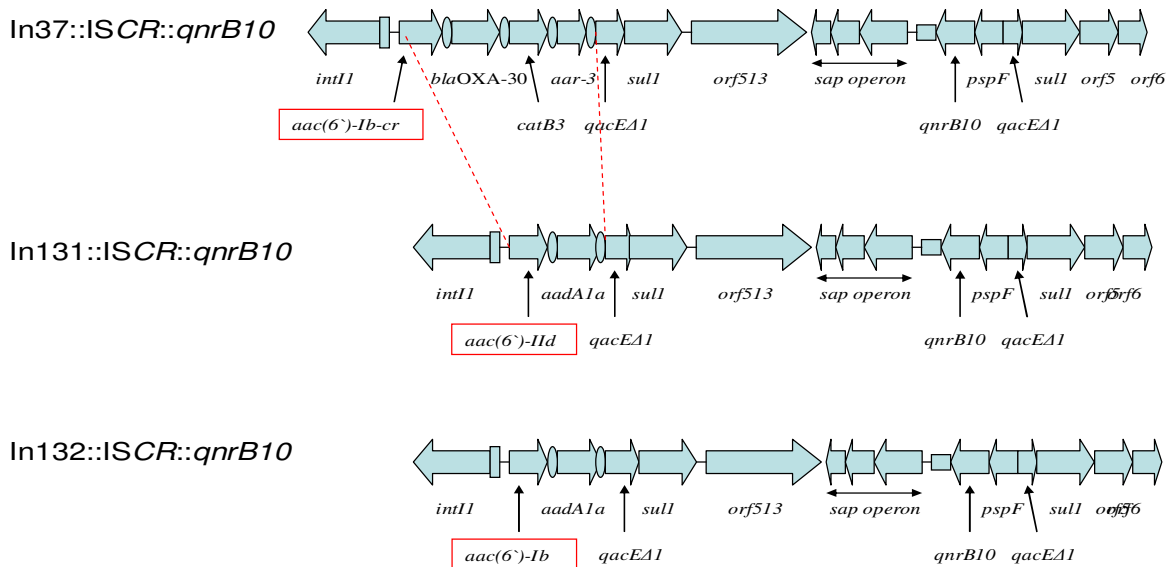
Figure 12. Genetic environment of the *qnrB10* gene (adapted from Quiroga *et al*. 2007).

The AAC(6`)-Ib-cr enzyme

Bacterial enzymes that modify antimicrobial agents have principally coevolved with the antimicrobial agent on which they act (Robiscek *et al*. 2006). Therefore, selective enzymatic modification of fluoroquinolone by bacteria has been thought not to exist in nature since quinolones are fully synthetic and consequently the time for coevolving such quinolone-modifying enzymes is limited. However, a plasmid-mediated enzyme able to attack some quinolone agents has recently been discovered (Robiscek *et al*. 2006). This enzyme, AAC(6`)-Ib-cr, represents a new variant of a common aminoglycoside acetyltransferase, AAC(6`)-Ib, that reduces the activity of both the aminoglycosides kanamycin, tobramycin, and amikacin and the fluoroquinolones ciprofloxacin and norfloxacin and hence stands for an unpredictable new mechanism of quinolone resistance.

The -cr variant of AAC(6`)-Ib has two amino acid changes, Trp102Arg and Asp179Tyr, which together are necessary and sufficient for the enzyme's ability to acetylate ciprofloxacin (Robiscek *et al*. 2006). This enzyme reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substitute. Ciprofloxacin and norfloxacin are the only fluoroquinolones with diminished activity in the presence of *aac(6`)-Ib-cr* since they are the only compounds harboring an unsubstituted piperazinyl group.

AAC(6`)-Ib-cr plays an important role in fluoroquinolone-resistance. First of all, the prevalence of *aac(6`)-Ib-cr* has been reported to be common in clinical isolates of Gram-negative bacteria (Nordman & Poirel. 2005). For example, 51% of the ciprofloxacin-resistant clinical *E. coli* isolates collected from China had *aac(6`)-Ib-cr*(Robiscek *et al*. 2006). The prevalence of *aac(6`)-Ib-cr* in this collection exceeded that of *qnr* by over six folds. Secondly, although the degree of resistance conferred by *aac(6`)-Ib-cr* is low, when both *qnr* and *aac(6`)-Ib-cr* are present in the same cell, the level of resistance is increased to a value near the clinical breakpoint for susceptibility. Thirdly and maybe most importantly, the presence of *aac(6`)-Ib-cr* increased substantially the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin (Robiscek *et al*. 2006).

2. Aims of this study

- Evaluate the prevalence of *aac(6')-Ib*, *qnrA*, *qnrB*, and *qnrS* genes among *E. coli* and *K. pnumoniae* clinical isolates from Norway and Sweden.
- Analyse the susceptibility profile of the *qnr*-positive isolates.
- Sequencing the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* for the *qnr*-positive isolates to detect chromosomal mutations that could be linked to quinolone resistance.
- Evaluate the mobility of the *qnr*-genes by transconjugation and transformation experiments.
- Characterize the *qnr*-carrying plasmids with respect to number, size, and incompatibility group.
- Analyse the genetic environment of the detected *qnr* genes.

2. Materials and methods

Strain collections

A total of 487 clinical isolates of *E. coli* (426) and *Klebsiella* spp. (61) was chosen for this study. Resistance to nalidixic acid (MIC > 16 μg/ml) and resistance or reduced susceptibility to ciprofloxacin (MIC ≥ 0.125 μg/ml) were the criteria used for selecting these isolates. The clinical MIC breakpoints for nalidixic acid and ciprofloxacin were used as defined by NWGA (Norwegian Working Group on Antibiotics) or EUCAST (European Committee on Antimicrobial Susceptibility Testing) (appendix A, table 2).

Overall, the study included 472 nalidixic acid-resistant, 12 nalidixic acid-susceptible but with reduced susceptibility to ciprofloxacin, and 3 nalidixic acid-susceptible but ciprofloxacin-resistant isolates. The isolates taken in this study were drawn from five different collections (Table 4). Two collections, Kronoberg collection and NORM collection, contained clinical isolates from Sweden and Norway representing the common bacterial population; they together comprised 370 isolates. The rest, 117 isolates, was picked from three different K-res (the Reference Centre for Detection of Antimicrobial Resistance) studies; the AmpC study, Ullevål ESBL study, and Norwegian ESBL study representing a collection of clinical isolates with other resistance mechanisms. The five collections are:

1- Kronoberg collection (n= 152)

This collection included 152 consecutive *E. coli* clinical isolates from urine specimens collected between June 2004 and January 2005 in Kronoberg County, Sweden (Bengtsson *et al*. 2007). 151 isolates were nalidixic acid-resistant and only 1 isolate was nalidixic acid susceptible but showed reduced susceptibility to ciprofloxacin (appendix A, table 3).

2- NORM collection 2005 (n= 218)

This collection included 165 *E. coli* and 53 *Klebsiella* spp. consecutive clinical isolates from blood or urine specimens collected during 2005 from several clinical microbiology laboratories in Norway (NORM/NORM-VET 2006 report). 207 isolates were resistant to nalidixic acid (161 *E. coli* and 46 *Klebsiella* spp.) and 11 isolates (4 *E. coli* and 7 *Klebsiella* spp.) were nalidixic acid-susceptible, 2 of them were resistant to ciprofloxacin and 9 showed reduced susceptibility to ciprofloxacin (appendix A, table 4).

3- *E. coli* AmpC study (n= 13)

13 nalidixic acid-resistant *E. coli* clinical isolates from the *E. coli* AmpC study were selected and screened (appendix A, table 5). All the isolates of the *E. coli* AmpC study had an AmpC phenotypic susceptibility profile and were firstly collected to analysis their genetic AmpC profile (Haldorsen *et al*. 2007).

4- Ullevål ESBL study (n= 60)

60 nalidixic acid-resistant clinical isolates (59 *E. coli* and 1 *K. pnumoniae*) from the Ullevål ESBL study were included in this study (appendix A, table 6).

5- Norwegian ESBL study (n= 44)

44 clinical isolates (33 *E. coli* and 11 *K. pnumoniae*) from the Norwegian ESBL study were included in this study (Tofteland *et al*. 2007). Of these isolates, 41 isolates were nalidixic acid-resistant (33 *E. coli* and 8 *K. pnumoniae*) and 3 nalidixic acid-susceptible *Klebsiella* spp. (two isolates were ciprofloxacin-resistant and 1 isolate showed reduced-susceptibility to ciprofloxacin) (appendix A, table 7).

Control isolates

Strains with known *qnr* genes (*E. coli* A2-67 containing *qnrA* gene, *E. coli* A3-22 with *qnrB* gene, *E. coli* A3-20 with *qnrS* gene, and *E. coli* A3-21 with *aac(6')-Ib-cr*

gene) were used as positive controls while reaction mixes without DNA template served as negative controls for the polymerase chain reactions (PCR). In addition, the rifampicin-resistant *E. coli* J53-2 and the chemically competent *E. coli* TOP'10 (Invitrogen, USA) were used as the recipient strains in transconjugation and transformation experiments, respectively.

Table 4. Summary of the five collections included in this study.

| Collection | Number of isolates | *E. coli** | *Klebsiella* spp.* | NAL-Resistant | NAL-Susceptible | |
					CIP-reduced-susceptible	CIP-resistant
Kronoberg collection	152	152	0	151	1	0
NORM collection	218	165	53	207	9	2
E. coli AmpC study	13	13	0	13	0	0
Ullevål ESBL study	60	59	1	60	0	0
Norwegian ESBL study	44	33	11	41	2	1
Total	487	422	65	472	12	3

* All isolates were identified at participating sites by routine procedures performed at each laboratory.

Preparation of stock cultures

Stock cultures were prepared of all the clinical isolates examined in this study using glycerol as the osmotic protector.

Procedure:
1. 8 to 10 colonies from an overnight incubated green agar plates (appendix C) were inoculated in 1 ml freeze broth (appendix C).
2. The broth was homogenized on a vortex mixer and stored at -70°C.
3. Control green agar plates were inoculated and incubated at 37°C overnight to check for contamination.
4. If the control plate showed contamination, the strain was purified by re-incubating it on green agar plate.

Isolation of DNA from bacterial cells

Procedure for isolating the DNA using the robot GenoM™-48 (Geno Vision, USA):

1. An inoculum of 0.5 McFarland was prepared in 200 µl of 0.9 % NaCl.
2. The sample tubes were placed in the robot which performed all steps of isolation, including sample lysis, binding to the GenoPrep™ beads, washing steps, and the elution.
3. When the robot was finished, the screw caps were replaced and samples were stored at 2 to 8º C.

Procedure for rapid isolating of DNA:

1. 8 to 10 colonies of bacteria were dissolved in 1 ml TE-buffer (appendix C) in an Eppendrof tube.
2. The tubes were spun at 5000 rpm for 5 min.
3. The pellets were re-suspended in 100 µl TE-buffer.
4. The tubes were boiled for 10 min at 100º C.
5. Then they were spun at 5000 rpm for 5 min (Eppendrof tubes were placed on ice).
6. The supernatants were transferred to new clean Eppendrof tubes.
7. Samples were stored at 2 to 8º C.

Polymerase Chain Reaction (PCR)

In 1983, Kary Mullis invented a process of amplifying the number of specific nucleic acid fragments present in a sample (Mullis. 1990). This process was termed Polymerase Chain Reaction (PCR) and has become the most common technique used in medical and biological research laboratories for isolating and exponentially amplifying a specific region of DNA (Wolcott. 1992). To carry out a PCR experiment, four fundamental components are required (Brown. 2002).

These components include:
1. DNA template containing the region of the DNA fragment to be amplified.
2. DNA polymerase used to synthesize a DNA copy of the region to be amplified.
3. A pair of oligonucleotide primers complementary to the DNA regions at the 5` and 3` ends of the DNA region that is to be amplified.
4. A supply of Deoxynucleotide triphosphates (dNTPs) from which the DNA polymerase builds the new DNA.

The PCR reaction most commonly follows three steps (Brown. 2002). In the denaturation step, the PCR reaction is heated to a temperature of around 95 °C where the hydrogen bonds of the double helix are broken resulting in single-stranded molecules to be used as templates. In the annealing step, the reaction temperature is lowered to around 50-60°C so that the primers can anneal to the single-stranded DNA template forming short segments of double-stranded DNA where the polymerase attaches and begins DNA synthesis. During the extension/elongation step, the DNA polymerase synthesizes new DNA strands complementary to the DNA template strands. A temperature of 72°C, the optimum for *Taq* polymerase, is generally used at this step.

In this first cycle of the PCR, a pair of long PCR products is synthesized from each strand of the target DNA (Brown. 2002). These polynucleotides have identical 5′ ends but random 3′ ends representing positions where DNA synthesis terminates by chance. By repeating the cycle of denaturation-annealing- synthesis, the long products act as templates for new DNA synthesis, giving rise to short amplicons whose 5′ and 3′ ends are both set by the primer positions (figure 13).
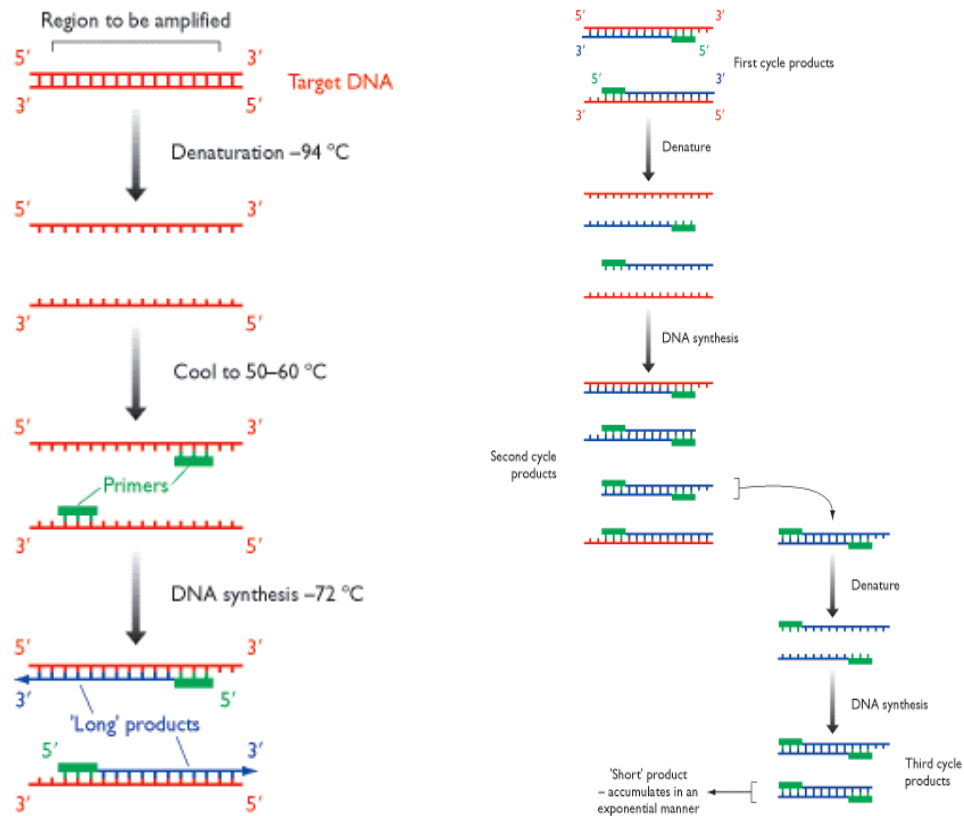
Figure 13. The first cycle of denaturation-annealing-synthesis results in two long products. Then, the next cycle leads to four products two of which are identical to the first cycle products and two of which are made entirely of new DNA. During the third cycle, the latter give rise to short products which, in subsequent cycles, accumulate in an exponential fashion (Brown. 2002).

When the PCR reaction components are mixed at room temperature (below the optimal primer annealing temperature), non-specific primer annealing may take place and extension products possibly will be formed. These products can later be amplified in the PCR, resulting in non-specific PCR products (Birch *et al.* 1996). In hot-start PCR technique, one essential reagent is withheld from the PCR mixture until the PCR machine has reached a temperature that favours specific primer annealing (Birch *et al.* 1996). This can be achieved either by mechanically separating one reagent or by blocking the polymerase enzyme with antibodies. Hot start PCRs have greatly improved specificity, sensitivity, and yield (Birch *et al.* 1996).

In this study, hot-start PCRs were performed by using the JumpStart™ REDTaq®
ReadyMix™ PCR Reaction Mix (Sigma-Aldrich LP, USA). The antibody-inactivated
polymerase enzyme employed in this study helped in minimizing non-specific
amplifications without compromising the desired high target yield as its activity is
fully restored during the first denaturation cycle of the PCR reaction.

PCR-based detection of *16SrDNA*

The *16SrDNA*-PCR provides a quick check of the quality of the DNA extraction and
can be used as a positive control for other PCRs (Ivanova *et al*. 2007). Strong
16SrDNA-PCR products indicate that DNA templates are generally of high quality
while unsuccessful amplification of *16SrDNA* reflects poor DNA isolation, DNA
degradation, or the presence of PCR inhibitors. Universal primers, to give PCR
product of about 1500 bp, were used (Weisburg *et al*. 1991) (appendix A, table 8).

Procedure:

1. PCR Mastermix:

Reagent	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix* (Sigma-Aldrich LP, USA):	500 µl
16SrDNA-F primer 50 pmole/µl (Eurogentec S.A., Belgium):	8 µl
16SrDNA-R primer 50 pmole/µl (Eurogentec S.A., Belgium):	8 µl
ddH2O:	384 µl

* http://www.sigmaaldrich.com/sigma/bulletin/P0982bul.pdf

2. For each reaction, 2.5 µl template was added to 22.5 µl PCR Mastermix.
3. The PCR thermocycler was programmed (appendix A. table 9).

Multiplex PCR-based screening for *qnrA*, *qnrB*, and *qnrS*

In multiplex PCR more than one target sequence are amplified by including more than
one pair of primers in the reaction (Elnifro *et al*. 2000). Multiplex PCR has the
potential to produce considerable savings of time and effort within the laboratory
without compromising test utility. In addition using several uniplex PCRs can be

limited by the availability of adequate volume of the test samples, PCR templates. Screening for the presence of *qnrA*, *qnrB*, and *qnrS* genes was done by modification of previously described PCR protocol (Robicsek *et al* 2006). The amplification was done using JumpStart REDTaq ReadyMix ReactionMix and using specific primers for *qnrA*, to give a 516-bp product, for *qnrB*, to give a 469-bp product, and for *qnrS*, to give a 417-bp product (Robicsek *et al* 2006) (appendix A, table 8). The conditions were altered to 95°C for 5 minutes then 30 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 40 seconds, then 72°C for 4 minutes (appendix A, table 9).

Procedure:

1. PCR Mastermix:

Reagent	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	12.5 µl
qnrA-F.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
qnrA-R.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
qnrB-F.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
qnrB-R.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
qnrS-F.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
qnrS-R.multiplex primer 50 pmole/µl (Eurogentec S.A., Belgium)	1 µl
ddH2O	5.5 µl

2. For each reaction, 1 µl template was added to 24 µl PCR Mastermix.
3. The PCR thermocycler was programmed (appendix A. table 9).

PCR-based screening for *aac(6')-Ib*

Screening for the presence of *aac(6')-Ib* gene was done by modification of previously described PCR protocol (Park *et al*. 2006). The amplification was done using JumpStart REDTaq ReadyMix ReactionMix and using specific primers for *aac(6')-Ib* to give a 482-bp product (Park et al, 2006) (appendix A, table 8). The PCR conditions were altered to 95°C for 5 minutes then 30 cycles of 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 40 seconds, then 72°C for 4 minutes (appendix A, table 9).

Procedure:

1. PCR Mastermix:

Reagent	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	12.5 µl
aac(6')-Ib-F primer 50 pmole/µl (Eurogentec S.A., Belgium):	1 µl
aac(6')-Ib-R primer 50 pmole/µl (Eurogentec S.A., Belgium):	1 µl
ddH2O:	9.5 µl

2. For each reaction, 1 µl template was added to 24 µl PCR Mastermix.

3. The PCR thermocycler was programmed (appendix A. table 9).

Agarose gel electrophoresis

Gel electrophoresis is a simple, rapid and highly sensitive method that is widely used to separate DNA and RNA molecules by size (Lodish *et al*. 2000). Because the rates at which these molecules travel are inversely proportional to their molecular weight, gel electrophoresis can also be used to estimate the unknown size of nucleic acid molecules by comparison with the migration of molecules of known size, called molecular ladders. DNA and RNA nucleic acids are negatively charged so they migrate through the gel in the direction toward the positive electrode of the electric field (Lodish *et al*. 2000). The gel acts as a sieve; it normally impedes the movement of larger molecules. Therefore, smaller molecules move faster along the gel matrix than larger molecules and so molecules of different length are separated by size.

Procedure:

1. 1.5 % agarose (Bio-Rad, USA) in 0.75x TBE buffer (appendix C) was made (the agarose was dissolved by boiling the solution in microwave oven).

2. 0.5 µg/ml EtBr (Sigma-Aldrich LP, USA) was added for staining the DNA molecules.

3. The agarose-EtBr solution was poured into the gel tray of the electrophoresis apparatus containing the combs and allowed to set for about 20 minutes.

4. 5 µl of each PCR product was loaded into the gel wells.

45

5. 5 µl of 1 KB plus DNA molecular size marker (Invitrogen, USA) (appendix C, figure 1) was loaded into the flanking wells.
6. The electrophoresis was run at 120V for approximately 1 hour and 15 minutes.
7. The gel was visualized on GelDoc system (BioRad, USA) and stored on disks as TIFF files.

Test repeatability

A test may be said to be repeatable when it is repeated by the same person or instrument on the same item and under the same conditions and it re-yields the same results or the variation in the results is smaller than some agreed limit (Taylor *et al*, 1994). According to NIST (National Institute of Standards and Technology), repeatability conditions include:

1. The same measurement procedure
2. The same observer
3. The same measuring instrument, used under the same conditions
4. The same location
5. Repetition over a short period of time.

For this study, all the *qnr-* and *aac(6')-Ib*-positive as well as a randomly chosen *qnr-* and *aac(6')-Ib*-negative clinical isolates were re-tested for presence/absence of *qnr* and *aac(6')-Ib* genes, respectively.

Identification of the *aac(6')-Ib-cr* variant by restriction digestion

Restriction endonucleases are enzymes that recognize specific DNA sequences and cleave the DNA in or close to the recognition site (Snyder & Champness. 2007). The restriction endonucleases are made only by bacteria where they are mostly associated with a methylating activity. The methylating activity modifies the bacterial own DNA and turns it immune to cutting while the restriction activity plays a role in protecting these bacteria from invading foreign unmodified phage DNA. These enzymes are

classified into three groups. The enzymes of the three groups differ in the relationship between their cutting and methylating activities. The type II enzymes serve as leading tools in molecular biology since the sequences recognized by many of them are palindromic, resulting in complementary sticky ends that are practical for DNA cloning (Snyder & Champness. 2007). More than 3500 type II restriction endonucleases with over 200 different specificities have been isolated (Roberts *et al*. 2005).

Restriction enzyme identification of the *aac(6')-Ib-cr* variant was done as previously described (Park *et al*. 2006). However, *Bts*CI restriction enzyme (New England Biolabs, USA) which has the same recognition site as *Bst*F5I used by Park *et al*. 2006 was used in this study (figure 14). This recognition site is only present in the *aac(6')-Ib* wild-type gene while the *aac(6')-Ib-cr* gene variant lacks it (Park *et al*. 2006). Subsequently, when the positive *aac(6`)-Ib*-PCR products were purified and treated with *Bts*CI, only those of wild-type gene were digested into two fragment products while those of the *-cr* variant were not been cut.

5` GGATG   NN ˅  3`

3` CCTAC ˅ NN   5`
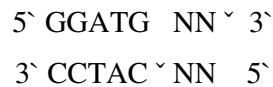
Figure 14. The recognition sequence of restriction enzymes *Bts*CI and *Bst*F5I
(Restriction Enzyme database, http://rebase.neb.com)

Procedure:

1. All the positive *aac(6')-Ib*-PCR products detected in this study were purified according to the E.Z.N.A™ Cycle-Pure Kit Spin Protocol (Omega Bio-tek, USA) (appendix D, kit 1) and used as template for the restriction digestion reaction.

2. Restriction digestion Mastermix:

Reagent	Amount
NEB Buffer4 (10×)* (New England Biolabs, USA):	2 µl
*Bts*CI ** (New England Biolabs, USA):	1 µl
ddH2O:	12 µl

* http://www.neb.com/nebecomm/products/productB7004.asp

** http://www.neb.com/nebecomm/products/productR0647.asp

3. For each reaction, 5 µl template was added to 15 µl restriction digestion Mastermix.

4. Digestion was carried out for 2 hours at 50°C.

5. Gel electrophoresis was performed as mentioned above. However, 4 µl Loading Buffer 6X (Promega, USA) was added to each digestion product and then 15 µl of each digestion product was loaded for gel electrophoresis.

DNA Sequencing

Determining the precise sequence of nucleotides of a DNA fragment is the ultimate characterization of its genetic structure (Griffiths *et al.* 1999). The sequencing method most commonly used nowadays was developed by Fred Sanger (Sanger *et al.* 1977). It is based on DNA synthesis in the presence of dideoxynucleotides, which differ from normal deoxynucleotides in that they lack a 3′-hydroxyl group.

In the traditional dideoxy sequencing methods, four parallel base-specific reactions should be conducted using a mix of all four dNTPs and a small proportion of one of the four ddNTPs (Griffiths *et al.* 1999). DNA synthesis proceeds normally until, by chance, DNA polymerase inserts a dideoxynucleotide instead of the normal deoxynucleotide. When a dideoxynucleotide is added to the growing DNA strand, it terminates the chain elongation because there is no 3′-OH for the next nucleotide to be attached. Therefore, in any given tube, various truncated chain lengths will be produced. These fragments are next separated by electrophoresis of the four samples in four lanes on an acrylamide gel. By scanning up the gel and analyzing the bands, the nucleotide order can be determined (figure 15).
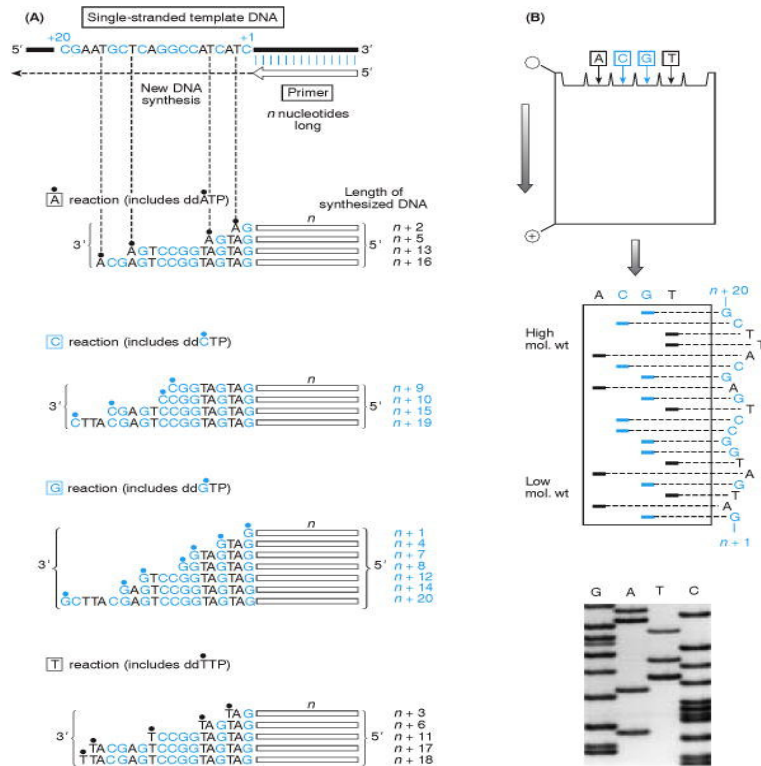
Figure 15. Dideoxy DNA sequencing relies on synthesizing new DNA strands from a single-stranded DNA template and random incorporation of a base-specific dideoxynucleotide to terminate chain synthesis. (A) Principle of dideoxy sequencing. (B) Conventional DNA sequencing (Griffiths *et al*. 1999).

The recent procedures have employed a different fluorescent color emitter for each of the four reactions and have used automated fluorescence detection machines (Strachan & Read. 1999). As a result, all four reactions can be loaded into a single lane and a monitor will detect and record the fluorescence signal as the DNA passes through a fixed point in the gel (figure 16).

(A)

1 2 3 4 5 6

Computer

Output

Laser

Gel

Detector

(B)

TATAAAACAT TTTAAAAGC TAGTACCCAGTACCT TCTAGT TCCAAAGCCCAATGTT GTTCACTATGGTTCACAATGGGACCA
140   150   160   170   180   190   200   210   220
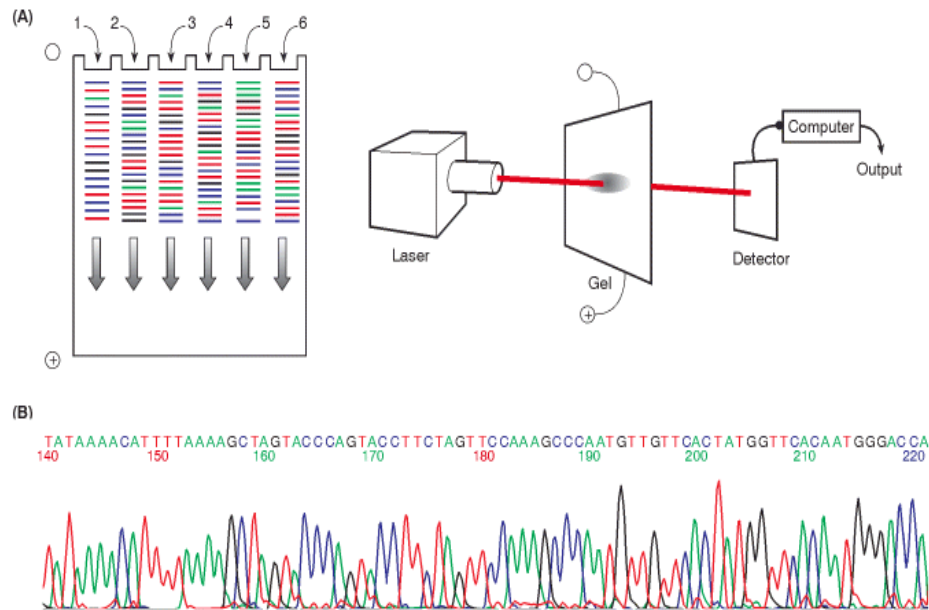
Figure 16. Automated DNA sequencing using fluorescent primers. (A) Principles of automated DNA sequencing. (B) Example of DNA sequence output (Strachan & Read. 1999).

Sequencing of the *qnr-* and *aac(6`)-Ib-cr* positives

Sequencing was performed in this study by using the BigDye v3.1 sequencing chemistry (Applied Biosystems, USA). To confirm the identification of *qnrB*, *qnrS* genes detected by multiplex PCR screening and to determine to which variant those *qnrB* and *qnrS* genes belong, same primers used in the *qnr*-multiplex PCR screening were used for sequencing both strands of the detected *qnrS* and *qnrB* genes (Robicsek et al, 2006). To confirm the identification of *aac(6')-Ib-cr* genes (detected by PCR screening and identified by restriction digestion), sequencing of one strand of the *aac(6')Ib* gene was performed using primer *aac(6')Ib*-SEQ (Park *et al*. 2006) (appendix A, table 8).

Procedure:

1. The positive *qnr*-multiplex PCR products, all positive *aac(6')-Ib* PCR products identified by restriction as *aac(6')-Ib*-cr, and 10 positive *aac(6')-Ib* PCR products identified by restriction as *aac(6')-Ib* wild-type were purified (appendix D, kit1) and used as template for sequencing.

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2. Sequencing master mix:

Reagent	Amount
Big-Dye v3.1 (Applied Biosystems, USA):	2 µl
Sequencing buffer (Applied Biosystems, USA):	3 µl
Primer 3.2 pmol/µl:	1 µl
ddH2O:	12 µl

3. For each reaction, 2 µl template was added to 18 µl master mix:

4. The PCR thermocycler was programmed (appendix A. table 9).

5. Nucleotide sequencing was performed at the Sequencing core facility.

6. Searching for nucleotide sequence homology was performed using BLAST available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

7. Nucleotide and deduced amino acid sequences were aligned, compared with published sequences of *qnrS1*, *qnrS2*, *qnrB1* to *qnrB17*, *aac(6')-Ib*, and *aac(6')-Ib-cr* using the lasergene software package (DNASTAR) and the online software ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2).

Etest susceptibility testing

Etest is an extensively used method for antibiotic susceptibility testing of a wide variety of microorganisms (Pfaller *et al*. 1999). The Etest strips contain known gradients of antimicrobial agents and calibrated in a special teqnique to give a stable-gradient agar diffusion, allowing quantitative measurement of the minimal inhibitory concentrations (MICs) of the agents (Pfaller *et al*. 1999). The MIC is the lowest concentration of the antibiotic that results in inhibition of visible growth (*i.e.* colonies on a plate or turbidity in broth culture) under standard conditions (Mayer. 2007).

Antibiotic susceptibility for the *qnrS-* and *qnrB*-positive clinical isolates detected in this study as well as *E. coli* J53-2 and *E. coli* TOP'10 which was used as a recipients in the transfer experiments was determined using Etest (AB BIODISK, Solna, Sweden) on Mueller-Hinton (MH) agar plates (appendix C) with 16 to 20 hour of incubation at 37°C. The isolates were tested against: nalidixic acid, norfloxacin,

ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin, gatifloxacin, ampicillin, amoxicillin–clavulanic acid, cefoxitin, cefuroxime, cefotaxime, ceftazidime, ceftazidime–clavulanic acid, piperacillin, piperacillin–tazobactam, cefepime, cefepime/clavulanic acid, amikacin, gentamicin, tobramycin, imipenem, meropenem, trimethoprim, and trimethoprim–sulfamethoxazol.

Procedure:
1. Etest strips kept in - 20°C were taken out the freezer and allowed to achieve room temperature.
2. Colonies from an overnight green agar plate were suspended in 3 ml 0.9 % NaCl and the turbidity was measured by a densitometer and adjusted to 0.45 – 0.55 McFarland.
3. A sterile cotton swab was dipped into the bacterial suspension; excess fluid was removed by pressing the swab against the tube wall.
4. The surface of an MH agar plate (appendix C) was swabbed using of a rotator.
5. A control spread to check for contaminants was performed.
6. The surface was let to dry for 10-15 min.
7. Etest strips were applied, ensuring that no air bubbles were trapped underneath them.
8. The MH agar plates were incubated overnight at 37°C, in stables of 5 plates.
9. The minimum inhibitory concentration (MIC) values were read at the points where the bacterial population is completely inhibited, according to the Etest reading guide (http://www.abbiodisk.com/pdf/etm_html/03_etm.htm).

VITEK 2, identification and susceptibility testing

Automated systems for bacterial identification and susceptibility testing have been used for more than two decades (Ling *et al.* 2001). The VITEK 2 system (bioMérieux, USA) is widely used system that exploits the fluorescence-based technology. VITEK 2 system has several advantages such as being a closed system that minimizes unwanted cross-contamination or environmental contamination. It also has a recheck system that can detect and cease operation if a specimen card is misplaced on the

specimen cartridge (the Smart Carrier). Furthermore, the VITEK 2 system improves efficiencies of routine clinical laboratories since it is able to handle dozens of specimens automatically in relatively short time (Ling *et al.* 2001).

The *qnrS-* and *qnrB*-positive clinical isolates detected in this study as well as *E. coli* J53-2 which was used as a recipient in the conjugation test were all tested. The VITEK®2 Gram-negative susceptibility cards (AST-NO29) evaluate the following antibiotics: nalidixic acid, ciprofloxacin, ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime, cefoxitin, cefotaxime, cefpirome, cefpodoxime, cephalothin, cefuroxime, cefuroxime axetil, aztreonam, meropenem, mecillinam; nitrofurantoin, gentamicin, tobramycin, and trimethoprim-sulfamethoxazole.

Procedure:
1. The VITEK 2 Gram-negative identification (ID-GNB) and the VITEK 2 Gram-negative susceptibility (AST-NO29) cards were taken out the refrigerator and allowed to achieve room temperature.
2. A Smart Carrier containing room for 14 tubes and 14 cards were placed.
3. The identity numbers of the bacterial strains were manually entered in the "Accession number" field.
4. Colonies from an overnight green agar plate was suspended in 3 ml 0.45 % NaCl (bioMérieux, USA) and the turbidity was measured by a densitometer and adjusted to 0.52 – 0.63 McFarland.
5. A control spread to check for contaminants was performed.
6. The inoculum tubes were placed at the first positions while empty tubes were placed at the following positions.
7. The ID-GNB card barcode labels were scanned and the cards were placed in the card positions next to the inoculum tubes.
8. The AST-NO29 card barcode labels were scanned and the cards were placed in the card positions next to the empty tubes.
9. The Smart Carrier containing the inoculum tubes, empty tubes, ID-GNB cards, and AST-NO29 cards were placed in VITEK 2 where each test card was automatically filled with a bacterial suspension and automatic identification and susceptibility testing was performed by kinetic fluorescence measurement every 15 min. The software then analyzed the data and reported the results.

Agar disk diffusion susceptibility testing

In the disk diffusion test, the bacterial isolate is inoculated uniformly onto the surface of an agar plate and a filter disk impregnated with a standard amount of an antibiotic is applied to the surface of the plate, resulting in a gradient of the antibiotic surrounding the disk (Mayer. 2007). Following incubation, a bacterial lawn appears on the plate and zones of inhibition of bacterial growth would be present around the antibiotic disk. The test is performed under standardized conditions hence the size of the inhibition zone is dependent on the degree of sensitivity of the microorganism to the antibiotic (Mayer. 2007).

In this study, susceptibility of the *qnr*-positive isolates to nalidixic acid and ciprofloxacin was measured using 30μg nalidixic acid and 10μg ciprofloxacin paper disks (Oxoid Ltd, UK).

Procedure:
1. The paper disks were taken out from the refrigerator and allowed to achieve room temperature.
2. Colonies from an overnight green agar plate were suspended in 3 ml PBS (appendix C) and the turbidity was measured by a densitometer and adjusted to 0.45 – 0.55 McFarland.
3. One drop of the solution was diluted in 5 ml PBS.
4. A sterile cotton swab was dipped into the bacterial suspension; excess fluid was removed by pressing the swab against the tube wall.
5. The surface of an Iso-Sensitest Agar plate (appendix C) was swabbed by the use of a rotator.
6. A control spread to check for contaminants was performed.
7. 30μg nalidixic acid and 10μg ciprofloxacin paper disks were applied onto the surface.
8. The plates were pre-incubated in room temperature for 30 minutes, then at 37°C overnight.
9. The diameters of the inhibition zone were measured.

Analysis of the quinolone resistance determining regions (QRDRs)

Alterations in the targets for quinolones due to chromosomal mutations in their genes are responsible for the majority of known cases of quinolone resistance (Gruger *et al.* 2004). These mutations are particularly encountered within conserved regions of *gyrA*, the gene encoding the A subunit of DNA gyrase, and *parC*, the gene encoding the homologous A subunit of topoisomerase IV (Robicsek *et al.* 2006). These hot spots for quinolone resistance have been termed the quinolone resistance determining regions (QRDR) and furthermore specific amino acid substitutions involved in the development of quinolone resistance have been described at specific positions at these regions (Ruiz. 2003) (table 5).

Table 5. Mutations described in GyrA and GyrB subunits of quinolone-resistant strains of *E. coli* (Ruiz. 2003).

Codon		Wild amino acid*	Mutations described*
GyrA	51^b	Ala	Val
	67^b	Ala	Ser
	81	Gly	Cys, Asp
	82^b	Asp	Gly
	83	Ser	Leu, Trp, Ala, Val
	84	Ala	Pro, Val
	87	Asp	Asn, Gly, Val, Tyr, His
	106^b	Gln	Arg, His
ParC	78	Gly	Asp
	80	Ser	Ile, Arg
	84	Glu	Lys, Val, Gly

*Asn/N: Asparagine, Asp/D: Aspartic acid, Ser/S: Serine, Leu/L: Leucine, Gly/G: Glycine, Glu/E: Glutamic acid, Arg/R: Arginine, Ile/I: Isoleucine. ^{b} Only described in mutants obtained *in vitro*.

Mutations in the quinolone resistance determining region (QRDR) of the genes *gyrA* and *parC* in *qnr*-positive isolates were evaluated by PCR and sequencing (Lavilla S. *et al.* 2008). The PCR amplifications were performed using DNA extracted from the *qnr*-positive isolates as template and using primer sets (gyrA6 and gyrA631R) to give a 620-bp product (Weigel *et al.* 1998) and (parCF and parCR) to give a 559-bp product (Qiang *et al*, 2002) (appendix A, table 8). Nucleotide and deduced amino acid sequences were aligned, compared with wild-type sequences of *gyrA* (GenBank

accession no. NP_416734) and *parC* (GenBank accession no. NP_417491) and analyzed using the online software ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) and the lasergene software package (DNASTAR).

Procedure:

1. PCR Mastermixes:

Reagent	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	250 µl
Primer-F 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
Primer-R 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
ddH2O:	192 µl

2. For each reaction, 2.5 µl template was added to 22.5 µl PCR Mastermix

3. The PCR thermocycler was programmed (appendix A. table 9).

4. Gel electrophoresis was done in a 1.5 % agarose gel containing 0.5 µg/ml EtBr at 120V for 1 hour in 0.75X TBE.

5. The PCR products were purified according to the E.Z.N.A™ Cycle-Pure Kit Spin Protocol (Omega Bio-tek, USA) (appendix D, kit 1) and used as template for sequencing.

6. Sequencing Mastermixes were prepared:

Reagent	Amount
BigDye v3.1 (Applied Biosystems, USA):	2 µl
Sequencing buffer (Applied Biosystems, USA):	3 µl
Primer 3.2 pmol/µl:	1 µl
ddH2O:	8 µl

7. For each reaction, 6 µl template was added to 14 µl Sequencing Mastermix

8. The PCR thermocycler was programmed (appendix A. table 9).

9. Sequencing was performed at the Sequencing core facility.

10. Nucleotide sequences were aligned and analyzed using the online software ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) and the lasergene software package (DNASTAR).

Transfer of quinolone resistance

Transconjugation and transformation experiments were performed to examine the ability to horizontally transfer resistance to quinolones conferred by *qnrS* or *qnrB* genes. Conjugation experiments involving the 8 *qnr*-positive isolates detected in this study were performed by the liquid mating assay (Sambrook *et al.* 1989). Rifampicin-resistant *E. coli* J53-2 was used as the recipient strain and Luria-Bertani (LB) agar plates (appendix C) containing rifampicin 100 µg/ml, sodium azide 100 µg/ml, sodium azide 50 µg/ml, ampicillin 100 µg/ml, ampicillin 20 µg/ml, ceftazidim 4 µg/ml, or nalidixic acid 6 µg/ml (Sigma-Aldrich LP, USA) were used for selection as required. Transformation experiments were performed for *qnr*-positive isolates for which no transconjugants were obtained. Plasmid DNA was extracted from the donor strains and introduced into chemically competent *E. coli* TOP'10 (Invitrogen, USA). Transformants were selected on Luria-Bertani plates supplemented with ampicillin 100 µg/ml, ampicillin 20 µg/ml, or nalidixic acid 6 µg/ml for selection as required.

Transconjugation experiment

Procedure:
1. The ability of the donor isolates to grow on 100 µg/ml rifampicin-containing LB agar plates was examined.
2. Green agar plates were inoculated from donors and the recipient and incubated over night at 37°C.
3. 5 ml LB-broth media (appendix C) were inoculated with 4-5 colonies from the agar plates and incubated over night at 37°C, shaking.
4. The overnight cultures were diluted 100 folds and incubated at 37°C, shaking until the cultures were in exponential growth (OD_{600}: 0.3-0.5). This was obtained after approximately 2 hours of incubation.
5. The donor and the recipient were mixed in a ratio 1:9 (0.5 ml of the donor was added to 4.5 ml of the recipient) and the incubation was continued at 37°C, very slow shaking (25-50 rpm).
6. After 60 minutes, samples were withdrawn and diluted in 0.9 % saline solution to 10^{-1}, 10^{-2}, 10^{-5}, and 10^{-6}.

7. 100µl aliquots from dilutions 10^{-1} and 10^{-2} were spread on LB agar plates selecting for transconjugants and 100µl aliquots from dilutions 10^{-5} and 10^{-6} were spread on LB plates selecting for donors and on LB plates selecting for recipients. Plates were incubated at 37°C over night.

8. Plates were counted and transconjugation frequencies were calculated by dividing the number of transconjugants by the number of donors or by the number of reciepients.

9. Transconjugants were inoculated on LB agar plates containing 0.06 µg/ml ciprofloxacin to screen for transconjugants that co-acquired ciprofloxacin resistance of ≥ 0.06 µg/ml.

10. Plasmid isolation from transconjugants was performed according to the E.Z.N.A™ Plasmid Miniprep Protocol (Omega Bio-tek) (appendix D, kit 2).

11. Detection of *qnrS* and *qnrB* genes in transconjugants was carried out by multiplex PCR amplification of *qnrA*, *qnrB*, and *qnrS* using same primers and following same procedure had already applied during the screening except for using isolated plasmid as template.

12. Extended susceptibility test on *qnr* positive transconjugants was performed using Etests and VITEK 2 as described above.

13. Stock cultures for transconjugants were prepared.

Transformation experiment

Procedure:
1. For each transformation, one 50 µl vial of One Shot® *E. coli* TOP'10 cells (Invitrogen, USA) was thawed on ice.

2. 2 µl of isolated plasmid was pipetted directly into the vial of competent cells and mixed by tapping gently.

3. The vial was incubated on ice for 30 min.

4. The vial was incubated for 30 seconds at 42ºC in Eppendorf thermomixer device.

5. 250 µl of pre-warmed S.O.C medium was added to the vial.

6. The vial was incubated at 37ºC for 1 hour with shaking at 225 rpm.

7. 50 µl from the transformation vial was spread on labelled LB agar plate supplied with the selecting antibiotic.

8. The plate was incubated at 37ºC overnight.

9. The number of colonies growing on the overnight plate was counted.

10. Transformants were inoculated on LB-plates containing 0.06 µg/ml ciprofloxacin to screen for transcformants that co-acquired ciprofloxacin resistance of ≥ 0.06 µg/ml.

14. Plasmid isolation from transformants was performed according to the E.Z.N.A™ Plasmid Miniprep Protocol (Omega Bio-tek) (appendix D, kit 2).

15. Detection of *qnrS* gene in transformants was carried out by multiplex PCR amplification of *qnrA*, *qnrB*, and *qnrS* using same primers and following same procedure had already applied during the screening except for using isolated plasmid as template.

11. Extended susceptibility test on *qnr* positive transformants was performed using Etest and VITEK 2 as described above.

12. Stock cultures for transformants were prepared

Plasmid analysis

To reliably estimate the number and molecular sizes of small and large plasmids, two techniques were applied; in the first technique, plasmid DNA was isolated and separated by agarose gel electrophoresis. Two bacterial strains containing plasmids of known size were used as standard size markers. In the second technique, total DNA was digested with S1 nuclease and subjected to pulsed-field gel electrophoresis (S1–PFGE). Appropriate linear DNA markers were used as size markers. Then, plasmid DNA was transferred from the agarose gel onto positively charged nylon membranes and hybridized with specific probe for *qnrS1* obtained by PCR with same primers used for screening.

Plasmid extraction-horizontal gel electrophoresis

Examination of plasmid DNA can be simply performed by plasmid DNA extraction followed by direct agarose gel electrophoresis of extracted DNA (Woodford *et al.* 1994). Most the commercial kits for plasmid DNA isolation exploit a silica membrane column. The procedure generally includes: (1) alkaline-sodium dodecyl sulfate (SDS) lysis of the bacterial cells, (2) application of the lysate onto silica that adsorbs plasmid DNA, (3) removal of contaminants by wash step, and (4) elution of bound plasmid DNA in TE buffer (http://www.omegabiotek.com/protocols/PlasmidMini.pdf).

In this study, the E.Z.N.A™ Plasmid Miniprep kit (Omega Bio-tek) was used for extracting the plasmid DNA from the *qnr*-positive clinical isolates, their transconjugants/transformants, *Escherichia coli* strains NCTC 50192 (containing plasmids of 148, 63.5, 36, and 7 kb in size) and NCTC 50193 (containing plasmids of 48.3, 7.8, 5.2, 4.5, 3.3, 2.6, 2.3, and 1.8 kb in size). The unknown plasmids were separated on agarose gel in parallel with the known plasmids used as standard size markers.

Procedure:
1. Plasmid DNA was isolated according to the E.Z.N.A™ Plasmid Miniprep Protocol I (Omega Bio-tek) (appendix D, kit 2).
2. For gel electrophoresis, 0.8 % agarose gel with 0.5µg/ml EtBr was used.
3. 3 µl loading buffer was added to15 µl of each plasmid DNA product.
4. Plasmids isolated from *E. coli* strains NCTC 50193 and NCTC 50192 were used as standard size markers.
5. The electrophoresis was run at 120V for approx. 3 hours.
6. The gel was visualized on GelDoc system (BioRad, USA) and stored on disks as TIFF files.

S1-PFGE plasmid analysis

S1 nuclease, isolated from the mold *Aspergillus oryzae*, is an endonuclease that attacks only single-stranded DNA (Wiegand *et al*. 1975). When a supercoiled plasmid is treated with S1 nuclease, the enzyme will introduce breaks at regions that have a single-stranded character, as a result of this nicking, supercoiling is lost and the plasmid is converted into full-length linear molecule (Barton *et al*. 1995). Since isolation and size determination of supercoiled large plasmids could be problematic, the facility of S1 nuclease was used and a method was described for detecting and estimating the sizes of large bacterial plasmids in the presence of genomic DNA (Barton *et al*. 1995). The procedure included (1) lysis of bacterial cells embedded in agarose plugs, (2) S1 nuclease treatment to convert the plasmids into unit-length linear molecules, and (3) pulsed-field gel electrophoresis (PFGE) of linearized plasmid samples together with an appropriate marker.

Procedure:

Preparation of PFGE plugs:

1. Green agar plates were streaked and incubated over night at 37ºC.
2. 5-10 colonies were inoculated into Falcon tubes with 5 ml BHI-media (appendix C) and incubated over night at 37ºC, shaking.
3. 50 μl of the cultures were transferred into Falcon tubes with 5 ml BHI-media and incubated for 4 hours at 37ºC, shaking.
4. The tubes were centrifuged at 3500 rpm for 10 min.
5. The supernatant was removed with a Pasteur pipette, and the pellet was re-suspended in 1 ml cold PIV-buffer (appendix C).
6. 495 μl of the suspension was transferred to an Eppendorf tube and 5 μl lysozyme (100 mg/ml) (Sigma-Aldrich LP, USA) was added. The tubes were mixed well by vortexing and equilibrated in water bath at 50 ºC.
7. 500 μl of 2% low melting point (LMP)-agarose (appendix C) was added.
8. The agarose/bacteria solution was mixed well by vortexing, and then the mixture was rapidly transferred to the plug mould.
9. The plug was let to solidify at 4 ºC for 15 min.
10. The plug was transferred to Eppendorf tubes containing 1.8 ml lysis buffer (appendix C) and incubated for 2 hours at 37 ºC, slow shaking.

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11. The lysisbuffer was removed and the plug was washed in 1 ml ddH2O for 15 min.

12. The water was removed and 1 ml ESP-buffer containing 50 μg/ml proteinase K-solution (appendix C) and incubated overnight at 50 ºC.

13. The proteinase K-solution was removed and the plug was washed in 1 ml TE-buffer for 30 min twice.

S1 nuclease plasmid linearization:

14. A thin slice of the plug was cut out and transferred to an Eppendorf tube.

15. The plug was washed in 1 ml 1M Tris-HCL (pH7.5) for 30 min (so EDTA was removed).

16. The plug was washed in 125 μl of S1-nuclease buffer (Sigma-Aldrich, USA) for 30 min.

17. The plug was transferred into 125 μl of S1-nuclease master mix and incubated at 37ºC for 25 min. S1-nuclease Master Mix:

Reagent	Amount
S1-nuclease buffer (10X) (Sigma-Aldrich, USA)	12.5 μl
S1-nuclease (50 U/μl) (Sigma-Aldrich, USA)	0.5 μl
dd H2O:	112 μl

18. The S1-nuclease master mix was removed and the plug was washed twice in 1 ml cold TE-buffer.

Pulsed field gel electrophoresis (PFGE):

19. The electrophoresis chamber was filled with 1900 ml of 0.5X TBE-buffer. The circulation was turn on and the cooler was set to 15 ºC.

20. 1 % agarose gel was prepared; 1 g agarose (Bio-Rad, USA) was added to 100 ml 0.5X TBE buffer and dissolved by boiling in the microwave.

21. The gel equipment was prepared and the gel was let to solidify for 30 min at room temperature.

22. The plugs were added after removing the comb.

23. Low Range PFG Marker (New England Biolabs, USA) was used as standard marker (appendix C, figure 2).

24. The gel was released from the mould and all excess agarose was removed from the sides and under the black support.

25. The gel with the support plate was placed into the frame in the electrophoresis chamber.

26. The PFGE device was programmed and the electrophoresis was run.

Program parameters	
Pulse time: initial pulse-final pulse	1-12 sec
Total runtime	15 hours
Volt	6 v/cm
Angle	120º
Buffer temperature	15 ºC
Gel running buffer	0.5X TBE

27. When the run was done, the gel was stained in EtBr-solution (500 ml ddH2O + 50 µl EtBr) for 45 min.

28. The gel was photographed then it was de-stained in ddH2O for 50 min.

29. The TBE-buffer was removed from the chamber and replaced by water. The circulation was run for few minutes then the water was removed and all remaining liquid was wiped off.

Southern blot

A Southern blot is a method named after its inventor Edwin Southern and used to transfer the size-separated DNA, generally by agarose gel electrophoresis, to a filter membrane (Southern E.M, 1975). DNA transfer is frequently followed by probe hybridization and is mostly used to check for the presence of a specific DNA sequence. In this study, the TurboBlotter™ system was used for transferring the plasmid DNA separated by S1-PFGE into Nytran® SuPerCharge Nylon Membranes (Whatman®, UK) (figure 17).

Figure 17. TurboBlotter

(http://www.whatman.com/References/TurboBlotter.pdf)

Procedure:

<u>Denaturation of DNA gels:</u>

1. The gel was incubated in depurination buffer (Appendix C) for 2 X 30 min.

2. The gel was incubated in denaturing buffer (Appendix C) for 2 X 30 min.

3. The gel was washed in neutralizing buffer (Appendix C) for 15 min.

<u>Transfer:</u>

4. The transfer membrane (Nytran® SuPerCharge nylon) was soaked in ddH2O for 15 min.

5. The "stack tray" of transfer device was placed on bench, making sure it was level.

6. 20 sheets of dry GB004 blotting paper (thick) were placed in stack tray.

7. 4 sheets of dry 3MM Chr blotting paper (thin) were placed on top of stack.

8. One sheet of prewet 3MM Chr blotting paper in neutralizing buffer was placed on stack.

9. Transfer membrane was placed on stack.

10. The membrane was covered with agarose gel; the gel was cut to the size of the membrane, making sure there were no air bubbles between the gel and the membrane.

11. The top surface of the gel was wetted with neutralizing buffer, and 3 sheets of 3MM Chr blotting paper, presoaked in neutralizing buffer, were placed on top of the gel.

12. The "buffer tray" of the transfer device was attached to the bottom tray, using the circular alignment buttons to align both trays.

13. The buffer tray was filled with about 125 ml neutralizing buffer.

14. The transfer was started by connecting the gel stack with the buffer tray using the precut "buffer wick" (included in each blotter stack), presoaked in neutralizing buffer.

15. The wick was placed across the stack so that the short dimension of the wick completely

16. The blotting stack was covered and both ends of the long dimension extend into the buffer tray.

17. The "wick cover" was placed on top of the stack to prevent evaporation. The edges of the wick were checked out to be immersed in the transfer buffer.

18. The transfer was continued for 2 hours.

Immobilization:

19. The membrane was placed on a fresh sheet of dry 3MM Chr blotting paper to remove any excess of neutralizing buffer.

20. The blot was exposed to a source of UV light (254 nm) for a total dose of 120 mJ/cm2 for a damp membrane (by doing so the DNA was covalently bound to the membrane (cross-linking the molecule to the nylon matrix in the presence of UV light).

Storage:

21. The blot was stored at 4 °C.

Labeling and purification of probe

Procedure:

Making a template for the probe:

1. Extracted plasmid DNA from clinical isolates K38-15 was used as template for multiplex-PCR amplification of *qnr* genes.

2. The PCR Master Mix, PCR reaction, PCR primers, and PCR conditions were same as in the *qnr* PCR-based screening.

3. The gel electrophoresis was performed in a 1.5% agarose gel containing 0.05 0.5 µg/ml EtBr at 120 V for 1 Hour in 0.75X TBE and a photo of the gel was taken on GelDoc system (BioRad, USA) and stored on disks as TIFF files.

4. PCR product was purified according to the E.Z.N.A™ Cycle-Pure Kit Spin Protocol (Omega Bio-tek, USA) (appendix D. kit1) and used as template for the probe.

Template labeling (DIG DNA labeling and detection kit, Roche Applied Science, Germany):

5. 15 µl from each template was taken. For a control labeling reaction, 10 µl of double distilled water was added to 5 µl of control DNA 2 (vial 2).

6. The DNA was denatured by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath.

7. Master Mix: Hexanucleotide Mix, $10 \times$ (vial 5): 2 µl

      dNTP Labeling Mix (vial 6): 2 µl

      Klenow enzyme labeling grade (vial 7): 1 µl

8. For each Reaction, 15 µl sample was added to 5 µl Mastermix.

9. The tubes were mixed and centrifuged briefly.

10. Tubes were incubated overnight at 37°C.

11. The reaction was stopped by heating to 65° C for 10 min.

Determination of labeling efficiency (DIG DNA labeling and detection kit, Roche Applied Science, Germany):

12. The labeled probes and the DIG-labeled control DNA (vial4) were diluted 10^{-1} to 10^{-5} times in ddH2O. A 10^{-1} dilution of the DIG-labeled control DNA corresponds to 0.5 µg/ml.

13. 1 μl of each dilution was spotted in a row on a nylon membrane and left to air dry for 20 minutes.

14. The membrane was transferred into a plastic container with 20 ml Maleic acid buffer (appendix C) and incubated under shaking for 2 min at 20° C.

15. The membrane was incubated for 30 min in 20 ml Blocking solution (appendix C).

16. The membrane was incubated for 30 min in 20 ml Antibody solution (appendix C).

17. The membrane was washed twice with 20 ml Washing buffer (appendix C) for 15 min each time.

18. The membrane was equilibrated in 20 ml Detection buffer (appendix C) for 3 min.

19. The membrane was incubated in 2 ml freshly prepared Color substrate solution (aapendix C) in an appropriate plastic box in the dark.

20. The color was developed fast and in 5 min the desired spot intensities were achieved and the reaction was stopped by taking the membrane out the bag and washing it in plastic container with sterile double distilled water for 5 min.

21. A photo was taken on GelDoc system (BioRad, USA) and stored on disks as TIFF files.

22. The intensity of the spots of the labeled probes was compared to the control and the amount of DIG-labeled DNA was calculated.

Hybridization

Two strands of DNA, RNA, or one strand DNA and one strand RNA are held together forming double-stranded helix, provided that their sequences are complementary. This process is called hybridization (Synder & Champness. 2007). The common aim of hybridization assays is to use a DNA or RNA sequence (probe) to identify a particular complementary DNA or RNA sequence (target) among thousands of other sequences. The basis of the standard nucleic acid hybridization assays currently in use involves (1) immobilizing the target DNA on a solid support, such as a membrane made of nitrocellulose or nylon, (2) adding labeled probe that will attach to the immobilized target DNA, (3) removing the solution containing unbound probe DNA, (4) extensive

washing and drying in preparation for detection, and finally (5) detecting the signal of the probe fixed to its complementary target (Synder & Champness. 2007). The objective of the hybridization assay in this study was to identify the location of *qnrS* and *qnrB* genes in the eight *qnr*-positive clinical isolates as well in the five *qnr*-positive transconjugants and transformants.

Procedure:

1. The hybridization-incubator and 20 ml prehybridization solution (appendix C) were pre-warmed to 68°C. The filter was pre-hybridized for 1-2 h in a hybridization tube.
2. The required amount of probe was diluted in a small volume of hybridization solution (appendix C) and boiled for 10min for denaturation. The probe was quickly cooled in ice water and 8 ml of pre-warmed hybridization solution were added to it. The tube was mixed thoroughly (bubbles were avoided).
3. The pre-hybridization solution was removed and the probe/hybridization solution was added to the filter. Hybridization was done for 16-20h.
4. The filter was washed in 100ml 2 x stringency-buffer (appendix C) 2 x 5 min at 68°C then in 100 ml 0.5 x stringency buffer (appendix C) 2 x 15 min at 68°C.

Detection of hybridization (DIG luminescence detection kit for nucleic acid, Roche Applied Science, Germany):

1. The membrane was rinsed briefly for 5 min in Wash buffer at room temperature.
2. The wash buffer was replaced with 100 ml 1% Blocking solution and the membrane was incubated with agitation for 30 min.
3. The blocking solution was replaced with 30 ml of Antibody solution and the membrane was incubated with agitation for 30 min.
4. Container was shifted. The membrane was washed in 100 ml Wash buffer for 15 min twice.
5. The filter was equilibrated in 30ml Detection buffer for 2-5 min.
6. The membrane was incubated in 2 ml freshly prepared Color substrate solution in an appropriate plastic box in the dark.

7. The color was developed fast and in 5 min the desired spot intensities were achieved and the reaction was stopped by washing the membrane with sterile ddH2O for 5 min.

8. A photo was taken on GelDoc system (BioRad, USA) and stored on disks as TIFF files

PCR-based plasmid typing

Identification and classification should always be based on traits that are universally present and are constant (Couturier *et al.* 1988). In plasmids, these criteria are best met by the genetic elements concerned with initiation of replication, control of replication, and partitioning. Plasmids have traditionally been classified according to their incompatibility with other plasmids, a property that is related to their replication and/or partitioning systems. The basis of incompatibility typing is that two plasmids sharing common replication and/or partitioning elements are unable to proliferate stably in the same cell line (Johanson *et al.* 2007). Although the incompatibility grouping method has been an important tool to identify and classify plasmids, this method can not be applied to a large number of strains; its application has been limited by the laborious and time-consuming work required. Therefore, a PCR-based replicon typing (inc/rep PCR-based typing) method has recently been developed for classification of plasmids occurring in members of the *Enterobacteriaceae* (Carattoli *et al.* 2005). Currently, there are 26 known Inc groups occurring among the *Enterobacteriaceae*, a number which has remained static for several years (Johanson *et al.* 2007). In this method, 5 multiplex- and 3 simplex-PCRs are performed to recognize 18 replicon types (FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA) representative of the major plasmid groups circulating among the *Enterobacteriaceae* (Carattoli *et al.* 2005).

Identification of the inc/rep type of the plasmids extracted from the eight *qnr*-positive isolates, and from four *qnr*-positive transconjugants/transformant was performed as previously described (Carattoli *et al.* 2005). The PCR amplifications were done using plasmid DNA extracted from the *qnr*-positive strains as template (appendix D. kit 2),

JumpStart REDTaq Ready Mix Reaction Mix (Sigma-Aldrich LP, USA), and specific primers (Carattoli *et al*. 2005) (appendix A, table 8).

Procedure:

1. Mastermixes for the multiplex-PCRs:

Reagent	Amount
JumpStart™ REDTaq® Ready Mix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	250 µl
Primer1-F (50 pmole/µl), Primer1-R (50 pmole/µl), Primer2-F (50 pmole/µl), Primer2-R (50 pmole/µl), Primer3-F (50 pmole/µl), and Primer3-R (50 pmole/µl) (Eurogentec S.A., Belgium):	4 µl each
ddH2O:	176 µl

2. Mastermixes for the simplex-PCRs:

Reagent	Amount
JumpStart™ REDTaq® Ready Mix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	500 µl
Primer-F 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
Primer-R 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
ddH2O:	192 µl

3. For each reaction, 2.5 µl template was added to 22.5 µl PCR Mastermix.
4. The PCR thermocycler was programmed (appendix A. table 9).
5. Gel electrophoresis was done in a 1.5 % agarose gel containing 0.5 µg/ml EtBr at 120V for 1 hour in 0.75X TBE.

Determination of the genetic environment of *qnrS1*

qnrS1 has been reported to be located upstream to truncated *orfB* of IS*2*, *bla*$_{LAP}$, and *pbp*-3 and downstream to an open reading frame coding for a 213 amino acid polypeptide (Poirel *et al*. 2007). Therefore, the genetic environment of *qnrS1* was analyzed by targeted PCR and sequencing. Targeted PCR amplifications were carried out using DNA extracted from the *qnrS*-positive isolates as template and six different sets of primers (qnrS-3`-ext and orfB-B), (qnrS-3`-ext and LAP-1A), (qnrS-3`-ext and pbp3-B), (qnrS-5`-ext and 213B), (*qnrS*-R.multiplex and LAP-1.Kres), and (*qnrS*-F.multiplex and Sea15.Kres) (appendix A, table 8). *qnrS1* has also been reported to be

located upstream to Tn3 (Kehrenberg *et al*. 2006). Thus, for the strains in which *bla*~LAP-1~ and *pbp*-3 were absent, further two PCR amplification were carried out with two sets of primers (qnrS-3`-ext with tnpRF/tnpRF2) and (qnrS-3`-ext with TEM-R mod TW) (appendix A, table 8). PCR amplification was followed by extracting and purifying the gel bands and sequencing the two strands of the DNA fragment using the same primers.

Procedure:

1. PCR Mastermixes:

Reagent	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich LP, USA):	250 µl
Primer-F 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
Primer-R 50 pmole/µl (Eurogentec S.A., Belgium):	4 µl
ddH2O:	192 µl

2. For each reaction, 2.5 µl template was added to 22.5 µl PCR Mastermix
3. The PCR thermocycler was programmed (appendix A. table 9).
4. Gel electrophoresis was done in a 0.8 % agarose gel containing 0.5 µg/ml EtBr at 120V for 1 hour and 15 minutes in 0.75X TBE.
5. Amplified DNA was extracted and purified from the agarose gel according to the QIAquick SpinHandbook for QIAquick Gel Extraction Kit (QIAGEN, Germany) (appendix D, kit 3) and used as template for sequencing.
6. Sequencing Mastermixes:

Reagent	Amount
BigDye v3.1 (Applied Biosystems, USA):	2 µl
Sequencing buffer (Applied Biosystems, USA):	3 µl
Primer 3.2 pmol/µl:	1 µl
ddH2O:	8 µl

7. For each reaction, 6 µl template was added to 14 µl Sequencing Mastermix
8. The PCR thermocycler was programmed (appendix A. table 9).
9. Searching for nucleotide sequence homology was performed using BLAST available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).
10. Nucleotide sequences were aligned and analyzed using the lasergene software package (DNASTAR).

3. Results

Prevalence of the *qnr* genes

Screening for the *qnrA*, *qnrB*, and *qnrS* genes among the clinical isolates of *E. coli* and *Klebsiella* spp. included in this study was done by a multiplex PCR (Robicsek *et al* 2006). The amplification products were provisionally identified as *qnr*-positive from their sizes in EtBr-stained agarose gels and the positive results were confirmed by direct sequencing of PCR products on both strands. The nucleotide and amino acid sequences were aligned and compared with published sequences of the *qnrS* and *qnrB* variants.

Overall, *qnr*-genes were identified in 8/487 (1.6%) of the clinical isolates (figure 18). The prevalence of *qnr* genes was 0.7% (3/422) and 7.7% (5/65) among *E. coli* and *Klebsiella* spp. isolates, respectively. Six isolates contained the *qnrS1* gene, one isolate contained the *qnrB1* gene, and one isolate contained *qnrB7*. The *qnrA* gene was not detected in any of the clinical isolates. Four *qnrS1* genes were detected in the NORM and Kronoberg collections representing the common population of clinical isolates in Norway and Sweden, whereas the other four *qnr* genes (two *qnrS1*, one *qnrB1*, and one *qnrB7*) were detected in clinical isolates from the Norwegian ESBL study, one of the three K-res collection representing a resistant population of clinical isolates in Norway (tables 6 and 7).

Prevalence of the *aac(6`)-Ib-cr* gene

Screening for the *aac(6')-Ib* gene among the *E. coli* and *Klebsiella* spp. clinical isolates was done by a simplex PCR (Park *et al.* 2006). The amplification products were provisionally identified as *aac(6')-Ib*-positive from their sizes in EtBr-stained agarose gels. The *aac(6')-Ib*-positive PCR products were subjected to *Bst*CI digestion to identify the *aac(6')-Ib-cr* variant. Digestion products that yielded only one band in EtBr-stained agarose gels were assigned to the *aac(6')-Ib-cr* variant while those yielding two bands were identified as *aac(6')-Ib* wild-type genes. The results were confirmed by sequencing PCR products on one strand to verify the two amino acid changes (Arg102Trp and Tyr179Asp) that characterize the *aac(6')-Ib-cr* variant.

The *aac(6')-Ib* gene was detected in 15.4 % (75/487) of the clinical isolates while the *aac(6')-Ib-cr* variant was detected in 14.1 % (69/487) of the isolates, representing 92 % (69/75) of the detected *aac(6')-Ib*. The prevalence of *aac(6')-Ib-cr* was 15.6% (66/422) and 4.6% (3/65) among *E. coli* and *Klebsiella* spp. clinical isolates, respectively (table 6) (features of the *aac(6`)-Ib*-positive isolates are presented in appendix A, table 10). Interestingly, two isolates showed partial digest with *Bst*CI suggesting that they could harbor both the wild type *aac(6')-Ib* and the *-cr* variant. The sequence data showed the presence of both cytosine (C) and thymidine (T) nucleotide peaks corresponding to Trp and Arg amino acids (at position 102) and hence confirming this.

While the prevalence of *aac(6')-Ib-cr* was only 0.5 % (1/152) and 5% (11/218) in the Kronoberg and NORM collections, respectively, it was 48.7 % (57/117) among the clinical isolates from K-res collections (the AmpC and ESBL studies). The *aac(6')-Ib-cr* gene was detected in 50 % (4/8) of the *qnr*-positive isolates (figure 18) while it was detected in only 14.8 % (71/479) of the *qnr*-negative isolates (table 6).

Table 6. Prevalence of the *qnr* and *aac(6')-Ib-cr* genes

Collection/species	Number of isolates	*qnr*	*qnr* variant	*aac(6`)-Ib*	*aac(6`)-Ib-cr*
Kronoberg collection	152	1	*qnrS1*	2	1
NORM collection	218	3	*qnrS1*	11	11
E. coli AmpC study	13	0	-	1	1
Ullevål ESBL study	60	0	-	41	39
Norwegian ESBL study	44	4	2 *qnrS1*,1 *qnrB1*, and 1 *qnrB7*	20	17
E. coli	422	3	*qnrS1*	68	66
Klebsiella spp.	65	5	3 *qnrS1*,1 *qnrB1*, and 1 *qnrB7*	7	3
Total	487	8 (1.6%)	6 *qnrS1*,1 *qnrB1*, and 1 *qnrB7*	75	69 (14.1%)

Table 7. Features of the *qnr*-positive clinical isolates.

Ref. nr	Species*	*qnr* variant	*aac(6`)-Ib-cr*	MIC(μg/ml) NAL	MIC(μg/ml) CIP	ESBL type
K38-15	*E. coli*	*qnrS1*	-	16	0.5	-
K4-13	*K. pneumoniae*	*qnrS1*	*aac(6`)-Ib-cr*	8	2	CTX-M gr.1 SHV-14
K4-43	*E. coli*	*qnrS1*	*aac(6`)-Ib-cr*	≥256	≥32	CTX-M gr. 9
K39-31	*K. pneumoniae*	*qnrS1*	-	16	0.5	-
K40-47	*K. pneumoniae*	*qnrS1*	-	64	4	-
K40-79	*E. coli*	*qnrS1*	*aac(6`)-Ib-cr*	≥256	≥32	-
K2-78	*K. pneumoniae*	*qnrB7*	-	16	1	SHV-5/5a/or12
K8-5	*K. pneumoniae*	*qnrB1*	*aac(6`)-Ib-cr*	≥256	≥32	CTX-M gr.1

* All *qnr*-positive isolates were re-identified by VITEK.
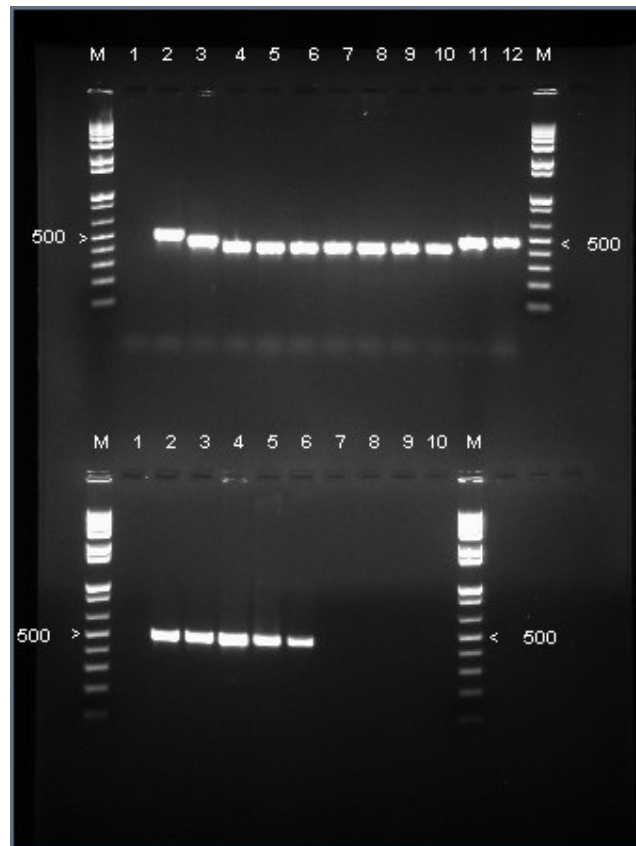
Figure 18. (Top). Agarose gel electrophoresis of *qnr*-multiplex PCR products of the *qnr*-positive clinical isolates. Lanes: 1, negative control; 2, *E. coli* A2-67 (*qnrA* positive control); 3, *E. coli* A3-22 (*qnrB* positive control); 4, *E. coli* A3-20 (*qnrS* positive control); 5, K38-15; 6, K4-13; 7, K4-43; 8, K39-31; 9, K40-47; 10, K40-79; 11, K2-78; and 12, K8-5. (Bottom). Agarose gel electrophoresis of *aac(6')-Ib*-simplex PCR products of the *qnr*-positive clinical isolates. Lanes: 1, negative control; 2, *E. coli* A3-21 (*aac(6')-Ib* positive control); 3, K4-13; 4, K4-43; 5, K40-79; 6, K8-5; 7, K38-15; 8, K39-31; 9, K40-47; and 10, K2-78. M, molecular size marker (1 KB plus ladder, Invitrogen, USA).

Resistance profiles of the *qnr*-positive isolates

Antimicrobial susceptibility testing on the *qnr*-positive isolates was performed using Etest, VITEK® 2, and agar disc diffusion. The clinical MIC breakpoints defined by NWGA (Norwegian Working Group on Antibiotics), EUCAST (European Committee on Antimicrobial Susceptibility Testing), or BSAC (the British Society for

Antimicrobial Chemotherapy) and the zone breakpoints defined by SRGA (the Swedish Reference Group for Antibiotics) were used (appendix A, table 2).

Four of the *qnr*-positive isolates (three *qnrS1*- and one *qnrB1*-) showed resistance to nalidixic acid and flouroquinolones with MICs ranging from 64 to ≥256 μg/ml and from 4 to ≥ 32 μg/ml, respectively. On the other hand, the other four *qnr*-positive isolates (three *qnrS1*- and one *qnrB7*-) were susceptible to nalidixic acid (MIC ≤ 16 μg/ml), one of these isolates (K4-13) was ciprofloxacin-resistant (MIC, 2 μg/ml) while the other three showed reduced susceptibility to ciprofloxacin (MIC, 0.25-0.5 μg/ml) (table 8).

All the *qnr*-positive isolates were resistant to ampicillin with MICs ranging from 32 to ≥ 256 μg/ml. Three of the four *qnr*- and ESBL-positive isolates (K4-13, K2-78, and K8-5) were, as expected, resistant to the oxyimino-cephalosporins (cefuroxime, cefotaxime, ceftazidime, and cefepime) while susceptible to the cephamycins (cefoxitin) and to the combination β-lactams/clavulanic acid (amoxicillin/clavulanic acid, and piperacillin/tazobactam). The fourth *qnr*- and ESBL-positive isolate, K4-43, was resistant to the oxyimino-cephalosporins (cefuroxime, cefotaxime, ceftazidime, and cefepime) but was furthermore resistant to cefoxitin, amoxicillin/clavulanic acid, and piperacillin/tazobactam (table 8).

All the *qnr*- and *aac(6`)-Ib-cr*-positive clinical isolates (K4-13, K4-43, K8-5, and K40-79) showed reduced susceptibility or resistance to amikacin and tobramycin. Three of these *aac(6`)-Ib-cr*-positive isolates were also resistant to gentamycin while only one was gentamycin-susceptible. On the other hand, the *qnr*-positive but *aac(6`)-Ib-cr*-negative clinical isolates (K38-15, K2-78, K39-31, and K40-47) were susceptible to amikacin, tobramycin, and gentamycin. 4/4 of the *aac(6`)-Ib-cr*-positive and 1/4 of the *aac(6`)-Ib-cr*-negative clinical isolates were furthermore resistant to trimethoprim and trimethoprim-sulfmethoxazole (table 8) (results of VITEK® 2 and Agar disk diffusion susceptibility testing are presented in appendix A, tables 11 and 12).

Table 8. MICs by Etest for the *qnr*-positive clinical isolates.

Antibiotic*	MIC (µg/ml)							
	K38-15	K4-13	K4-43	K39-31	K40-47	K40-79	K2-78	K8-5
NAL	16	8	≥256	16	64	≥256	16	≥256
CIP	0,5	2	≥32	0.25	4	≥32	0.5	≥32
NOR	2	8	≥256	2	16	≥256	2	≥256
OFX	2	2	≥32	2	32	≥32	2	≥32
LVX	1	1	≥32	1	4	≥32	1	32
MXF	1	2	≥32	1	8	≥32	2	≥32
GAT	0,5	1	≥32	0,5	4	≥32	0,5	32
AMK	2	8	8	2	1	8	2	8
TOB	0,5	32	≥256	0,5	0,25	64	0,5	32
GEN	0,5	64	128	0,5	0,25	1	0,5	64
AMP	≥256	≥256	≥256	32	32	≥256	≥256	≥256
AMC	8	16	32	2	2	16	4	16
PIP	128	≥256	≥256	8	4	256	≥256	≥256
TZP	1	16	≥256	2	4	4	4	12
FOX	4	4	256	4	16	8	8	8
CXM	4	≥256	≥256	4	8	8	32	≥256
CTX	0,125	256	64	0,064	0,125	0,25	8	256
CAZ	≤0.5	≥32	≥32	≤0.5	≤0.5	≤0.5	≥32	≥32
CCV	0,064	0,25	≥4	0,25	0,5	0,5	0,25	0,25
FEP	≤0.25	≥16	≥16	≤0.25	≤0.25	0,5	2	≥16
FCV	≤0.064	0,064	2	≤0.064	≤0.064	0,064	0,064	0,094
IPM	0,125	0,25	0,25	0,5	0,25	0,25	0,25	0,25
MEM	0,016	0,064	0,25	0,032	0,032	0,032	0,064	0,064
TMP	0,25	≥32	≥32	≥32	2	≥32	1	≥32
SXT	0,064	≥32	≥32	≥32	4	≥32	0,125	≥32

* NAL, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; OFX, ofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin; AMK, amikacin; TOB, tobramycin; GEN, gentamicin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; FOX, cefoxitin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CCV, ceftazidime/clavulanic acid; FEP, cefepime; FCV, cefepime/clavulanic acid; IPM, imipenem; MEM, meropenem; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole.

Analysis of the quinolone resistance determining regions (QRDRs)

To detect the presence of chromosomal mutations in the QRDRs of *gyrA* and *parC* genes in the *qnr*-positive isolates, PCR amplifications and sequencing were performed (Lavilla *et al*. 2008). The nucleotide and deduced amino acid sequences were aligned and compared with wild-type sequences.

Mutations L83S in *gyrA* and I80S in *parC* were detected in the three *qnr*-positive clinical isolates with high nalidixic acid- and ciprofloxacin-resistance (MIC $\geq$ 256 µg/ml and $\geq$ 32 µg/ml, respectively). Mutation N87D in *gyrA* was furthermore detected in two of these three isolates. No mutations were detected neither in the *K. pneumoniae* isolate K40-47 that was nalidixic acid- and ciprofloxacin-resistant but with lower MICs (64 µg/ml and 4 µg/ml, respectively) nor in the four clinical isolates with only reduced susceptibility to nalidixic acid and ciprofloxacin (table 9) (the amino acid alignments are presented in appendix B, figure 1).

Table 9. Mutations in QRDR of *gyrA* and *parC* and the corresponding MICs of nalidixic acid and ciprofloxacin in the *qnr*-positive clinical isolates.

Isolate	Species	MIC (µg/ml)		QRDR	
		NAL	**CIP**	*gyrA*	*parC*
K38-15	*E. coli*	16	0.5	-	-
K4-13	*K. pneumoniae*	8	2	-	-
K4-43	*E. coli*	$\geq$256	$\geq$32	L83S, N87D	I80S
K39-31	*K. pneumoniae*	16	0.5	-	-
K40-47	*K. pneumoniae.*	64	4	-	-
K40-79	*E. coli*	$\geq$256	$\geq$32	L83S, N87D	I80S
K2-78	*K. pneumoniae*	16	0.5	-	-
K8-5	*K. pneumoniae*	$\geq$256	$\geq$32	L83S	I80S

Transfer of quinolone resistance

The ability to horizontally transfer quinolone resistance conferred by *qnrS* or *qnrB* genes was examined by transconjugation and transformation experiments. Transconjugation was performed by the liquid mating assay (Sambrook *et al*. 1989) using rifampicin-resistant *E. coli* J53-2 as the recipient strain. For transformation, plasmid DNA was extracted from the donor strains and introduced into chemically competent *E. coli* TOP'10 recipients.

The *qnrS1* gene was successfully transferred by transconjugation from 3/8 of the *qnr*-positive isolates using ampicillin 100 µg/ml for selection. The transconjugation frequencies varied between 1 x 10^{-4} and 5.8 x 10^{-4} Transconjugant per donor (appendix A, table 13). In addition, transformation experiments were successful with plasmid extracted from 2/5 of the non-transconjugable *qnr*-positive isolates, one of the donors was *qnrS1*-positive and the other one was *qnrB7*-positive. Co-transfer of the *aac(6`)-Ib-cr* gene was achieved from 2/3 of the *qnr*- and *aac(6`)-Ib-cr*-positive donor isolates (table 10).

Table 10. Transfer of *qnr* and the co-transfer of *aac(6`)-Ib-cr* genes.

Donor	PMQR	Transfer experiment	Transfer of *qnr*	Co-transfer of *aac(6`)-Ib-cr*
K38-15	*qnrS1*-positive	transconjugation	+	Not applicable
K4-13	*qnrS1*- and *aac(6`)-Ib-cr*-positive	transconjugation	+	+
K4-43	*qnrS1*- and *aac(6`)-Ib-cr*-positive	transformation	+	-
K39-31	*qnrS1*-positive	-	-	-
K40-47	*qnrS1*-positive	-	-	-
K40-79	*qnrS1*- and *aac(6`)-Ib-cr*-positive	transconjugation	+	+
K2-78	*qnrB7*-positive	transformation	+	Not applicable
K8-5	*qnrB1*- and *aac(6`)-Ib-cr*-positive	-	-	-

By comparing the MICs for the transconjugants/transformants with those for the recipients (table 11) (results of VITEK® 2 susceptibility testing are presented in appendix A, table 14), it can be noticed that resistance to ampicillin and piperacillin (ampicillin was used for selection) and reduced susceptibility to fluoroquinolones were horizontally transferred from all the 5 donors. Interestingly, resistance to the β-lactams, cefuroxime, cefotaxime, and ceftazidime were horizontally transferred from the *qnrB7*-positive isolate (K2-78) but not from the *qnrS1*-positive isolates. For K4-13 as a donor, resistance or reduced susceptibility to ampicillin, piperacillin, fluoroquinolones, amikacin, tobramycin, trimethoprim, and trimethoprim-sulfamethoxazole were transferred as a bloc.

The horizontal transfer of *qnrS1* from K38-15, *qnrS1* from K4-43, and *qnrB7* from K2-78 as donors increased the MIC of nalidixic acid by 2, 4, and 2 folds, respectively, and of ciprofloxacin by 62, 83, and 10 folds, respectively (table 11). The horizontal transfer of both the *qnrS* and *acc(6`)-Ib-cr* genes from K4-13 as a donor increased the MIC of nalidixic acid, ciprofloxacin, amikacin, and tobramycin by 4, 125, 2, and 64 folds, respectively. While for K40-79, the transfer of *qnrS* and *acc(6`)-Ib-cr* increased the MIC of nalidixic acid and ciprofloxacin by 4 and ~62 folds, respectively, whereas there were no increase in the MIC of amikacyin and tobramycin (table 11).

Repeated transconjugation and transformation experiments using K8-5 (*qnrB1*-positive), K4-43, K40-47 and K39-31 (*qnrS1*-positive) as donors yielded negative results though different antibiotics were used for selection.

Table 11. MICs by Etest for the recipients *E. coli* J53-2 and *E. coli* TOP'10 and the *qnr*-positive transconjugants/transformants

Antibiotic*	MIC ((µg/ml)						
	TOP'10	J53-2	TC38-15	TC4-13	TF4-43	TC40-79	TF2-78
NAL	2	2	4	8	8	8	4
CIP	0.003	0.016	1	2	0.25	1	0.032
NOR	0.032	ND	4	8	1	4	0.125
OFX	0.032	0.064	1	1	1	2	0.064
LVX	0.006	0.016	0.5	0.5	0.5	1	0.032
MXF	0.006	0.032	0.5	0.5	0.25	1	0.064
GAT	0.003	0.032	0.5	0.5	0.25	0.5	0.032
AMK	2	2	2	4	2	2	2
TOB	0.5	0.25	0.5	16	0.5	0.25	0.5
GEN	0.5	0.5	0.5	0.5	0.5	0.5	0.5
AMP	4	16	≥256	≥256	≥256	≥256	≥256
AMC	4	8	16	24	8	16	8
PIP	2	2	128	64	64	64	128
TZP	0.5	2	4	16	2	4	2
FOX	8	8	16	16	64	16	16
CXM	8	16	16	16	8	16	32
CTX	0.125	0.25	0.5	0.5	0.125	0.25	4
CAZ	0.5	0,5	0.5	0.5	0.5	1	≥32
CCV	0.5	0,5	0.5	0.5	0.5	1	0.5
FEP	≤0.25	≤0.25	≤0.25	0.032	<0.25	≤0.25	≤0.25
FCV	≤0.064	0,094	0.125	0.125	0.064	≤0.064	≤0.064
IPM	0.25	0,25	0.25	0,25	0.25	0.25	0.25
MEM	0.032	0,064	0,032	0.032	0.064	0.064	0.032
TMP	ND	0.064	0.064	≥32	0.032	0.064	0.25
SXT	0.064	0,016	0.016	0.25	0.064	0.016	0.064

* NAL, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; OFX, ofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin; AMK, amikacin; TOB, tobramycin; GEN, gentamicin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; FOX, cefoxitin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CCV, ceftazidime/clavulanic acid; CEF, cephalothin; FEP, cefepime; FCV, cefepime/clavulanic acid; IPM, imipenem; MEM, meropenem; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole.

Plasmid analysis

For the *qnr*-positive isolates and their transconjugants/transformants, plasmid DNA was analyzed by plasmid isolation followed by gel electrophoresis (Woodford *et al.* 1994). Plasmid DNA from *E. coli* strains NCTC 50192 (four plasmids of 148, 63.5, 36, and 7 kb in size) and NCTC 50193 (eight plasmids of 48.3, 7.8, 5.2, 4.5, 3.3, 2.6, 2.3, and 1.8 kb in size) was used as standard size marker.

Clinical isolate K4-43 contained two plasmids of ≥ 150 kb in size. Also, isolate K40-47 contained two plasmids of about 45 kb and 65 kb in size. Each of isolates K38-15 and K40-79 contained only one plasmid of less than 10 kb whereas no plasmid DNA was detected for the other 4 clinical isolates and all the transconjugants/transformants (figure 19).
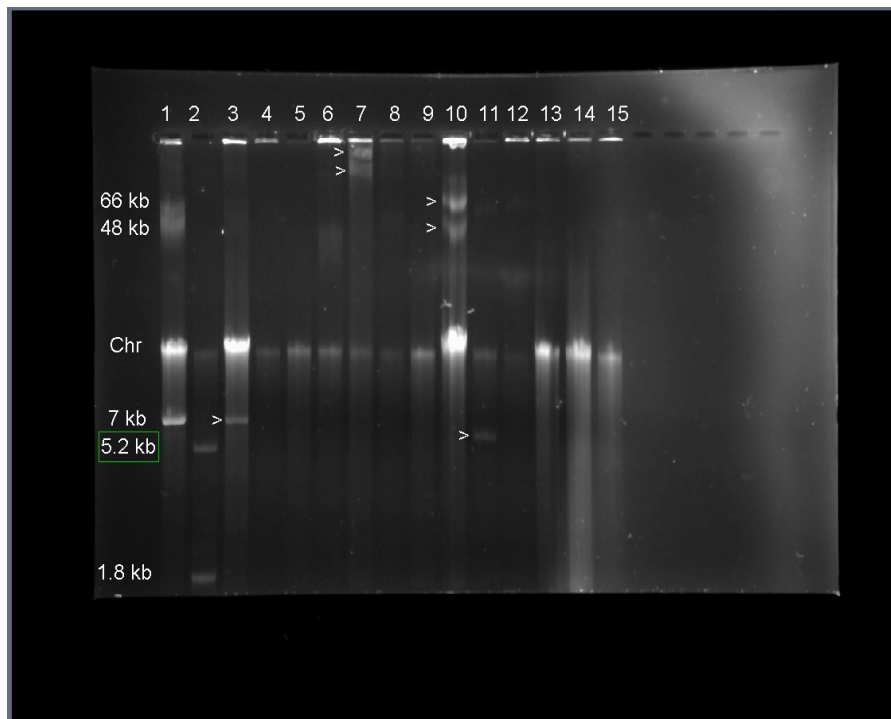
Figure 19. Agarose gel electrophoresis of isolated plasmid DNA from the *qnr*-positive clinical isolates, transconjugants, and transformants. Lanes: 1, *E. coli* NCTC 50192; 2, *E. coli* NCTC 50193; 3, K38-15; 4, TC38-15; 5, K4-13; 6, TCK4-13; 7, K4-43; 8, TF4-43; 9, K39-31; 10, K40-47; 11, K40-79; 12, TC40-79; 13, K2-78; 14, TF2-78; and 15, K8-5. Chr, chromosomal DNA. Plasmid DNA is indicated by >.

Plasmid analysis was also performed using S1-nuclease, to convert the plasmids into unit-length linear molecules, followed by pulsed-field gel electrophoresis (PFGE) with appropriate linear DNA markers (Barton *et al*. 1995). After that, plasmid DNA was transferred from the agarose gel onto positively charged nylon membranes and hybridized with PCR-generated probe specific for *qnrS1*.

Overall, each clinical isolate contained one to four plasmids ranging from 30 kb to $\geq$ 195 kb in size while each transconjugant/transformant contained only one or two plasmids between 100 kb and 190 kb in molecular size. *qnrS1* was located on a ~140 kb plasmid in three clinical isolates and their transconjugants/transformants (K38-15, TC38-15, K4-43, TF4-43, K40-79, and TC40-79) while it was located on a plasmid of approximately 175 kb in K4-13 and TC4-13. For K40-47, *qnrS1* was carried on plasmid of about 195 kb while for K39-31 it was not clear whether *qnrS1* was carried on a plasmid of $\geq$ 200 kb or it was chromosome-located (figure 20).

(A)

(B)
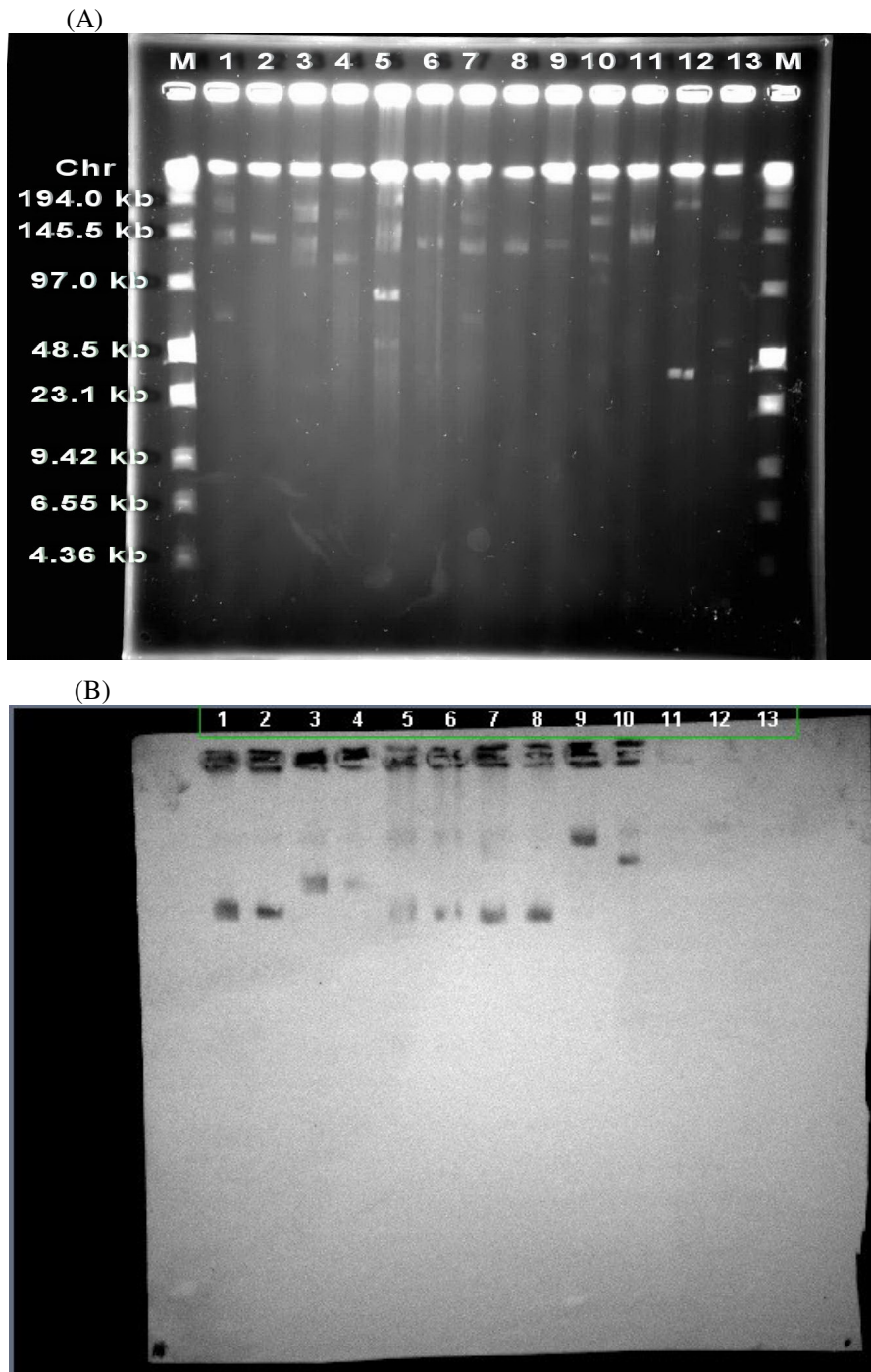
Figure 20. (A) Pulsed field gel electrophoresis of S1 nuclease–digested genomic DNA from the *qnr*-positive clinical isolates, transconjugants, and transformants. (B) Southern hybridization with *qnrS1* probe. Lanes: 1, K38-15; 2, TC38-15; 3, K4-13; 4, TC4-13; 5, K4-43; 6, TF4-43; 7, K40-79; 8, TC40-79; 9, K39-31; 10, K40-47; 11, K2-78; 12, K8-5; and 13, *E. coli* NCTC 50193. M, molecular size marker (Low Range PFG Marker, New England BioLabs, USA). Chr, chromosomal DNA.

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PCR-based plasmid typing

The inc/rep type of plasmid DNA isolated from the *qnr*-positive clinical isolates and their transconjugants/transformants was determined by performing 5 multiplex- and 3 simplex-PCRs (Carattoli *et al.* 2005). This PCR-based replicon typing method is able to recognize 18 replicon types (FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA), representing the major plasmid groups circulating among the *Enterobacteriaceae*.

Overall, 5 replicon types (N, F, FIIs, FIB, and I1-Iy) were detected with IncN being the most identified replicon type. IncN type was found in plasmid DNA isolated from K38-15, TC38-15, K4-13, TC4-13, K40-79, TC40-79, K4-43, and K40-47. Interestingly, the replicon type F was detected for all the transconjugants and transformants while it was detected in only 2/5 donors (K38-15 and K40-79). Plasmid DNA from K2-78 and K39-31 had solely the replicon type FIIs while plasmid DNA from K8-5 could not be assigned to any of the 18 replicon types (table 12).

Table 12. Rep/Inc types of the plasmid DNA

Isolate	Plasmid rep/inc type
K38-15	I1-Iy, N, FIB, F
TC38-15	N, F
K4-13	N
TC4-13	N, F
K4-43	I1-Iy, N
TF4-43	F
K40-79	N, F
TC40-79	N, F
K39-31	FIIs
k40-47	I1-Iy, N
K2-78	FIIs
K8-5	-

Determination of the genetic environment of *qnrS1*

Analyzing the genetic surrounding of *qnrS1* was performed by PCR amplifications followed by sequencing with primers targeting the *qnrS1* gene and the DNA elements that have been previously reported to be in its vicinity (*orf-B* of IS*2*, *bla*$_{\text{LAP-1}}$, *pbp-3*, and *orf* encoding a 213-aminoacid polypeptide) (Poirel *et al*. 2007) (figure 21).

The truncated insertion sequence IS*Ecl2* was detected downstream to *qnrS1* in all the six *qnrS1*-positive strains. The *bla*$_{\text{LAP-1}}$ and *pbp*-3 genes were detected further downstream in three isolates (K4-13, K39-31, and K40-47) while they were absent in the other three isolates (K38-15, K40-79, and K4-43). In addition, *Δres*, *orf259*, and *orf213* were detected upstream to *qnrS1* in 5/6 of the *qnrS1*-positive strains. In K4-43, the presence of *Δres* and *orf259* was detected by PCR and confirmed by sequencing while the presence of *orf213* could only be expected according to gel electrophoresis of the PCR product yet it was not confirmed by sequencing since sequencing yielded bad results. In K38-15, a deletion of about 350 bp was detected in the *Δres* gene.

For the three strains, K40-79, K38-15, and K4-43, in which *bla*$_{\text{LAP-1}}$ and *pbp*-3 were absent, effort was done to link the *qnrS1* with the *bla*$_{\text{TEM}}$-containing transposon Tn3 (Kehrenberg *et al*. 2006) (figure 21). Primers targeting the *qnrS1* gene and the *tnpR*, and *bla*$_{\text{TEM}}$ genes were used for both PCR and sequencing. Using the (qnrS-3`-ext with tnpRF/tnpRF2) set of primers, a band of only 1300 bp was detected by gel electrophoresis of the PCR amplicons of the three isolates while the expected size of this PCR amplicon was > 3000 bp. The nucleotide sequence of these amplicons matched the *qnrS1-orfB* region (figure 21). No bands were detected with the (qnrS-3`-ext with TEM-R mod TW) set of primers.
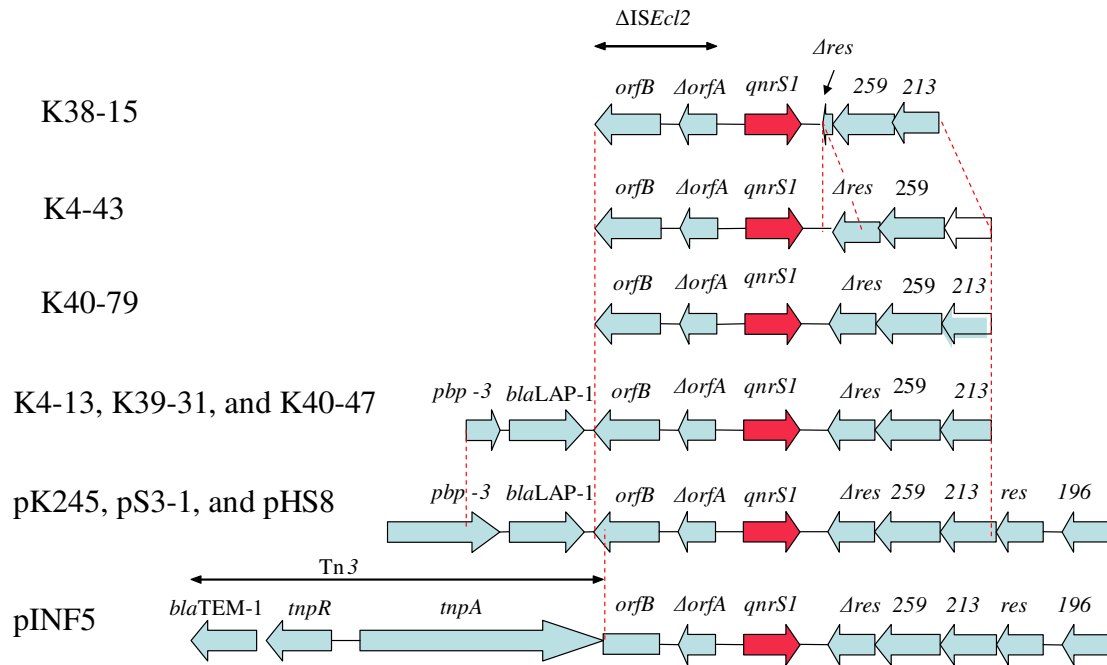
Figure 21. Schematic diagram comparing the *qnrS*-containing region found in the 6 *qnrS1*-positive strains; K38-15, K4-13, K4-43, K39-31, K40-47, and K40-79 with that in pS3-1 from *E. cloacae* (Poirel *et al*. 2007), and pINF5 from *S. enterica* serovar Infantis (Kehrenberg *et al*. 2006). Open reading frames are represented by arrows with the arrow head indicating the direction of transcription. The red vertical lines show regions of similarity.

5. *Discussion*

Resistance to antimicrobial agents can take place in four major ways; by altering drug targets, reducing drug accumulation, protecting drug targets, and enzymatic drug modification (McDermott *et al.* 2003). *Enterobacteriaceae* resist quinolones by all of these ways. While chromosomal mutations in genes coding for DNA gyrase and topoisomerase IV and genes coding for outer membrane and efflux proteins are mostly responsible for this resistance, plasmid-mediated quinolone resistance (PMQR) involving the Qnr proteins protecting DNA gyrase and topoisomerase IV from quinolones and the AAC(6´)-Ib-cr enzyme able to modify some fluoroquinolones has lately been described (Robicsek *et al.* 2006).

In this study, the aim was to evaluate the prevalence of *qnr* and *aac(6')-Ib-cr* genes among *E. coli* and *Klebsiella* spp. clinical isolates obtained from Norway and Sweden between 2003 and 2005 and showed resistance to nalidixic acid and/or reduced-susceptibility to ciprofloxacine. The clinical isolates were taken from five collections which can be sorted into two main groups; the first group (the Kronoberg and NORM collections) included consecutive clinical isolates representing the common bacterial population, while the second group contained clinical isolates picked from three collections at K-res (the *E. coli* AmpC, Ullevål ESBL, and Norwegian ESBL studies) representing a bacterial population with other resistance mechanisms.

Prevalence of the *qnr* genes

Overall, prevalence of the *qnr* genes 1.6 % (8/487) in the *E. coli* and *Klebsiella* spp. clinical isolates collected from Norway and Sweden and included in this study was low. This was well consistent with a study conducted in Denmark showing that only 1.63 % (2/122) of nalidixic acid-resistant *E. coli* isolates was *qnr*-positive (Cavaco *et al.* 2007). Low prevalence of *qnr* genes has also been reported from France and Canada. In France, the prevalence of *qnr* genes was 1.6 % (2/125) among ESBL-

producing *E. coli* and *Klebsiella* spp. isolates (Poirel *et al*. 2006 and Cattoir *et al*. 2007) while in Canada only about 1 % (5/550) of ciprofloxacin and/or tobramycin resistant *E. coli* and *Klebsiella* spp. isolates were *qnr*-positive (Pitout *et al*. 2008). Nevertheless, high prevalence has also been detected in other parts of the world such as Spain (5 %) (Lavilla *et al*. 2007), China (8 %) (Jiang *et al*. 2008) and the United States (15 %) (Robicsek *et al*. 2006). However, the comparison should be taken with caution since different criteria for selecting the bacterial isolates were used in these studies (appendix A, table 15). For instance, resistance to tobramycin was used as a selection criterion in the Spanish study which probably under-estimated the real prevalence (Lavilla *et al*. 2007).

In this study, the criteria used for selecting these strains included: (1) resistance to nalidixic acid (MIC > 16 μg/ml) and (2) resistance or reduced susceptibility to ciprofloxacin (MIC ≥ 0.125 μg/ml). These criteria were chosen since QnrA1, the first PMQR determinant to be discovered, increased the MIC of nalidixic acid to clinically resistant levels (Tran & Jacoby. 2002) and because a ciprofloxacin MIC of ≥ 0.125 μg/ml is the minimum expected for *Enterobacteriaceae* containing a *qnr* gene (Robicsek *et al*. 2006). However, it is noteworthy that several *qnr*-positive isolates have increasingly been detected in nalidixic acid-susceptible isolates (Cano *et al*. 2006, Cavaco *et al*. 2007, and Hopkins *et al*. 2007) and so the true prevalence of Qnr determinants could be underestimated in this study.

The *qnr* genes were detected in only 0.7 % (3/422) of the *E. coli* while in 7.7% (5/65) of the *Klebsiella* spp. clinical isolates. The higher prevalence of *qnr* genes in *Klebsiella* spp. isolates than in *E. coli* isolates was also noticed in other studies conducted in France (Poirel *et al*. 2006), USA (Robiscek *et al*. 2006), Spain (Lavilla *et al*. 2007), and recently China (Jiang *et al*. 2008). In France, for example, *qnr* was detected in 0.63 % (3/472) and 7 % (5/70) among the *E. coli* and *Klebsiella* spp. isolates, respectively.

qnrS and *qnrB* were detected in six and two isolates, respectively, while *qnrA* was not found in any of the isolates. The dominance of *qnrS* and *qnrB* in our strain collections is similar to other studies from Europe (Chen *et al*. 2006 and Poirel *et al*. 2006). On the contrary, *qnrA* gene was highly detected in a selected collection of blood culture

isolates of *Enterobacteriaceae* resistant to both ciprofloxacin and cefotaxime in UK (Corkill *et al*. 2006). Furthermore, *qnrA1* was the most prevalent *qnr* gene in a most recent Spanish study (Lavilla *et al*. 2008) where *qnrA1* was detected in 14 of 305 ESBL-producing enterobacterial isolates whereas only one *qnrS* and no *qnrB* were detected.

The amino acid and nucleotide sequences of all the *qnrS* genes, *qnrB* from K8-5, and *qnrB* from K2-78 were 100% identical to those of *qnrS1* (GenBank reference number DQ485529.1), *qnrB1* (GenBank reference number DQ777878.1), and *qnrB7* (GenBank reference number ABW03156.3), respectively. However, the *qnrB*-positive PCR products that were sequenced did not cover the whole gene therefore amino acid changes could be present outside this region (the amino acid alignments are presented in appendix B, figures 2, 3, 4, and 5).

Only two *qnrS1* genes were detected in ESBL-producers, according to their phenotypic profile and to the Norwegian ESBL study data (Tofteland *et al*. 2007), while the other four *qnrS1* genes were detected in non-ESBL-producing clinical isolates, according to their phenotypic profile. This was not unanticipated since similar finding was reported in studies from France and Korea where the *qnrS1* gene was mostly identified in non-ESBL strains (Poirel *et al*. 2006 and Park *et al*. 2007, respectively). Furthermore, although the *qnrS1* gene and genes encoding for extended-spectrum β-lactamases (ESBLs) have frequently been associated in same clinical isolates, they have been identified on different plasmids (Chen *et al*. 2006 and Hopkins *et al*. 2007). On the other hand, the detection of QnrB determinants in two ESBL-positive strains was also highly expected given that co-presence of *qnrB* and ESBL genes on same plasmids has been regularly reported (Jacoby *et al*. 2006, Robicsek *et al*. 2006, Cattoir *et al*. 2007, and Pai *et al*. 2007).

Interestingly, three *qnrS1*- and one *qnrB7*-positive isolates were found in nalidixic acid-susceptible isolates. This has been found in other studies as mentioned above. The only reduced-susceptibility to quinolones conferred by *qnr* genes raises the concern that these genes could spread insidiously. Subsequently, a substantial population of clinical *Enterobacteriaceae* on the threshold of high-level resistance

would continue to be treated and exposed to quinolones (Gay *et al*. 2006). Thus, although the *qnr* genes might not allow a population of bacteria to survive in the presence of a quinolone, they enhance the selection of resistant mutants from the population (Robicsek *et al*. 2006).

Prevalence of the *aac(6`)-Ib-cr* gene

The prevalence of *aac(6`)-Ib-cr* among the selected *E. coli* and *Klebsiella* spp. clinical isolates was 14.1 % (69/487). This finding was comparable to or lower than what was been found in other studies; the prevalence of *aac(6`)-Ib-cr* was 11.3 % (62/549) among ciprofloxacin- and/or tobramycin-resistant *E. coli* and *Klebsiella spp*. clinical isolates from Canada (Pitout *et al*. 2008) and 9.9 % (36/365) among ESBL-producing *E. coli* and *K. pnumoniae* isolates from six provinces in China (Jiang *et al*. 2008) while it was 51% among ciprofloxacin-resistant clinical isolates of *E. coli* isolated from Shanghai, China (Robicsek *et al*. 2006).

In this study 92 % of the *aac(6`)-Ib* were of the *aac(6')-Ib-cr* variant. This is also observed in many other studies where 58 %, 84 %, and 100% of the *aac(6`)-Ib* were of the *aac(6')-Ib-cr* variant (Jiang *et al*. 2008, Robicsek *et al*. 2006, and Samuelsen *et al*. 2007). The high percentage of this variant mostly reflect a long-time presence of under-screened *aac(6')-Ib-cr*. Interestingly, two isolates harbored both the wild type *aac(6')-Ib* and the *-cr* variant. This was similar to what been found in *E. coli* and *Klebsiella* spp. clinical isolates from Russia where four isolates were identified to hold both the *aac(6')-Ib-cr* variant and the *aac(6')-Ib* wild-type (Samuelsen *et al*. 2007).

While *aac(6')-Ib-cr* was found in only 3% (12/400) from the two collections representing the common bacterial population, it was detected in 48.7 % (57/117) of clinical isolates from the *E. coli* AmpC and ESBL studies. The high association between the PMQR genes (both the *qnr* and *aac(6`)-Ib-cr* genes) and genes encoding for ESBLs or AmpC β-lactams may somewhat explain the prevalence of enterobacterial clinical isolates resistant to both flouroquinolones and β-lactams,

mostly due to the co-selection of these bacteria by use of either agent alone (Gay *et al*. 2006). Furthermore, this association could be clinically significant since the therapeutic options for treatment of the increasingly encountered ciprofloxacin- and β-lactam-resistant *E. coli* are limited.

It is worth mentioning that *aac(6')-Ib-cr* variant was detected more commonly among the *qnr*-positive than *qnr*-negative isolates; it was detected in 50 % (4/8) and 14.8 % (71/479) of the *qnr*-positive and the *qnr*-negative isolates, respectively. A comparable prevalence was reported from China where the *aac(6')-Ib-cr* variant was 55.2 % among *qnr*-positive and only 6 % among *qnr*-negative isolates (Jiang *et al*. 2008). This could be explained by the co-existence of the *qnr* and *aac(6`)-Ib-cr* genes on the same plasmid (Jiang *et al*. 2008). Nevertheless, this co-existence has been reported for *qnrA* and *qnrB* genes (Jiang *et al*. 2008 and Quiroga *et al*. 2007, respectively) while in this study *qnrS1* was the most prevalent *qnr* gene.

Resistance profiles of the *qnr*-positive isolates

Four of the *qnr*-positive isolates were resistant to antimicrobials of four different classes; ß-lactams, aminoglycosides, quinolones and sulfonamides and were classified as multi-drug resistant isolates (Sahm *et al*. 2001). The prevalence of such multi-drug resistant clinical isolates could be due to either the spread of a successful single or few clonal groups (Manges *et al*. 2001) or the presence of transferable R-plasmids in these isolates (Shehabi *et al*. 2004). In this study, transferable R-plasmids were detected in 5/8 of the *qnr*-positive isolates suggesting that plasmids are responsible for the spread. Although the clonality of the isolates were not investigated, it is unlikely that the isolates are clonal since they are from different strain collections and from two countries.

The ESBL-producing clinical isolate K4-43 was resistant to the oxyimino-cephalosporins (cefuroxime, cefotaxime, ceftazidime, and cefepime) but was furthermore resistant to cephamycins (cefoxitin), amoxicillin/clavulanic acid, and

piperacillin/tazobactam as well. This finding was explained by the high impermeability of K4-43 according to the Norwegian ESBL study data.

The four *aac(6`)-Ib-cr*-positive clinical isolates (K4-13, K4-43, K8-5, and K40-79) showed reduced susceptibility or resistance to amikacin and tobramycin. This can be clearly clarified by the fact the AAC(6`)-Ib-cr variant is able to modify these aminoglycosides though to a less degree compared with the AAC(6`)-Ib wild-type gene (Robiscek *et al*. 2006). The *aac(6`)-Ib-cr*-positive clinical isolates were in addition resistant to trimethoprim, and trimethoprim-sulfmethoxazole. The facts that *aac(6`)-Ib-cr* has mostly been found in complex integrons (Wang *et al*. 2003) and that the *3′-CS* region of such integrons contains the *sul1* gene encoding resistance to sulphonamides (Bennett. 1999) explain the associated resistant to trimethoprim, and trimethoprim-sulfmethoxazole. The considerable association between *aac(6`)-Ib-cr* and gentamycin resistance (3/4 of the isolates) was unexpected finding since *aac(6`)-Ib-cr* does not confer resistance to gentamycin (Robicsek *et al*. 2006). The same unanticipated finding was reported from USA (Park *et al*. 2006). The explanation could simply be that the isolates harbor other resistance determinants affecting gentamycin.

Analysis of the quinolone resistance determining regions (QRDRs)

PMQR determinants have always been reported to confer only low level of resistance or just reduced susceptibility to nalidixic acid and fluoroquinolones (Robicesk *et al*. 2006). Whereas, the most important mechanism for acquiring high levels of quinolone resistance has constantly developed by the acquisition of chromosomal point mutations in the QRDRs of target genes particularly *gyrA* and *parC* (Fendukly *et al*. 2003).

The presence of double mutations in the *gyrA* gene has been reported to be responsible for resistance to quinolones (Fendukly *et al*. 2003). In addition, the presence of single or double mutations in the *parC* gene seems to play a complementary role in increasing this quinolone resistance (Fendukly *et al*. 2003). In

this study, two *qnr*-positive *E. coli* clinical isolates (K4-43 and k40-79) were found to have high level of quinolone resistance. Double mutations L83S and N87D in the *gyrA* gene with the addition of a single mutation I80S in the *parC* gene were detected in both these strains.

For K8-5, the *qnr*-positive *K. pneumoniae* clinical isolate with high level of quinolone resistance, a single mutation L83S in the *gyrA* gene and a single mutation I80S in the *parC* gene were detected. This finding is in accordance with previously published report where only one or two mutations in the *gyrA* gene are sufficient to explain the quinolone resistance (Fendukly *et al*. 2003). Once more, the presence of mutations in the *parC* gene seems to play a complementary role in increasing this quinolone resistance (Fendukly *et al*. 2003).

No mutations were detected in the QRDRs of *gyrA* and *parC* genes neither in K40-47 which was a *qnr*-positive quinolone-resistant *K. pneumoniae* clinical isolate but showed lower MICs of nalidixic acid (64 μg/ml) and ciprofloxacin (4 μg/ml) than the three isolates mentioned above nor in the four nalidixic acid-susceptible clinical isolates (K38-15, K2-78, K4-13, and K39-31). For isolate K40-47, the low-level resistance to nalidixic acid could be due to the presence of mutations in QRDRs of the *gyrB* or *parE* genes though these mutations are much less frequent and less significant than those in the QRDRs of *gyrA* and *parC* genes (Everett *et al*. 1996). In addition, decreased accumulation of quinolone could be the basis for this quinolone resistance (Ruiz. 2003). Decreased quinolone accumulation is generally associated with (1) alterations in membrane permeability usually due decreased expression of porins and/or (2) the over-expression of efflux systems capable of pumping out quinolones. This finding was encountered in previously published study conducted by Fendukly *et al*. where a *K. pneumoniae* clinical isolate with MIC of 128 μg/ml and 0.38 μg/ml for nalidixic acid and ciprofloxacin, respectively, was lacking any mutations in the *gyrA* and *parC* genes (Fendukly *et al*. 2003).

Transfer of quinolone resistance

Success in transferring the *qnrS* and *qnrB* genes by transconjugation has been frequently reported (Hata *et al*. 2005 and Hu *et al*. 2008). In addition, transfer of these *qnr* genes can also be performed by transformation (Lavilla *et al*. 2007). On the other hand, both of *qnrS* and *qnrB* genes have also been detected on non-transconjugative plasmids (Cavaco *et al*. 2007 and Cattoir *et al*. 2007).

In this study, transfer of low-level quinolone resistance conferred by *qnr* genes was successful from 5/8 of the *qnr*-positive isolates. Three *qnrS1* genes were transferred by transconjugation, while one *qnrS1* and one *qnrB7* genes were transferred by transformation. Additionally, the co-transfer of the *aac(6`)-Ib-cr* gene was also detected from 2/4 of the isolates. The success in transferring *qnr* genes using 100 mg/L ampicillin as selection pressure indicates that both the *qnr* gene and a ß-lactamase gene are carried on same plasmid. Furthermore, resistance to the β-lactams, cefuroxime, cefotaxime, and ceftazidime was horizontally transferred from the *qnrB7*-positive isolate (K2-78) but not from the *qnrS1*-positive isolates. This finding was not unpredicted since the co-presence of *qnrB* and ESBL-encoding genes on same plasmids has been regularly reported (Jacoby *et al*. 2006, Robicsek *et al*. 2006, Cattoir *et al*. 2007, and Pai *et al*. 2007) while, in contrast, the *qnrS1* gene and genes encoding for extended-spectrum β-lactamases (ESBLs) have frequently been identified on different plasmids (Chen *et al*. 2006 and Hopkins *et al*. 2007).

The co-transfer of *aac(6`)-Ib-cr* again imply that this gene could be located at the same plasmid with the *qnr* and ß-lactamase genes. In the study conducted by Jiang *et al*, southern hybridization indicated that *qnrA*, *aac(6`)-Ib-cr*, and ESBL-encoding genes were always located on the same plasmids (Jiang *et al*. 2008). However, the association between these genes on same plasmids was not confirmed in this study.

On the other hand, two *qnrS1* genes and one *qnrB1* gene were neither transconjugable nor transformable. The failure in transferring these *qnr* genes indicates that they are located on non-transferable plasmid. Nevertheless, a chromosomal location for *qnr* genes has been suggested by some studies (Rodriguez-Martinez *et al*. 2006 and

Cavaco *et al*. 2007). This could be the case for K39-31 where repeated transconjugation and transformation experiments yielded negative results though different antibiotics were used for selection and the *qnrS1* probe could be hybridized with chromosomal DNA (figure 20).

It has been reported that transfer of the *qnrS* or *qnrB* gene resulted in a 3 and 8 folds increase in the MIC of nalidixic acid and between 8 to 62.5 folds in the MIC of ciprofloxacin (Chen *et al*. 2006, Gay *et al*. 2006, Kehrenberg *et al*. 2006, Poirel *et al*. 2006, Hu *et al*. 2008, Jacoby *et al*. 2006, Pai *et al*. 2007, Kehrenberg *et al*. 2007). Comparable results were detected in this study where the *qnr* genes increased the MIC for nalidixic acid by 2 to 4 folds and for ciprofloxacin by 10 to 125 folds. *qnrS* conferred higher fluoroquinolone MICs than *qnrB* (table 11). This has also been observed in other studies (Cattoir *et al*. 2007 and Hu *et al*. 2008).

The differences in resistance levels to qiunolones among transconjugants have regularly been reported and could be caused by different gene expression, or different gene copy number (Robicsek *et al*. 2006). Different levels of expression of *qnrA* gene among transconjugants have been observed for the *qnrA* gene (Rodriguez-Martinez *et al*. 2006). The higher ciprofloxacin-resistance levels among transconjugants from K4-13 (table 10) can be explained by the co-transfer of the *aac(6`)-Ib-cr* gene. The presence of *aac(6`)-Ib-cr* on certain *qnrA* plasmids from clinical *E. coli* collected in Shanghai provided about four-fold further higher levels of ciprofloxacin resistance (Robicsek *et al*. 2006). Furthermore, the co-transfer of *aac(6`)-Ib-cr* could be the explanation for the increase in MIC of amikacin (2 folds), and tobramycin (> 10 folds) among the transconjugants from K4-13 (table 10). However, the *aac(6`)-Ib-cr* gene was also co-transferred from K40-79 as a donor but the increase in ciprofloxacin MIC was comparable and there was no increase in MIC of amikacin or tobramycin. The only explanation could be that *aac(6`)-Ib-cr* was transferred but not expressed.

Plasmid analysis

Analyzing plasmid DNA by doing plasmid isolation followed by gel electrophoresis has been widely used (Corkill *et al*. 2005, Jiang *et al*. 2008). In this study this method did not give satisfying results. Only two plasmids were detected in K4-43 and K40-47, one plasmid in K38-15 and K40-79, and no plasmid DNA was detected for the other four clinical isolates and all the transconjugants/transformants. For *E. coli* strain NCTC 50192, only three bands representing plasmids of 63.5, 36, and 7 kb in size were detected while the band representing a 148 kb plasmid was missed. For *E. coli* strain NCTC 50193, only two bands representing plasmids of 5.2, and 1.8 kb in size were detected while 6 bands representing plasmids of 48.3, 7.8, 4.5, 3.3, 2.6, and 2.3 kb in size were all not detected. The absence of those bands in the gel photo in this study was in line with the finding of very week bands in gel photos from previously published studies (Woodford *et al*. 1994 and Cattoir *et al*. 2007). Furthermore, it was reported in another study from Spain that larger plasmids could not be visualized by using this method while they were detected by using the S1-PFGE method (Martinez *et al*. 2005).

Plasmid analysis was also performed by the S1-PFGE method where the *qnr*-positive clinical isolates, transconjugants, and transformants contained one to four plasmids ranging in size from 30 kb to ≥ 195 kb. By southern blotting and hybridization with PCR-generated a probe specific for *qnrS1,* the *qnrS1* genes were located on at least three different plasmid backbones according to the diversity in their sizes (140 kb, 175 kb, and 195 kb) (figure 20) which implies a successful inter-plasmid dissemination of the *qnrS1* gene itself. The variability in the molecular size of the *qnr*-carrying plasmids has frequently been reported (Veldman *et al*. 2008 and Hopkins *et al*. 2007)

PCR-based plasmid typing

The IncN replicon type was detected in *qnrS*-positive plasmid DNA from K38-15, TC38-15, K4-13, TC4-13, K40-79, TC40-79, K4-43, and K40-47. In this respect, *qnrS1*-positive plasmids isolated from *Salmonella* isolates from the UK (Hopkins *et al*. 2007) and from the Netherlands (Veldman *et al*. 2008) also carried the IncN replicon type. Furthermore, the *qnrS1-* positive plasmid pINF5 from *Salmonella enterica* from Germany was hypothesized to have a pMUR050-like ancestor which is an IncN plasmid (Hata *et al*. 2005). However, the association between *qnrS1* and IncN on same plasmids needs to be confirmed by hybridization experiments using an IncN probe.

The replicon type FIIs was assigned to the *qnrB7*-positive plasmid DNA from K2-78 given that it was the only type detected by PCR. The *qnrB1*-positive plasmid DNA from K8-5 was not positive for any of the 18 replicon types. Several plasmids isolated from *Salmonella enterica* from the UK as well as the pAH0376 plasmid from *Shigella flexneri* from China were also negative for all these 18 replicons (Hopkins *et al*.2007). However, those isolates were all *qnrS1*-positive. There is no explanation why replicon type F was present in plasmid DNA from four transconjugants/transformants while it was detected in only two donors.

Determination of the genetic environment of *qnrS1*

The nucleotide sequences of the region closely surrounding *qnrS1* in all the six *qnrS1*-positive clinical isolates detected in this study showed ≥ 99 % identity to the *qnrS1*-containing region in plasmids pINF5 (AM234722.1) (Kehrenberg *et al*. 2006), pK245 (DQ449578.1) (Chen *et al*. 2006), pS3-1 (Poirel *et al*. 2007), pHS8 (EF683584.1) (Hu *et al*. 2008) except that a 350 bp deletion was detected in K38-15. The area was of about 4200 bp in size and contained ΔIS*Ecl2* (*orfB-ΔorfA*), *qnrS1*, *Δres*, *orf259*, and *orf213* (figure 21). An additional 99 % nucleotide identity was detected between the region further downstream to *qnrS1* in K4-13, K39-31, and K40-47 and the parallel region in plasmids pK245 (DQ 449578.1) (Chen *et al*. 2006), pS3-1 (Poirel *et al*.

2007), pHS8 (EF 683584.1) (Hu *et al.* 2008). The area was of about 1150 bp in size and contained *pbp*-3, and *bla*$_{LAP-1}$ (figure 21).

The similarity of the *qnrS1*-containing area in plasmids from this study and from several other studies indicates a potential common origin of the region as a unit. This region could be contained in a composite transposon bounded by two flanking IS26 elements, which explains its mobility (Chen *et al.* 2006). On the other hand, the universal association between *qnrS1* and the ΔIS*Ecl2* could play a role in the dissemination of this gene itself probably by mediating an inter-plasmid insertional or recombinational genetic event. However, a recombination event appears more likely sine the insertion target site of the ΔIS*Ecl2* element was detected upstream to *qnrS1* suggesting that its insertion had occurred individually (Poirel *et al.* 2006)

For the three strains, K40-79, K38-15, and K4-43, in which *bla*$_{LAP-1}$ and *pbp*-3 were absent, attempts were done to link the *qnrS1* with Tn3 (Kehrenberg *et al.* 2006). A band of 1300 bp was detected in the PCR amplicons of all the three isolates but the nucleotide sequence was again matching the *qnrS1-orfB* region. By looking in deep it was found that the *orfB* of the ΔIS*Ecl2* also encodes a resolvase enzyme so similarity between its DNA sequence and *tnpR* DNA sequence explains why the tnpRF/tnpRF2 primers annealed with *orfB* instead of *tnpR* resulting in those 1300 bp bands. However, no band was detected with the (qnrS-3`-ext with TEM-R mod TW) set of primers and *bla*$_{TEM-1}$. First of all, this could be because conventional PCR amplifications were performed and that the predicted distance between *qnrS1* and *bla*$_{TEM-1}$ is > 5000 bp (Kehrenberg *et al.* 2006) and so using long-range PCR kits would have been more appropriate. Secondly, this finding may simply suggest that Tn*3* is absent in these 3 isolates. Although most studies on the genetic environment of *qnrS1* have detected either (*bla*$_{LAP-1}$ with *pbp*-3) or *bla*$_{TEM-1}$-containing Tn*3* downstream *qnrS1*, their absence has recently been reported in plasmid TPqnrS-1a isolated from a multi-resistant *Salmonella Typhimurium* strain from the UK (Kehrenberg *et al.* 2007).

Concluding remarks

The prevalence of *qnr* (1.6%) and *aac(6')-Ib-cr* (14.1%) determinants among Norwegian and Swedish clinical isolates of *E. coli* and *Klebsiella* spp. with resistance to nalidixic acid and/or resistance/reduced susceptibility to ciprofloxacin was low. The *qnr*-genes were more prevalent in *Klebsiella* spp. (7.7%) than in *E. coli* (0.7%).

While *qnrS1* was detected in six isolates and was the most common *qnr* gene, each of *qnrB1* and *qnrB7* was detected in only one isolate. The *qnrS1* genes were located on at least three different plasmid backbones; two transconjugative plasmids of about 140 kb and 175 kb and one non-transconjugative plasmid of about 195 kb. Nevertheless, the *qnrS1*-containing area contand matching structures in the six isolates.

The global distribution of the *qnr* genes, their presence in several enterobacterial species, and the identification of several *qnr* variants suggest that they have had a substantial evolutionary history. The presence of *qnrS1* gene in several enterobacterial species also indicates a successful dissemination of the *qnrS1*-carrying plasmids. Furthermore, the presence of *qnrS1* gene on different plasmid backbones implies a successful dissemination of the *qnrS1* gene itself which could result from association with insertion sequence elements.

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Appendix A

Table 1. All *qnrA*, *qnrB*, and *qnrS* variants have been detected so far.

qnr gene	Amino acid comparison	Bacterial isolate	Location	GenBank accession number	Authors
qnrA1	The first identified *qnr* gene	*K. pneumoniae*	USA	AY070235	Martinez-Martinez *et al*. 1998 Tran & Jacoby. 2002
qnrA2	Few amino acid substitutions from *qnrA1*	*K. oxytoca*	China	AY675584	Li *et al*. Unpublished
qnrA3 (Natural reservoir of *qnrA*)	Few amino acid substitutions from *qnrA1*	*S. algae*	France	DQ058661	Poirel *et al*. 2005
qnrA4 (Natural reservoir of *qnrA*)	Few amino acid substitutions from *qnrA1*	*S. algae*	France	DQ058662	Poirel *et al*. 2005
qnrA5 (Natural reservoir of *qnrA*)	Few amino acid substitutions from *qnrA1*	*S. algae*	France	DQ058663	Poirel *et al*. 2005
qnrA6	Few amino acid substitutions from *qnrA1*	*P. mirabilis*	France	DQ151889	Cambau *et al*. 2006
qnrS1	59% amino acid identity to *qnrA1*	*S. flexneri*	Japan	AB187515	Hata *et al*. 2005
qnrS2	92% amino acid identity to *qnrS1*	Plasmid pGNB2	Germany	DQ460733	Bonemann *et al*. 2006
	91.3% amino acid identity to *qnrS1*	*S. enterica* serotype Anatum	USA	DQ485530	Gay *et al*. 2006
qnrVS1 (Natural reservoir of *qnrS*)	83.9 % and 87.6 % amino acid identity to *qnrS1* and *qnrS2*, respectively.	*V. splendidus*	USA	EAP95542 (EMBL-Bank)	Cattoir *et al*. 2007
qnrVS2 (Natural reservoir of *qnrS*)	83.1 % and 87.1 % amino acid identity to *qnrS1* and *qnrS2*, respectively. 4 amino acid substitutions from *qnrVS1*	*Vibro* spp.	France	EAQ55748 (EMBL-Bank)	Cattoir *et al*. 2007
qnrB1	40% amino acid identity to *qnrA1*	*K. pneumoniae*	India/USA	DQ351241	Jacoby *et al*. 2006
qnrB2	5 amino acid substitutions from *qnrB1*	*C. koseri*	USA	DQ351242	Jacoby *et al*. 2006
qnrB3	99% amino acid identity to *qnrB1*	*E. coli*	USA	DQ303920	Robicsek *et al*. 2006
qnrB4	94% amino acid identity to *qnrB1*	*E. coli*	USA	DQ303921	Robicsek *et al*. 2006
qnrB5	95.6% amino acid identity to *qnrB1*	*S. enterica* serotype Berta	USA	DQ303919	Gay *et al*. 2006
qnrB6	Similar to *qnrB3*	*E. aerogenes*	China	EF517946	Ma *et al*. unpublished
qnrB7	97% amino acid identity to *qnrB1*	*E. cloacae*	France	EU043311	Cattoir *et al*. 2007
qnrB8	96% amino acid identity to *qnrB6*	*C. freundii*	France	EU043312	Cattoir *et al*. 2007
qnrB9	Not-determined	*C. freundii*	France	EF653270	Rodriguez-Zulueta *et al*. unpublished

Table 1. All *qnrA*, *qnrB*, and *qnrS* variants have been detected so far, cont.

qnr gene	Amino acid comparison	Bacterial isolate	Location	GenBank accession number	Authors
qnrB10	98% amino acid identity to *qnrB5*	*C. freundii*	Argentina	DQ631414	Quiroga *et al.* 2007
	Not-determined	*C. freundii*	Spain	EU136182	Sanchez-Cespedes *et al.* unpublished
qnrB11	Not-determined	*C. freundii*	Spain	EU136183	Sanchez-Cespedes *et al.* unpublished
qnrB12	98.9% amino acid identity to *qnrB9*	*C. werkmanii*	Germany	AM774474	Kehrenberg *et al.* 2008
qnrB13	Not-determined	*C. freundii*	Korea	EU273756	Tamang *et al.* unpublished
qnrB14	Not-determined	*C. freundii*	China	EU325573	Wang et al. unpublished
qnrB15	Not-determined	*C. freundii*	Korea	EU302865	Tamang *et al.* unpublished
qnrB16	Not-determined	*C. freundii*	Spain	AM919398	Gonzalez-Lopez *et al.* unpublished
qnrB17	Not-determined	*C. freundii*	Spain	AM919399	Gonzalez-Lopez *et al.* unpublished

Table 2. Clinical MIC and zone breakpoints

Antibiotic	Reference	Clinical MIC breakpoints (µg/ml)	
		Susceptible	Resistant
Nalidixic acid	NORM*	≤ 16	> 16
Ciprofloxacin	EUCAST**	≤ 0.5	> 1
Ofloxacin	EUCAST	≤ 0.5	> 1
Norfloxacin	EUCAST	≤ 0.5	> 1
Levofloxacin	EUCAST	≤ 1	> 2
Moxifloxacin	EUCAST	≤ 0.5	> 1
Amikacin	EUCAST	≤ 8	> 16
Tobramycin	EUCAST	≤ 2	> 4
Gentamicin	EUCAST	≤ 2	> 4
Ampicillin	BSAC***	≤ 16	> 16
Amoxicillin/clavulanic acid	BSAC	≤ 16	> 16
Piperacillin	EUCAST	≤ 8	> 16
Piperacillin/tazobactam	EUCAST	≤ 8	> 16
Cefuroxime	EUCAST	≤ 8	> 8
Cetriaxone	EUCAST	≤ 1	> 2
Ceftazidime	EUCAST	≤ 1	> 8
Cefotaxime	EUCAST	≤ 1	> 2
Cefoxitin	BSAC	≤ 8	> 8
Meropenem	EUCAST	≤ 2	> 8
Imipenem	EUCAST	≤ 2	> 8
Trimethoprim	EUCAST	≤ 2	> 4
Trimethoprim sulfamethoxazole	EUCAST	≤ 2	> 4
Antibiotic	**Reference**	**Clinical zone breakpoints (mm)**	
		Susceptible	Resistant
Nalidixic acid 30 µg	SRAG****	> 20	≤16
Ciprofloxacin 10 µg	SRAG	> 20	≤16

* NWGA (Norwegian Working Group on Antibiotics), according to the NORM/NORM-VET 2006 report, ** EUCAST (the European Committee on Antimicrobial Susceptibility Testing) http://www.escmid.org/sites/index_f.aspx?par=2.4, *** BSAC (the British Society for Antimicrobial Chemotherapy) http://www.bsac.org.uk/_db/_documents/version_6.1.pdf, **** SRAG (the Swedish Reference Group for Antibiotics) http://www.srga.org/ZONTAB/zontab2b.htm

Table 3. Bacterial isolates of the Kronoberg collection.

Nr.	Ref. nr	Lab. nr	Species	Material	NAL profile*	CIP profile*
1	K37-01	04-64719	*E. coli*	urine	>256 R	>32 R
2	K37-02	04-35036	*E. coli*	urine	64 R	0.064 S
3	K37-03	04-67291	*E. coli*	urine	>256 R	0.25 S
4	K37-04	04-74539	*E. coli*	urine	>256 R	0.5 S
5	K37-05	04-74492	*E. coli*	urine	>256 R	>32 R
6	K37-06	04-77210	*E. coli*	urine	>256 R	>32 R
7	K37-07	05-5648	*E. coli*	urine	>256 R	>32 R
8	K37-08	05-4791	*E. coli*	urine	>256 R	0.125 S
9	K37-09	04-73969	*E. coli*	urine	128 R	0.25 S
10	K37-10	04-69398	*E. coli*	urine	>256 R	16 R
11	K37-11	04-75431	*E. coli*	urine	>256 R	>32 R
12	K37-12	04-74872	*E. coli*	urine	>256 R	32 R
13	K37-13	04-73296	*E. coli*	urine	>256 R	0.25 S
14	K37-14	04-52450	*E. coli*	urine	>256 R	>32 R
15	K37-15	04-56221	*E. coli*	urine	>256 R	0.25 S
16	K37-16	04-58589	*E. coli*	urine	128 R	0.25 S
17	K37-17	04-35296	*E. coli*	urine	>256 R	0.25 S
18	K37-18	04-37984	*E. coli*	urine	>256 R	0.25 S
19	K37-19	04-41416	*E. coli*	urine	>256 R	>32 R
20	K37-20	04-45097	*E. coli*	urine	>256 R	8 R
21	K37-21	04-48663	*E. coli*	urine	>256 R	>32 R
22	K37-22	04-61100	*E. coli*	urine	>256 R	32 R
23	K37-23	04-64385	*E. coli*	urine	>256 R	0.25 S
24	K37-24	04-34725	*E. coli*	urine	>256 R	0.25 S
25	K37-25	04-37622	*E. coli*	urine	Not determined	Not determined
26	K37-26	04-41151	*E. coli*	urine	>256 R	>32 R
27	K37-27	04-43618	*E. coli*	urine	32 R	0.064 S
28	K37-28	04-48586	*E. coli*	urine	128 R	0.25 S
29	K37-29	04-53744	*E. coli*	urine	>256 R	0.25 S
30	K37-30	04-56355	*E. coli*	urine	>256 R	>32 R
31	K37-31	04-58593	*E. coli*	urine	>256 R	>32 R
32	K37-32	04-61103	*E. coli*	urine	64 R	0.125 S
33	K37-33	04-78102	*E. coli*	urine	>256 R	>32 R
34	K37-34	04-77833	*E. coli*	urine	>256 R	1 I
35	K37-35	04-77588	*E. coli*	urine	>256 R	>32 R
36	K37-36	04-77275	*E. coli*	urine	>256 R	>32 R
37	K37-37	04-69026	*E. coli*	urine	>256 R	>32 R
38	K37-38	04-68894	*E. coli*	urine	Not determined	Not determined
39	K37-39	04-67984	*E. coli*	urine	>256 R	0.25 S
40	K37-40	04-37909	*E. coli*	urine	>256 R	>32 R
41	K37-41	04-41351	*E. coli*	urine	>256 R	>32 R
42	K37-42	04-43810	*E. coli*	urine	128 R	0.25 S
43	K37-43	04-48644	*E. coli*	urine	>256 R	0.5 S
44	K37-44	04-54371	*E. coli*	urine	256 R	0.125 S
45	K37-45	04-57421	*E. coli*	urine	>256 R	>32 R
46	K37-46	04-59168	*E. coli*	urine	>256 R	16 R
47	K37-47	04-61558	*E. coli*	urine	32 R	0.125 S
48	K37-48	04-65082	*E. coli*	urine	128 R	0.125 S
49	K37-49	04-39413	*E. coli*	urine	Not determined	Not determined
50	K37-50	04-42314	*E. coli*	urine	>256 R	2 R
51	K37-51	04-45827	*E. coli*	urine	64 R	0.125 S
52	K37-52	04-50457	*E. coli*	urine	>256 R	>32 R
53	K37-53	04-55415	*E. coli*	urine	>256 R	>32 R
54	K37-54	04-57880	*E. coli*	urine	>256 R	>32 R
55	K37-55	04-59861	*E. coli*	urine	128 R	0.125 S

Table 3. Bacterial isolates of the Kronoberg collection, cont.

Nr.	Ref. nr	Lab. nr	Species	Material	NAL profile*	CIP profile*
56	K37-56	04-63391	*E. coli*	urine	>256 R	16 S
57	K37-57	04-33244	*E. coli*	urine	128 R	0.125 S
58	K37-58	04-36171	*E. coli*	urine	>256 R	0.5 S
59	K37-59	04-39850	*E. coli*	urine	>256 R	0.25 S
60	K37-60	04-42645	*E. coli*	urine	256 R	0.25 S
61	K37-61	04-46789	*E. coli*	urine	>256 R	0.25 S
62	K37-62	04-51866	*E. coli*	urine	64 R	0.125 S
63	K37-63	04-55463	*E. coli*	urine	>256 R	0.25 S
64	K37-64	04-48328	*E. coli*	urine	>256 R	>32 R
65	K37-65	04-78174	*E. coli*	urine	>256 R	0.25 S
66	K37-66	04-78145	*E. coli*	urine	>256 R	4 R
67	K37-67	04-43417	*E. coli*	urine	128 R	0.25 S
68	K37-68	04-40350	*E. coli*	urine	128 R	0.064 S
69	K37-69	04-37147	*E. coli*	urine	>256 R	0.25 S
70	K37-70	04-34147	*E. coli*	urine	>256 R	>32 R
71	K37-71	04-32611	*E. coli*	urine	>256 R	>32 R
72	K37-72	04-35964	*E. coli*	urine	>256 R	16 R
73	K37-73	04-59170	*E. coli*	urine	64 R	0.125 S
74	K37-74	04-61779	*E. coli*	urine	>256 R	>32 R
75	K37-75	04-65529	*E. coli*	urine	32 R	0.125 S
76	K37-76	04-35340	*E. coli*	urine	128 R	0.125 S
77	K37-77	04-39100	*E. coli*	urine	>256 R	1 I
78	K37-78	04-41681	*E. coli*	urine	>256 R	1 I
79	K37-79	04-45139	*E. coli*	urine	>256 R	0.25 S
80	K37-80	04-49504	*E. coli*	urine	>256 R	>32 R
81	K37-81	04-54549	*E. coli*	urine	256 R	0.5 S
82	K38-01	04-57577	*E. coli*	urine	>256 R	>32 R
83	K38-02	04-59400	*E. coli*	urine	>256 R	16 R
84	K38-03	04-61781	*E. coli*	urine	>256 R	0.25 S
85	K38-04	04-65561	*E. coli*	urine	64 R	0.25 S
86	K38-05	04-52017	*E. coli*	urine	32 R	0.125 S
87	K38-06	04-47745	*E. coli*	urine	>256 R	0.25 S
88	K38-07	04-42672	*E. coli*	urine	>256 R	0.25 S
89	K38-08	04-39856	*E. coli*	urine	>256 R	0.25 S
90	K38-09	04-36547	*E. coli*	urine	>256 R	0.5 S
91	K38-10	04-54383	*E. coli*	urine	64 R	0.25 S
92	K38-11	04-57573	*E. coli*	urine	>256 R	>32 R
93	K38-12	04-58249	*E. coli*	urine	64 R	0.125 S
94	K38-13	04-41791	*E. coli*	urine	>256 R	>32 R
95	K38-14	04-45560	*E. coli*	urine	>256 R	0.25 S
96	K38-15	04-35623	*E. coli*	urine	16 S	0.5 S
97	K38-16	04-39396	*E. coli*	urine	256 R	0.5 S
98	K38-17	04-59461	*E. coli*	urine	32 R	0.064 S
99	K38-18	04-57824	*E. coli*	urine	>256 R	>32 R
100	K38-19	04-54826	*E. coli*	urine	>256 R	32 R
101	K38-20	04-49747	*E. coli*	urine	>256 R	0.5 S
102	K38-21	04-58203	*E. coli*	urine	>256 R	0.25 S
103	K38-22	04-55469	*E. coli*	urine	>256 R	0.25 S
104	K38-23	04-64075	*E. coli*	urine	>256 R	>32 R
105	K38-24	04-60150	*E. coli*	urine	64 R	0.064 S
106	K38-25	04-55841	*E. coli*	urine	>256 R	0.25 S
107	K38-26	04-52374	*E. coli*	urine	>256 R	>32 R
108	K38-27	04-48326	*E. coli*	urine	128 R	0.125 S
109	K38-28	04-43365	*E. coli*	urine	>256 R	8 R

Table 3. Bacterial isolates of the Kronoberg collection, cont.

Nr.	Ref. nr	Lab. nr	Species	Material	NAL profile*	CIP profile*
110	K38-29	04-64316	*E. coli*	urine	>256 R	16 R
111	K38-30	04-60831	*E. coli*	urine	>256 R	0.25 S
112	K38-31	04-63670	*E. coli*	urine	>256 R	16 R
113	K38-32	04-60057	*E. coli*	urine	>256 R	4 R
114	K38-33	04-57923	*E. coli*	urine	>256 R	0.25 S
115	K38-34	04-33757	*E. coli*	urine	>256 R	32 R
116	K38-35	04-40345	*E. coli*	urine	64 R	0.125 S
117	K38-36	04-37016	*E. coli*	urine	>256 R	>32 R
118	K38-37	04-33847	*E. coli*	urine	>256 R	0.25 S
119	K38-38	04-61795	*E. coli*	urine	>256 R	>32 R
120	K38-39	04-70413	*E. coli*	urine	>256 R	>32 R
121	K38-40	04-78983	*E. coli*	urine	256 R	32 R
122	K38-41	05-36	*E. coli*	urine	>256 R	0.25 S
123	K38-42	05-6086	*E. coli*	urine	>256 R	0.5 S
124	K38-43	04-69673	*E. coli*	urine	64 R	0.125 S
125	K38-44	05-5696	*E. coli*	urine	>256 R	32 R
126	K38-45	04-73270	*E. coli*	urine	>256 R	0.25 S
127	K38-46	04-73216	*E. coli*	urine	>256 R	>32 R
128	K38-47	04-72705	*E. coli*	urine	>256 R	0.25 S
129	K38-48	04-72655	*E. coli*	urine	64 R	0.064 S
130	K38-49	04-78775	*E. coli*	urine	>256 R	>32 R
131	K38-50	05-4059	*E. coli*	urine	>256 R	32 R
132	K38-51	05-3524	*E. coli*	urine	>256 R	>32 R
133	K38-52	05-2833	*E. coli*	urine	>256 R	0.25 S
134	K38-53	05-2523	*E. coli*	urine	256 R	0.25 S
135	K38-54	05-1794	*E. coli*	urine	>256 R	4 R
136	K38-55	05-1349	*E. coli*	urine	>256 R	>32 R
137	K38-56	04-66886	*E. coli*	urine	>256 R	8 R
138	K38-57	04-66705	*E. coli*	urine	>256 R	0.25 S
139	K38-58	04-72651	*E. coli*	urine	>256 R	>32 R
140	K38-59	04-66700	*E. coli*	urine	>256 R	>32 R
141	K38-60	04-66594	*E. coli*	urine	>256 R	>32 R
142	K38-61	04-77012	*E. coli*	urine	64 R	0.25 S
143	K38-62	04-76966	*E. coli*	urine	>256 R	>32 R
144	K38-63	04-65934	*E. coli*	urine	>256 R	>32 R
145	K38-64	04-72378	*E. coli*	urine	>256 R	>32 R
146	K38-65	04-72375	*E. coli*	urine	128 R	0.064 S
147	K38-66	04-71288	*E. coli*	urine	256 R	0.25 S
148	K38-67	04-71062	*E. coli*	urine	>256 R	32 R
149	K38-68	04-71060	*E. coli*	urine	>256 R	0.25 S
150	K38-69	04-70948	*E. coli*	urine	>256 R	4 R
151	K38-70	05-4670	*E. coli*	urine	>256 R	16 R
152	K38-71	05-4490	*E. coli*	urine	>256 R	0.125 S

* MICs (µg/ml) were determined by E-test; R, resistant; I, intermediate; and S, susceptible

Table 4. Bacterial isolates of the NORM collection.

Nr.	Ref nr.	Species	Sample	Location	NAL profile*	CIP profile**
1	K40-06	*E. coli*	Blood	Bodø	6 R	32 R
2	K40-07	*K. pneumoniae*	Blood	Bodø	6 R	0,5 S
3	K40-08	*K. pneumoniae*	Blood	Bodø	11 R	0,25 S
4	K40-09	*K. pneumoniae*	Blood	Bodø	16 R	0,125 S
5	K40-45	*E. coli*	Blood	Levanger	6 R	32 R
6	K40-46	*E. coli*	Blood	Levanger	9 R	0,064 S
7	K40-47	*Klebsiella spp.*	Blood	Levanger	10 R	0,125 S
8	K40-48	*K. pneumoniae*	Blood	Levanger	19 R	0,125 S
9	K40-49	*K. pneumoniae*	Blood	Levanger	6 R	0,25 S
10	K40-50	*K. pneumoniae*	Blood	Levanger	6 R	0,5 S
11	K39-21	*E. coli*	Blood	Molde	6 R	32 R
12	K39-22	*E. coli*	Blood	Molde	6 R	0,008 S
13	K39-23	*E. coli*	Blood	Molde	6 R	2 R
14	K39-24	*E. coli*	Blood	Molde	6 R	0,25 S
15	K39-25	*E. coli*	Blood	Molde	6 R	0,064 S
16	K39-26	*K. pneumoniae*	Blood	Molde	6 R	2 R
17	K39-27	*Klebsiella spp.*	Blood	Molde	6 R	0,125 S
18	K39-28	*K. pneumoniae*	Blood	Molde	6 R	0,5 S
19	K39-29	*K. pneumoniae*	Blood	Molde	16 R	0,064 S
20	K40-10	*E. coli*	Blood	Ålesund	6 R	4 R
21	K40-11	*E. coli*	Blood	Ålesund	6 R	4 R
22	K40-12	*E. coli*	Blood	Ålesund	6 R	0,125 S
23	K40-13	*E. coli*	Blood	Ålesund	6 R	32 R
24	K40-14	*E. coli*	Blood	Ålesund	6 R	32 R
25	K40-15	*E. coli*	Blood	Ålesund	6 R	0,25 S
26	K40-16	*E. coli*	Blood	Ålesund	19 R	0,016 S
27	K40-17	*E. coli*	Blood	Ålesund	6 R	32 R
28	K40-18	*K. pneumoniae*	Blood	Ålesund	19 R	0,032 S
29	K40-70	*E. coli*	Blood	Førde	6 R	32 R
30	K40-71	*E. coli*	Blood	Førde	6 R	0,25 S
31	K40-72	*E. coli*	Blood	Førde	6 R	32 R
32	K40-73	*Klebsiella spp.*	Blood	Førde	6 R	1 R
33	K40-74	*K. pneumoniae*	Blood	Førde	16 R	0,125 S
34	K40-19	*E. coli*	Blood	Haugesund	6 R	0,25 S
35	K40-20	*E. coli*	Blood	Haugesund	6 R	0,25 S
36	K40-21	*E. coli*	Blood	Haugesund	7 R	0,125 S
37	K40-22	*E. coli*	Blood	Haugesund	7 R	0,25 S
38	K40-23	*E. coli*	Blood	Haugesund	6 R	32 R
39	K40-24	*E. coli*	Blood	Haugesund	6 R	0,5 S
40	K40-25	*K. pneumoniae*	Blood	Haugesund	26 S	0,125 S
41	K40-26	*K. pneumoniae*	Blood	Haugesund	20 S	0,25 S
42	K40-27	*E. coli*	Blood	Stavanger	6 R	0,25 S
43	K40-28	*E. coli*	Blood	Stavanger	6 R	32 R
44	K40-29	*E. coli*	Blood	Stavanger	8 R	0,125 S
45	K40-30	*E. coli*	Blood	Stavanger	6 R	32 R
46	K40-31	*E. coli*	Blood	Stavanger	6 R	0,125 S
47	K39-53	*E. coli*	Blood	Kristiansand	6 R	32 R
48	K39-54	*E. coli*	Blood	Kristiansand	6 R	1 R
49	K39-55	*E. coli*	Blood	Kristiansand	8 R	0,125 S

Table 4. Bacterial isolates of the NORM collection, cont.

Nr.	Ref nr.	Species	Sample	Location	NAL profile*	CIP profile**
50	K39-56	*E. coli*	Blood	Kristiansand	12 R	0,25 S
51	K39-57	*E. coli*	Blood	Kristiansand	6 R	32 R
52	K39-58	*E. coli*	Blood	Kristiansand	6 R	0,25 S
53	K39-59	*Klebsiella spp.*	Blood	Kristiansand	9 R	0,25 S
54	K39-60	*K. pneumoniae*	Blood	Kristiansand	21 S	0,125 S
55	K39-61	*K. pneumoniae*	Blood	Kristiansand	11 R	0,25 S
56	K39-12	*E. coli*	Blood	Telelab	6 R	0,25 S
57	K39-15	*E. coli*	Blood	Telelab	6 R	1 R
58	K39-16	*E. coli*	Blood	Telelab	6 R	0,25 S
59	K39-17	*E. coli*	Blood	Telelab	6 R	0,25 S
60	K39-18	*E. coli*	Blood	Telelab	6 R	32 R
61	K39-19	*E. coli*	Blood	Telelab	6 R	0,5 S
62	K40-32	*E. coli*	Blood	Tønsberg	6 R	32 R
63	K40-33	*E. coli*	Blood	Tønsberg	6 R	32 R
64	K40-34	*E. coli*	Blood	Tønsberg	6 R	32 R
65	K40-35	*E. coli*	Blood	Tønsberg	10 R	0,25 S
66	K40-36	*K. pneumoniae*	Blood	Tønsberg	15 R	0,125 S
67	K40-37	*E. coli*	Blood	Drammen	6 R	32 R
68	K40-38	*E. coli*	Blood	Drammen	6 R	32 R
69	K40-39	*E. coli*	Blood	Drammen	6 R	0,25 S
70	K40-40	*E. coli*	Blood	Drammen	6 R	16 R
71	K40-41	*K. pneumoniae*	Blood	Drammen	15 R	0,25 S
72	K40-42	*K. oxytoca*	Blood	Drammen	19 R	0,064 S
73	K40-43	*K. pneumoniae*	Blood	Drammen	14 R	0,125 S
74	K40-44	*K. pneumoniae*	Blood	Drammen	7 R	0,5 S
75	K39-03	*E. coli*	Blood	AHUS	7 R	0,125 S
76	K39-04	*E. coli*	Blood	AHUS	6 R	0,5 S
77	K39-05	*E. coli*	Blood	AHUS	6 R	32 R
78	K39-06	*E. coli*	Blood	AHUS	6 R	0,5 S
79	K39-08	*K. pneumoniae*	Blood	AHUS	14 R	0,25 S
80	K39-09	*K. pneumoniae*	Blood	AHUS	18 R	0,125 S
81	K39-10	*K. pneumoniae*	Blood	AHUS	14 R	0,25 S
82	K39-11	*K. pneumoniae*	Blood	AHUS	20 S	0,125 S
83	K39-73	*E. coli*	Blood	Haukeland	6 R	32 R
84	K39-74	*E. coli*	Blood	Haukeland	6 R	1 R
85	K39-75	*E. coli*	Blood	Haukeland	6 R	32 R
86	K39-76	*E. coli*	Blood	Haukeland	15 R	0,064 S
87	K39-77	*E. coli*	Blood	Haukeland	6 R	32 R
88	K39-78	*E. coli*	Blood	Haukeland	6 R	32 R
89	K39-79	*E. coli*	Blood	Haukeland	6 R	4 R
90	K39-80	*E. coli*	Blood	Haukeland	6 R	32 R
91	K39-81	*K. pneumoniae*	Blood	Haukeland	6 R	4 R
92	K40-02	*K. pneumoniae*	Blood	Haukeland	18 R	0,25 S
93	K40-03	*K. pneumoniae*	Blood	Haukeland	6 R	0,5 S
94	K40-04	*K. pneumoniae*	Blood	Haukeland	22 S	0,125 S
95	K40-05	*E. coli*	Blood	Haukeland	6 R	8 R
96	K40-52	*E. coli*	Blood	Trondheim	6 R	0,25 S
97	K40-53	*E. coli*	Blood	Trondheim	6 R	0,25 S
98	K40-54	*E. coli*	Blood	Trondheim	6 R	32 R

Table 4. Bacterial isolates of the NORM collection, cont.

Nr.	Ref nr.	Species	Sample	Location	NAL profile*	CIP profile**
99	K40-56	*K. pneumoniae*	Blood	Trondheim	10 R	1 R
100	K40-58	*K. oxytoca*	Blood	Trondheim	13 R	0,125 S
101	K40-59	*K. pneumoniae*	Blood	Trondheim	13 R	0,125 S
102	K40-60	*K. pneumoniae*	Blood	Trondheim	11 R	0,25 S
103	K40-61	*K. oxytoca*	Blood	Trondheim	6 R	0,25 S
104	K40-62	*K. pneumoniae*	Blood	Tromsø	20 S	0,25 S
105	K40-63	*K. pneumoniae*	Blood	Tromsø	9 R	0,25 S
106	K40-64	*E. coli*	Blood	Tromsø	6 R	4 R
107	K40-68	*E. coli*	Blood	Tromsø	6 R	0,25 S
108	K40-69	*E. coli*	Blood	Tromsø	6 R	0,25 S
109	K39-31	*K. pneumoniae*	Blood	Rikshospitalet	22 S	0,5 S
110	K39-32	*E. coli*	Blood	Rikshospitalet	6 R	8 R
111	K39-33	*E. coli*	Blood	Rikshospitalet	21 S	0,125 S
112	K39-34	*E. coli*	Blood	Rikshospitalet	19 R	0,016 S
113	K39-35	*E. coli*	Blood	Rikshospitalet	18 R	0,016 S
114	K39-36	*E. coli*	Blood	Rikshospitalet	19 R	0,008 S
115	K39-37	*E. coli*	Blood	Rikshospitalet	6 R	32 R
116	K39-50	*E. coli*	Blood	Lillehammer	6 R	0,25 S
117	K39-51	*E. coli*	Blood	Lillehammer	15 R	1 R
118	K39-52	*E. coli*	Blood	Lillehammer	6 R	0,25 S
119	K39-30	*K. pneumoniae*	Blood	Bærum	16 R	0,125 S
120	K39-62	*E. coli*	Blood	Fredrikstad	6 R	32 R
121	K39-63	*E. coli*	Blood	Fredrikstad	6 R	0,125 S
122	K39-64	*E. coli*	Blood	Fredrikstad	6 R	32 R
123	K39-65	*E. coli*	Blood	Fredrikstad	6 R	32 R
124	K39-66	*E. coli*	Blood	Fredrikstad	6 R	32 R
125	K39-67	*E. coli*	Blood	Fredrikstad	19 R	0,125 S
126	K39-68	*E. coli*	Blood	Fredrikstad	6 R	0,125 S
127	K39-69	*K. pneumoniae*	Blood	Fredrikstad	15 R	0,25 S
128	K39-70	*K. pneumoniae*	Blood	Fredrikstad	19 R	0,25 S
129	K40-75	*E. coli*	Blood	Aker	6 R	32 R
130	K40-76	*E. coli*	Blood	Aker	6 R	32 R
131	K40-77	*E. coli*	Blood	Aker	6 R	0,125 S
132	K40-78	*E. coli*	Blood	Aker	6 R	4 R
133	K40-79	*E. coli*	Blood	Aker	6 R	32 R
134	K40-80	*Klebsiella spp.*	Blood	Aker	6 R	0,5 S
135	K40-81	*Klebsiella spp.*	Blood	Aker	9 R	0,5 S
136	K43-05	*Klebsiella spp.*	Blood	Aker	6 R	1 R
137	K43-06	*Klebsiella spp.*	Blood	Aker	6 R	0,25 S
138	K39-38	*K. pneumoniae*	Blood	Ullevål	6 R	32 R
139	K39-39	*Klebsiella spp.*	Blood	Ullevål	8 R	0,5 S
140	K39-40	*K. pneumoniae*	Blood	Ullevål	9 R	0,5 S
141	K39-41	*K. pneumoniae*	Blood	Ullevål	16 R	0,125 S
142	K39-42	*K. pneumoniae*	Blood	Ullevål	15 R	0,25 S
143	K39-43	*E. coli*	Blood	Ullevål	6 R	16 R
144	K39-44	*E. coli*	Blood	Ullevål	8 R	0,064 S
145	K39-45	*E. coli*	Blood	Ullevål	11 R	0,25 S
146	K39-46	*E. coli*	Blood	Ullevål	9 R	0,125 S
147	K39-47	*E. coli*	Blood	Ullevål	6 R	32 R

Table 4. Bacterial isolates of the NORM collection, cont.

Nr.	Ref nr.	Species	Sample	Location	NAL profile*	CIP profile**
148	K39-48	*E. coli*	Blood	Ullevål	6 R	32 R
149	K39-49	*E. coli*	Blood	Ullevål	12 R	0,064 S
Nr.	**Ref nr.**	**Species**	**Sample**	**Location**	**NAL profile***	**CIP profile***
150	K42-48	*E. coli*	Urine 2005	Bodø	6 R	8 R
151	K42-49	*E. coli*	Urine 2005	Bodø	6 R	13 R
152	K42-17	*E. coli*	Urine 2005	Molde	6 R	29 S
153	K42-16	*E. coli*	Urine 2005	Molde	6 R	6 R
154	K42-15	*E. coli*	Urine 2005	Molde	19 R	29 S
155	K42-14	*E. coli*	Urine 2005	Molde	6 R	6 R
156	K42-50	*E. coli*	Urine 2005	Ålesund	6 R	18 R
157	K42-51	*E. coli*	Urine 2005	Ålesund	6 R	29 S
158	K42-52	*E. coli*	Urine 2005	Ålesund	6 R	31 S
159	K42-80	*E. coli*	Urine 2005	Førde	6 R	6 R
160	K42-81	*E. coli*	Urine 2005	Førde	6 R	12 R
161	K42-53	*E. coli*	Urine 2005	Haugesund	6 R	10 R
162	K42-54	*E. coli*	Urine 2005	Haugesund	6 R	29 S
163	K42-55	*E. coli*	Urine 2005	Stavanger	6 R	6 R
164	K42-56	*E. coli*	Urine 2005	Stavanger	6 R	6 R
165	K42-57	*E. coli*	Urine 2005	Stavanger	6 R	28 S
166	K42-38	*E. coli*	Urine 2005	Kristiansand	6 R	29 S
167	K42-39	*E. coli*	Urine 2005	Kristiansand	6 R	27 S
168	K42-11	*E. coli*	Urine 2005	Telelab	32 S	10 R
169	K42-58	*E. coli*	Urine 2005	Tønsberg	6 R	29 S
170	K42-59	*E. coli*	Urine 2005	Drammen	28 S	17 R
171	K42-60	*E. coli*	Urine 2005	Drammen	7 R	8 R
172	K42-61	*E. coli*	Urine 2005	Drammen	7 R	10 R
173	K42-62	*E. coli*	Urine 2005	Drammen	19 R	30 S
174	K42-63	*E. coli*	Urine 2005	Drammen	8 R	29 S
175	K42-64	*E. coli*	Urine 2005	Drammen	7 R	7 R
176	K42-65	*E. coli*	Urine 2005	Drammen	7 R	7 R
177	K42-36	*E. coli*	Urine 2005	Radiumhospitalet	6 R	6 R
178	K42-37	*E. coli*	Urine 2005	Radiumhospitalet	6 R	6 R
179	K42-01	*E. coli*	Urine 2005	AHUS	6 R	28 S
180	K42-02	*E. coli*	Urine 2005	AHUS	6 R	6 R
181	K42-03	*E. coli*	Urine 2005	AHUS	6 R	11 R
182	K42-06	*E. coli*	Urine 2005	AHUS	10 R	30 S
183	K42-07	*E. coli*	Urine 2005	AHUS	6 R	6 R
184	K42-09	*E. coli*	Urine 2005	AHUS	6 R	6 R
185	K42-41	*E. coli*	Urine 2005	Haukeland	6 R	36 S
186	K42-42	*E. coli*	Urine 2005	Haukeland	6 R	10 R
187	K42-45	*E. coli*	Urine 2005	Haukeland	6 R	25 S
188	K42-46	*E. coli*	Urine 2005	Haukeland	6 R	6 R
189	K42-66	*E. coli*	Urine 2005	Trondheim	6 R	8 R
190	K42-67	*E. coli*	Urine 2005	Trondheim	6 R	8 R
191	K42-70	*E. coli*	Urine 2005	Trondheim	6 R	32 S
192	K42-72	*E. coli*	Urine 2005	Trondheim	6 R	29 S
193	K42-73	*E. coli*	Urine 2005	Tromsø	6 R	8 R
194	K42-74	*E. coli*	Urine 2005	Tromsø	12 R	30 S
195	K42-75	*E. coli*	Urine 2005	Tromsø	10 R	30 S

Table 4. Bacterial isolates of the NORM collection, cont.

Nr.	Ref nr.	Species	Sample	Location	NAL profile*	CIP profile*
196	K42-79	*E. coli*	Urine 2005	Tromsø	6 R	26 S
197	K42-22	*E. coli*	Urine 2005	Rikshospitalet	6 R	25 S
198	K42-23	*E. coli*	Urine 2005	Rikshospitalet	6 R	6 R
199	K42-24	*K. oxytoca*	Urine 2005	Rikshospitalet	6 R	22 S
200	K42-25	*E. coli*	Urine 2005	Rikshospitalet	6 R	21 S
201	K42-26	*E. coli*	Urine 2005	Rikshospitalet	17 R	28 S
202	K42-27	*E. coli*	Urine 2005	Rikshospitalet	6 R	24 S
203	K42-28	*E. coli*	Urine 2005	Rikshospitalet	6 R	25 S
204	K42-29	*E. coli*	Urine 2005	Rikshospitalet	6 R	6 R
205	K42-30	*E. coli*	Urine 2005	Rikshospitalet	6 R	9 R
206	K42-31	*E. coli*	Urine 2005	Rikshospitalet	6 R	23 S
207	K42-18	*E. coli*	Urine 2005	Bærum	6 R	27 S
208	K42-19	*E. coli*	Urine 2005	Bærum	6 R	6 R
209	K42-20	*E. coli*	Urine 2005	Bærum	6 R	7 R
210	K42-21	*E. coli*	Urine 2005	Bærum	6 R	30 S
211	K42-40	*E. coli*	Urine 2005	Fredrikstad	6 R	28 S
212	K43-01	*E. coli*	Urine 2005	Aker	27 S	21 S
213	K43-02	*E. coli*	Urine 2005	Aker	6 R	14 R
214	K43-03	*E. coli*	Urine 2005	Aker	6 R	22 S
215	K43-04	*E. coli*	Urine 2005	Aker	6 R	6 R
216	K42-33	*E. coli*	Urine 2005	Ullevål	6 R	6 R
217	K42-34	*E. coli*	Urine 2005	Ullevål	6 R	12 R
218	K42-35	*E. coli*	Urine 2005	Ullevål	6 R	6 R

* Zones of inhibition (mm) determined by agar diffusion; R, resistant; I, intermediate; and S, susceptible, ** MICs (µg/ml) determined by E-test; R, resistant; I, intermediate; and S, susceptible

Table 5. Bacterial isolates of the *E. coli* AmpC collection.

Nr.	Ref. nr	Species	Material	NAL profile*	CIP profile*	AmpC profile
1	K2-67	*E. coli*	Urine	> 32 R	≥ 4 R	Plasmid-mediated
2	K2-68	*E. coli*	Nose	≥ 32 R	≥ 4 R	Chromosomal upregulation mutation
3	K4-37	*E. coli*	Blood	≥ 32 R	≥ 4 R	Chromosomal upregulation mutation
4	K5-20	*E. coli*	Urine	≥ 32 R	≥ 4 R	Both plasmid-mediated and chromosomal upregulation mutation
5	K5-41	*E. coli*	Urine	≥ 32 R	≤ 0.25 S	Plasmid-mediated
6	K5-63	*E. coli*	Urine	≥ 32 R	≥ 4 R	Plasmid-mediated
7	K15-8	*E. coli*	Blood	≥ 32 R	1 I	ND
8	K22-31	*E. coli*	Urine	≥ 32 R	0.5 S	Chromosomal upregulation mutation
9	K22-51	*E. coli*	Urine	≥ 32 R	2 R	ND
10	K25-65	*E. coli*	Blood	≥ 32 R	≥ 4 R	Chromosomal upregulation mutation
11	K26-7	*E. coli*	Urine	≥ 32 R	1 I	Plasmid-mediated
12	K26-21	*E. coli*	Urine	≥ 32 R	≥ 4 R	Plasmid-mediated
13	K26-35	*E. coli*	Urine	≥ 32 R	0.5 S	Plasmid-mediated

* MICs (µg/ml) were determined by E-test; R, resistant; I, intermediate; and S, susceptible.

Table 6. Bacterial isolates of Ullevål ESBL study.

Nr.	Ref.nr	Ref.nr UUS	Species	NAL profile*	CIP profile*	ESBL profile
1	K2-70	B 1364	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
2	K4-69	B 1418	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
3	K4-79	B 1436	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
4	K5-9	B 1468	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
5	K5-11	B 1472 st 1	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
6	K5-12	B 1473 st 2	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
7	K5-43	B 1502	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
8	K5-65	B 1509	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
9	K15-28	B 790	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
10	K15-29	12774	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
11	K15-30	13094	*E. coli*	≥ 32 R	≥ 4 R	SHV ESBL
12	K15-31	B 1046	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
13	K15-33	B 1079	*E. coli*	≥ 32 R	≥ 4 R	SHV ESBL
14	K15-35	12120	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
15	K15-36	B 547	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
16	K15-37	B 548	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
17	K15-38	B 549	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
18	K15-39	12515	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
19	K15-41	B 1130	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
20	K15-42	B 1131	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
21	K15-43	B 1141	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
22	K15-44	B 988	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
23	K15-45	13231	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
24	K15-46	13441	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
25	K15-47	B 1004	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
26	K15-48	13527	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
27	K15-49	B 265	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
28	K15-50	B 306	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
29	K15-52	B 459	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
30	K15-76	B 1781	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
31	K15-77	B 1782	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
32	K18-2	B 1871	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
33	K18-3	B 1641	*E. coli*	≥ 32 R	0.5 S	CTX-M ESBL
34	K18-4	B 1721	*E. coli*	≥ 32 R	0.5 S	CTX-M ESBL
35	K18-5	B 1722	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
36	K18-7	B 1761	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
37	K18-8	14901	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
38	K18-9	B 1724	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
39	K18-10	B 1723	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
40	K18-11	B 1845	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
41	K18-12	B 1844	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
42	K18-13	B 1830	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
43	K18-14	B 1935	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
44	K18-15	B 2162	*E. coli*	≥ 32 R	≥ 4 R	TEM ESBL
45	K18-16	B 1194	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
46	K18-17	B 1225	*K. pneumoniae*	≥ 32 R	1 I	SHV ESBL
47	K18-19	B 1253	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
48	K18-22	B 2132	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
49	K18-24	B 1911	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
50	K18-26	B 1868	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
51	K18-27	B 1866	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
52	K18-28	B 1590	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
53	K18-29	B 1589	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
54	K18-30	B 1591	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
55	K18-31	B 1604	*E. coli*	≥ 32 R	1 I	SHV ESBL

Table 6. Bacterial isolates of Ullevål ESBL study, cont.

Nr.	Ref.nr	Ref.nr UUS	Species	NAL profile*	CIP profile*	ESBL profile
56	K18-32	B 1962	*E. coli*	≥ 32 R	0.5 S	SHV ESBL
57	K18-33	B 1952	*E. coli*	≥ 32 R	2 R	CTX-M ESBL
58	K18-34	B 1981	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL
59	K18-35	B 1988	*E. coli*	≥ 32 R	1 I	CTX-M ESBL
60	K4-60	B 1413 st 3	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL

* MICs (µg/ml) were determined by E-test; R, resistant; I, intermediate; and S, susceptible

Table 7. Bacterial isolates of the Norwegian ESBL study.

Nr.	Ref. nr	Species	NAL profile*	CIP profile*	ESBL profile
1	K2-57	*K. oxytoca*	4 S	≥ 64 R	Not ESBL
2	K4-13	*K. pneumoniae*	8 S	2 R	ESBL+
3	K2-78	*K. pneumoniae*	16 S	1 I	ESBL+
4	K4-51	*E. coli*	≥32 R	≤ 0.25 S	ESBL+
5	K4-46	*E. coli*	≥32 R	1 I	ESBL+
6	K5-16	*E. coli*	≥32 R	1 I	ESBL+
7	K5-64	*E. coli*	≥32 R	1 I	ESBL+
8	K4-39	*E. coli*	≥32 R	2 R	ESBL+
9	K2-35	*E. coli*	≥32 R	≥4 R	Not ESBL
10	K2-63	*E. coli*	≥32 R	≥4 R	ESBL+
11	K2-64	*E. coli*	≥32 R	≥4 R	ESBL+
12	K4-40	*E. coli*	≥32 R	≥4 R	ESBL+
13	K4-43	*E. coli*	≥32 R	≥4 R	ESBL+
14	K4-45	*E. coli*	≥32 R	≥4 R	ESBL+
15	K4-76	*E. coli*	≥32 R	≥4 R	ESBL+
16	K4-81	*E. coli*	≥32 R	≥4 R	ESBL+
17	K5-8	*E. coli*	≥32 R	≥4 R	ESBL+
18	K5-13	*E. coli*	≥32 R	≥4 R	Not ESBL
19	K5-15	*E. coli*	≥ 32 R	≥4 R	Not ESBL
20	K5-21	*E. coli*	≥ 32 R	≥4 R	Not ESBL
21	K5-23	*E. coli*	≥ 32 R	≥4 R	ESBL+
22	K5-34	*E. coli*	≥ 32 R	≥4 R	ESBL+
23	K5-51	*E. coli*	≥ 32 R	≥4 R	ESBL+
24	K5-56	*E. coli*	≥ 32 R	≥4 R	ESBL+
25	K5-58	*E. coli*	≥ 32 R	≥4 R	ESBL+
26	K5-59	*E. coli*	≥ 32 R	≥4 R	ESBL+
27	K5-62	*E. coli*	≥ 32 R	≥4 R	ESBL+
28	K5-72	*E. coli*	≥ 32 R	≥4 R	Not ESBL
29	K5-76	*E. coli*	≥ 32 R	≥4 R	ESBL+
30	K5-78	*E. coli*	≥ 32 R	≥4 R	Not ESBL
31	K8-1	*E. coli*	≥ 32 R	≥4 R	ESBL+
32	K8-4	*E. coli*	≥ 32 R	≥4 R	ESBL+
33	K8-8	*E. coli*	≥ 32 R	≥4 R	ESBL+
34	K4-19	*K. pneumoniae*	≥ 32 R	≤0.25 S	Not ESBL
35	K5-45	*K. pneumoniae*	≥ 32 R	≤0.25 S	Not ESBL
36	K5-32	*K. pneumoniae*	≥ 32 R	0.5 S	Not ESBL
37	K5-6	*K. pneumoniae*	≥ 32 R	1 I	ESBL+
38	K4-49	*K. pneumoniae*	≥ 32 R	2 R	ESBL+
39	K4-61	*K. pneumoniae*	≥ 32 R	≥4 R	ESBL+

Table 7. Bacterial isolates of the Norwegian ESBL study, cont.

Nr.	Ref. nr	Species	NAL profile*	CIP profile*	ESBL profile
40	K4-77	*K. pneumoniae*	≥ 32 R	≥4 R	ESBL+
41	K2-55	*E. coli*	≥ 32 R	≥4 R	ESBL+
42	K4-31	*E. coli*	≥ 32 R	≥4 R	ESBL+
43	K5-60	*E. coli*	≥ 32 R	≥4 R	ESBL+
44	K8-5	*K. pneumoniae*	≥ 32 R	≥4 R	ESBL+

* MICs (µg/ml) were determined by E-test; R, resistant; I, intermediate; and S, susceptible

Table 8. Primers used in this study.

Name	DNA sequence 5`-3`	Target site	Amplicon size (bp)	Reference
aac(6')-Ib-F	ttgcgatgctctatgagtggcta	*aac(6')-Ib*	482	Park *et al.* 2006
aac(6')-Ib-R	ctcgaatgcctggcgtgttt			
aac(6')-Ib-SEQ	cgtcactccatacattgcaa	*aac(6')-Ib*	482	Park *et al.* 2006
qnrA-F.multiplex	atttctcacgccaggatttg	*qnrA*	516	Robicsek *et al.* 2006
qnrA-R.multiplex	gatcggcaaaggttaggtca			
qnrB-F.multiplex	gatcgtgaaagccagaaagg	*qnrB*	469	Robicsek *et al.* 2006
qnrB-R.multiplex	acgatgcctggtagttgtcc			
qnrS-F.multiplex	acgacattcgtcaactgcaa	*qnrS*	417	Robicsek *et al.* 2006
qnrS-R.multiplex	taaattggcaccctgtaggc			
gyrA6	cgaccttgcgagagaaat	QRDR of *gyrA*	620	Weigel *et al.* 1998
gyrA631R	gttccatcagcccttcaa			
parCF	tgaatttagggaaaacgccta	QRDR of *parC*	559	Qiang *et al*, 2002
parCR	gccacttcacgcaggttatg			
HI1 FW	ggagcgatggattacttcagtac	*parA-parB*	471	Carattoli *et al.* 2005
HI1 RV	tgccgtttcacctcgtgagta			
HI2 FW	tttctcctgagtcacctgttaacac	*iterons*	644	Carattoli *et al.* 2005
HI2 RV	ggctcactaccgttgtcatcct			
I1 FW	cgaaagccggacggcagaa	RNAI	139	Carattoli *et al.* 2005
I1 RV	tcgtcgttccgccaagttcgt			
X FW	aaccttagaggctatttaagttgctgat	*ori*g	376	Carattoli *et al.* 2005
X RV	tgagagtcaatttttatctcatgttttagc			
L/M FW	ggatgaaaactatcagcatctgaag	*repA,B,C*	785	Carattoli *et al.* 2005
L/M RV	ctgcaggggcgattctttagg			
N FW	gtctaacgagcttaccgaag	*repA*	559	Carattoli *et al.* 2005
N RV	gtttcaactctgccaagttc			
FIA FW	ccatgctggttctagagaaggtg	*iterons*	462	Carattoli *et al.* 2005
FIA RV	gtatatccttactggcttccgcag			
FIB FW	ggagttctgacacacgattttctg	*repA*	702	Carattoli *et al.* 2005
FIB RV	ctcccgtcgcttcagggcatt			
W FW	cctaagaacaacaaagcccccg	*repA*	242	Carattoli *et al.* 2005
W RV	ggtgcgcggcatagaaccgt			
Y FW	aattcaaacaacactgtgcagcctg	*repA*	765	Carattoli *et al.* 2005
Y RV	gcgagaatggacgattacaaaacttt			
P FW	ctatggccctgcaaacgcgccagaaa	*iterons*	534	Carattoli *et al.* 2005
P RV	tcacgcgccagggcgcagcc			
FIC FW	gtgaactggcagatgaggaagg	*repA2*	262	Carattoli *et al.* 2005
FIC RV	ttctcctcgtcgccaaactagat			
A/C FW	gagaaccaaagacaaagacctgga	*repA*	465	Carattoli *et al.* 2005
A/C RV	acgacaaacctgaattgcctcctt			
T FW	ttggcctgtttgtgcctaaaccat	*repA*	750	Carattoli *et al.* 2005
T RV	cgttgattacacttagctttggac			

Table 8. Primers used in this study, cont.

Name	DNA sequence 5`-3`	Target site	Amplicon size (bp)	Reference
FIIS FW	ctgtcgtaagctgatggc	*repA*	270	Carattoli *et al.* 2005
FIIS RV	ctctgccacaaacttcagc			
FrepB FW	tgatcgtttaaggaattttg	RNAI/*repA*	270	Carattoli *et al.* 2005
FrepB RV	gaagatcagtcacaccatcc			
K/B FW	gcggtccggaaagccagaaaac	RNAI	160	Carattoli *et al.* 2005
K RV	tctttcacgagcccgccaaa			
K/B FW	gcggtccggaaagccagaaaac	RNAI	159	Carattoli *et al.* 2005
B/O RV	tctgcgttccgccaagttcga			
qnrS-3`ext	gaactcgacggtttagatcc	Upstream *qnrS1*	2000	Poirel *et al.* 2007
213B	cgatgaatcaggctccagtc			
qnrS-F.multiplex	acgacattcgtcaactgcaa	Upstream *qnrS1*	1800	This study
Sea15.Kres	atgaggctgaaggtgctcat			
qnrS-5`ext	gcgaatgaatgtgcaagcgg	Downstream *qnrS1*	1300	Poirel *et al.* 2007
orfB-B	cagcagtcctgcgcgaagg			
qnrS-5`ext	gcgaatgaatgtgcaagcgg	Downstream *qnrS1*	3000	Poirel *et al.* 2007
pbp3b	cgtcctgaagtggcactgg			
qnrS-5`ext	gcgaatgaatgtgcaagcgg	Downstream *qnrS1*	3000	Poirel *et al.* 2007
LAP-1A	caatacaaagcacagaagacc			
qnrS-R.multiplex	taaattggcaccctgtaggc	Downstream *qnrS1*	2000	This study
LAP-1.Kres	gcggatgatgttcgttgtaa			
TEM-R mod TW	ccaatgcttaatcagtgagg	Downstream *qnrS1*		This study
tnpRF2	gcgcatgtcaatcaattcgg	Downstream *qnrS1*		This study
tnpRF	gatacagggtttcgcgactg	Downstream *qnrS1*		This study

Table 9. PCR programs applied in this study.

PCR	Initial denaturation	cycles	Denaturation	Primer annealing	Elongation	Final elongation
16S rDNA	95ºC / 1 min	30	95ºC / 30 sec	55ºC / 30 sec	72°C / 1 min	72°C / 7 min (then 4°C → ∞)
qnr multiplex	95ºC / 5 min	30	95ºC / 15 sec	55ºC / 15 sec	72ºC / 40 sec	72ºC / 4 min (then 4°C → ∞)
aac(6')-Ib	95ºC / 5 min	30	95ºC / 15 sec	58ºC / 15 sec	72ºC / 40 sec	72ºC / 4 min (then 4°C → ∞)
QRDR gyrA/parC	94ºC / 5 min	30	94ºC / 15 sec	55ºC / 15 sec	72ºC / 40 sec	72ºC / 4 min (then 4°C → ∞)
All multiplex and simplex PCRs for amplicon typing except F-simplex PCR	94ºC / 5 min	30	94ºC / 40 sec	60ºC / 20 sec	72ºC / 30 sec	72ºC / 5 min (then 4°C → ∞)
F-simplex PCR	94ºC / 5 min	30	94ºC / 40 sec	52ºC / 20 sec	72ºC / 30 sec	72ºC / 5 min (then 4°C → ∞)
Targeted PCRs (*qnrS1* genetic environment)	94ºC / 5 min	20	94ºC / 20 sec	58ºC / 20 sec	72ºC / 2 min and 30 sec	72ºC / 15 min (then 4°C → ∞)
Sequencing	96ºC / 5 min	25	96ºC / 10 sec	50ºC / 5 sec	60ºC / 4 min	(then 4°C → ∞)

Table 10. Features of the *aac(6`)-Ib*-positive isolates.

Nr.	Ref. nr	Species	NAL* profile	CIP* profile	ESBL status	Aac(6`)-Ib
1	K37-80	*E.coli*	>256 R	>32 R	Not determined	*aac(6`)-Ib-cr*
2	K38-23	*E.coli*	>256 R	>32 R	Not determined	Wild-type
3	K5-63	*E.coli*	≥ 32 R	≥ 4 R	Not determined	*aac(6`)-Ib-cr*
4	K2-70	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
5	K5-9	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
6	K5-11	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
7	K5-12	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
8	K5-43	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
9	K5-65	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
10	K15-28	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
11	K15-31	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
12	K15-36	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
13	K15-37	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
14	K15-38	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
15	K15-41	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
16	K15-42	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
17	K15-43	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
18	K15-45	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
19	K15-46	*E. coli*	≥ 32 R	1 I	CTX-M ESBL	*aac(6`)-Ib-cr*
20	K15-47	*E. coli*	≥ 32 R	1 I	CTX-M ESBL	*aac(6`)-Ib-cr*
21	K15-48	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
22	K15-50	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
23	K15-52	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
24	K15-76	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
25	K15-77	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
26	K18-2	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
27	K18-7	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
28	K18-9	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
29	K18-11	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
30	K18-12	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
31	K18-13	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
32	K18-14	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
33	K18-16	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
34	K18-17	*K. pneumoniae*	≥ 32 R	1 I	SHV ESBL	Wild-type
35	K18-19	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	both
36	K18-22	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
37	K18-24	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
38	K18-26	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
39	K18-27	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
40	K18-28	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
41	K18-30	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
42	K18-31	*E. coli*	≥ 32 R	1 I	SHV ESBL	Wild-type
43	K18-34	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
44	K4-60	*E. coli*	≥ 32 R	≥ 4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
45	K15-32	*E. coli*	≤ 2 S	≤ 0.25 S	TEM ESBL	Wild-type
46	K15-78	*E. coli*	≤ 2 S	≤ 0.25 S	CTX-M ESBL	*aac(6`)-Ib-cr*
47	K4-13	*K. pneumoniae*	8 S	2 R	CTX-M/SHV ESBL	*aac(6`)-Ib-cr*
48	K4-39	*E. coli*	≥32 R	2 R	ESBL+	*aac(6`)-Ib-cr*
49	K2-35	*E. coli*	≥32 R	≥4 R	Not ESBL	*aac(6`)-Ib-cr*
50	k2-63	*E. coli*	≥32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
51	k4-40	*E. coli*	≥32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
52	k4-43	*E. coli*	≥32 R	≥4 R	ESBL+	both
53	k4-45	*E. coli*	≥32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
54	k4-76	*E. coli*	≥32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*

Table 10. Features of the *aac(6`)-Ib*-positive isolates, cont.

55	k5-34	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
56	k5-51	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
57	k5-56	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
58	k5-59	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
59	k5-62	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
60	k8-1	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
61	k8-4	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
62	k5-6	*K. pneumoniae*	≥ 32 R	1 I	ESBL+	Wild-type
63	k4-49	*K. pneumoniae*	≥ 32 R	2 R	ESBL+	Wild-type
64	k4-77	*K. pneumoniae*	≥ 32 R	≥4 R	ESBL+	Wild-type
65	K2-55	*E. coli*	≥ 32 R	≥4 R	ESBL+	*aac(6`)-Ib-cr*
66	k8-5	*K. pneumoniae*	≥ 32 R	≥4 R	CTX-M ESBL	*aac(6`)-Ib-cr*
67	K40-30	*E. coli*	6 R**	32 R	Not determined	*aac(6`)-Ib-cr*
68	K39-60	*K. pneumoniae*	21 S**	0,125 S	Not determined	*aac(6`)-Ib-cr*
69	K40-38	*E. coli*	6 R**	32 R**	Not determined	*aac(6`)-Ib-cr*
70	K39-05	*E. coli*	6 R**	32 R**	Not determined	*aac(6`)-Ib-cr*
71	K40-79	*E. coli*	6 R**	32 R**	Not determined	*aac(6`)-Ib-cr*
72	K42-55	E. coli	6 R**	6 R**	Not determined	*aac(6`)-Ib-cr*
73	K42-56	E. coli	6 R**	6 R**	Not determined	*aac(6`)-Ib-cr*
74	K42-65	E. coli	7 R**	7 R**	Not determined	*aac(6`)-Ib-cr*
75	K42-36	E. coli	6 R**	6 R**	Not determined	*aac(6`)-Ib-cr*
76	K42-07	E. coli	6 R**	6 R**	Not determined	*aac(6`)-Ib-cr*
77	K43-04	E. coli	6 R**	6 R**	Not determined	*aac(6`)-Ib-cr*

* MICs (µg/ml) were determined by E-test; R, resistant; I, intermediate; and S, susceptible
** Zones of inhibition determined by agar diffusion; R, resistant; I, intermediate; and S, susceptible

Table 11. Results of the susceptibility testing by VITEK® 2 for the *qnr*-positive isolates

Antibiotic *	MIC (µg/ml)**							
	K38-15	**K4-13**	**K4-43**	**K39-31**	**K40-47**	**K40-79**	**K2-78**	**K8-5**
NAL	16 S	4 S	≥32 R	4 S	≥32 R	≥32 R	16 S	≥32 R
CIP	1 S	2 I	≥4 R	1 S	≥4 R	≥4 R	0.5 S	≥4 R
TOB	≤1 S	≥16 R	≥16 R	≤1 S	≤1 S	≥16 R	≤1 S	≥16 R
GEN	≤1 S	≥16 R	≥16 R	≤1 S	≤1 S	2 S	≤1 S	≥16 R
CXM	4 I	≥64 R	≥64 R	2 I	4 I	16 I	16 I	≥64 R
FOX	≤4 S	≤4 S	≥64 R	≤4 S	≤4 S	8 S	≤4 S	≤4 S
CTX	≤1 S	≥64 R	≥64 R	≤1 S	≤1 S	≤1 S	4 S	≥64 R
AMP	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R
AMC	4 S	16 I	≥32 R	≤2 S	≤2 S	16 I	8 S	16 I
TZP	≤4 S	8 S	≥128 R	≤4 S	≤4 S	8 S	≤4 S	8 S
CAZ	≤1 S	16 I	≥64 R	≤1 S	≤1 S	≤1 S	≥64 R	32 R
CPO	≤1 S	16 I	≥64 R	≤1 S	≤1 S	≤1 S	≤1 S	16 I
CPD	≤0.25 S	≥8 R	≥8 R	≤0.25 S	≤0.25 S	1 S	≥8 R	≥8 R
NIT	≤16 S	64 I	64 I	64 I	≥512 R	≤16 S	64 I	256 R
ATM	≤1 S	≥64 R	≥64 R	≤1 S	≤1 S	≤1 S	≥64 R	≥64 R
CEP	16 I	≥64 R	≥64 R	≤2 S	4 S	16 I	≥64 R	≥64 R
CXA	4 S	≥64 R	≥64 R	2 S	4 S	16 I	16 I	≥64 R
MEM	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S
TMP	≤0.5 S	≥16 R	≥16 R	≥16 R	1 S	≥16 R	≤0.5 S	≥16 R
SXT	≤20 S	≥320 R	≥320 R	≥320 R	≤20 S	≥320 R	≤20 S	≥320 R

* NAL, nalidixic acid; CIP, ciprofloxacin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CAZ, ceftazidime; FOX, cefoxitin; CTX, cefotaxime; CPO, cefpirome; CPD, cefpodoxime; CEP, cephalothin; CXM, cefuroxime; CXA, cefuroxime axetil; ATM, aztreonam; MEM, meropenem; nitrofurantoin; GEN, gentamicin; TOB, tobramycin; and SXT, trimethoprim-sulfamethoxazole. ** S, susceptible; I, intermediate; and R, resistant.

Table 12. Inhibition zone diameters obtained with nalidixic acid 30 µg and ciprofloxacin 10 µg discs for the *qnr*-positive clinical isolates

Clinical isolate	Zone diameter (mm)**	
	NAL30*	CIP10*
K38-15	18 I	28 S
K4-13	24 S	23 S
K2-78	18 I	29 S
K4-43	6R	6R
K8-5	6R	6R
K39-31	24 S	28 S
K40-47	8 R	16 R
K40-79	6R	6R

* NAL30, 30µg nalidixic acid disk; CIP10, 10µg ciprofloxacin disk.
** S, susceptible; I, intermediate; and R, resistant.

Table 13. Transconjugation frequencies

Donor clinical isolate	Transconjugation frequency*	
	Transconjugant per donor	Transconjugant per recipient
K38-15 (*E. coli*)	1.1×10^{-4}	0.8×10^{-4}
K4-13 (*K. pneumoniae*)	5.8×10^{-4}	2.9×10^{-4}
K40-79 (*E. coli*)	1×10^{-4}	1×10^{-4}

* transconjugation frequencies were calculated by dividing the number of transconjugants by the number of donors or by the number of reciepients.

Table 14. Results of the susceptibility testing by VITEK 2 for the recipient *E. coli* J53-2 and the *qnr*-positive transconjugants/transformants

Code*	Antibiotic	MIC (µg/ml)**				
		TC38-15	TC4-13	TF4-43	TC40-79	TF2-78
NAL	nalidixic acid	16 S	8 S	8 S	16 S	4 S
CIP	ciprofloxacin	1 S	2 I	≤0.25 S	1 S	≤0.25 S
TOB	tobramycin	≤1 S	8 I	≤1 S	≤1 S	≤1 S
GEN	gentamicin	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
CXM	cefuroxime	32 R	32 R	8 I	16 I	32 R
FOX	cefoxitin	≥64 R	16 I	8 S	32 R	8 S
CTX	cefotaxime	≤1 S	≤1 S	≤1 S	≤1 S	2 S
AMP	ampicillin	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R
AMC	amoxicillin/clavulanic acid	16 I	≥32 R	8 S	16 I	S 4
TZP	piperacillin/tazobactam	≤4 S	8 S	≤4 S	≤4 S	≤4 S
CAZ	ceftazidime	≤1 S	≤1 S	≤1 S	≤1 S	≥64 R
CPO	cefpirome	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
CPD	cefpodoxime	2 S	2 S	1 S	1 S	≥8 R
NIT	nitrofurantoin	32 S	≤16 S	≤16 S	32 S	≤16 S
ATM	aztreonam	≤1 S	≤1 S	≤1 S	≤1 S	≥64 R
CEP	cefalotin	≥64 R	16 I	16 I	≥64 R	≥64 R
CXA	cefuroxime axetil	32 R	32 R	8 I	16 I	32 R
MEM	meropenem	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S
TMP	trimethoprim	≤0.5 S	≥16 R	≤0.5 S	≤0.5 S	≤0.5 S
SXT	trimethoprim/sulfamethoxazole	≤20 S	≥320 R	≤20 S	≤20 S	≤20 S

* NAL, nalidixic acid; CIP, ciprofloxacin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CAZ, ceftazidime; FOX, cefoxitin; CTX, cefotaxime; CPO, cefpirome; CPD, cefpodoxime; CEP, cephalothin; CXM, cefuroxime; CXA, cefuroxime axetil; ATM, aztreonam; MEM, meropenem; nitrofurantoin; GEN, gentamicin; TOB, tobramycin; SXT, trimethoprim-sulfamethoxazole. ** S, susceptible; I, intermediate; and R, resistant.

Table 15. Prevalence of the *qnr* genes from different countries.

Country	Prevalence of *qnr* genes	Criteria for selecting the clinical isolates	Reference
Norway & Sweden	1.6 % (8/487)	nalidixic acid resistant and/or ciprofloxacin-reduced-susceptible *E. coli* and *klebsiella* spp. isolates from wild-type consecutive, ESBL-, or AmpC- collections	This study
Denmark	1.63 % (2/122)	nalidixic acid-resistant *E. coli* isolates	Cavaco *et al*. 2007
France	1.6 % (2/125)	ESBL-producing *E. coli* and *klebsiella* spp. isolates	Poirel *et al*. 2006 Cattoir *et al*. 2007
Canada	1 % (5/550)	ciprofloxacin and/or tobramycin resistant *E. coli* and *Klebsiella* spp. isolates	Pitout *et al*. 2008
Spain	5 % (15/305)	ESBL-producing enterobacterial isolates	Lavilla *et al*. 2007
USA	15 % (23/153)	ceftazidime-resistant and ciprofloxacin-reduced-susceptible *E. coli* and *klebsiella* spp. isolates	Robicsek *et al*. 2006
China	8 % (29/362)	ESBL-producing *E. coli* and *k. pnumoniae* isolates	Jiang *et al*. 2008

Appendix B

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QRDR/GyrA NP_416734  GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K40-47     GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K2-78      GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K4-13      GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K39-31     GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K38-15     GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLR 60
QRDR/GyrA K8-5       GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDIAVYDTIVRMAQPFSLR 60
QRDR/GyrA K4-43      GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDLAVYNTIVRMAQPFSLR 60
QRDR/GyrA K40-79     GLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDLAVYNTIVRMAQPFSLR 60
                     **********************************************↑***↑************


QRDR/GyrA NP_416734  YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K40-47     YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K2-78      YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K4-13      YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K39-31     YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K38-15     YMLVDGQGNFGSIDGDSAAQH 81
QRDR/GyrA K8-5       YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K4-43      YMLVDGQGNFGSIDGDSAA-- 79
QRDR/GyrA K40-79     YMLVDGQGNFGSIDGDSAA-- 79
                     *******************


QRDR/ParC NP_417491  NASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K2-78      NASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K4-13      NASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K40-47     NASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K39-31     NASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K38-15     ------------GKYHPXGDSXCXEAMVLMAQPFSYRYPLVDGQG 33
QRDR/ParC K8-5       NASAKFKKSARTVGDVLGKYHPHGDIACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K40-79     NASAKFKKSARTVGDVLGKYHPHGDIACYEAMVLMAQPFSYRYPLVDGQG 50
QRDR/ParC K4-43      NXSAKFKKSXRXVGDVLGKXHPHGDIACYEAMVXMAQPFSYRYPLVDGQG 50
                     ********↑*********************


QRDR/ParC NP_417491  NWGAPDDPKSFAAM 64
QRDR/ParC K2-78      NWGAPDDPKSFAAM 64
QRDR/ParC K4-13      NWGAPDDPKSFAAM 64
QRDR/ParC K40-47     NWGAPDDPKSFAAM 64
QRDR/ParC K39-31     NWGAPDDPKSFAAM 64
QRDR/ParC K38-15     NWGAPDDPKSFAAM 47
QRDR/ParC K8-5       NWGAPDDPKSFAAM 64
QRDR/ParC K40-79     NWGAPDDPKSFAAM 64
QRDR/ParC K4-43      NWGAPDDPKSFAAM 64
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Figure 1. Amino acid sequence alignment of the QRDR of GyrA and ParC of the *qnr*-positive isolates detected in this study in comparison with the published wild types. GenBank accession numbers for the wild types are shown. The ClustalW program was used for alignment.

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QnrB    K8-5         ------------------------------------------------------------
QnrB1   DQ777878.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB16  CAP45902.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB3   DQ30392      MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB6   EF523819.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB17  CAP45903.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIDNSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB14  ABX72044.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIDNSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB15  ABX72227.1   MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIESSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB2   AB281054.1   ------------MALALVGEKINRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 48
QnrB13  ABX72043.1   MTPLLYKKTSTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB8   EF526508.1   ------------MALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 48
QnrB11  EU136183.1   MTPLLYKKTSTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB7   ABW03156.3   ------------MALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 48
QnrB    K2-78        ------------------------------------------------------------
QnrB5   DQ303919.1   MTPLLYKNTGIDMTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB10  DQ631414.1   MLSLLYKNTGIDMTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDR 60
QnrB19  ACB12691.1   ------------MTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDR 48
QnrB9   EF653270.1   -----------MMTLALVGEKIDRNRFTGAKVENSTFFNCDFSGADLSGTEFIGCQFYDR 49
QnrB12  ABX72042.1   -----------MMTLALVGEKIDRNRFTGAKVENSTFFNCDFSGADLSGTEFIGCQFYDR 49
QnrB4   DQ303921.2   -----------MMTLALVGEKIDRNRFTGEKVENSTFFNCDFSGADLSGTEFIGCQFYDR 49


QnrB    K8-5         ---KGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 57
QnrB1   DQ777878.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB16  CAP45902.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB3   DQ30392      ESQKGCKFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB6   EF523819.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB17  CAP45903.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB14  ABX72044.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB15  ABX72227.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNANALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB2   AB281054.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 108
QnrB13  ABX72043.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB8   EF526508.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 108
QnrB11  EU136183.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB7   ABW03156.3   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 108
QnrB    K2-78        ---KGCNFSRAMLKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTR 57
QnrB5   DQ303919.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB10  DQ631414.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTR 120
QnrB19  ACB12691.1   ESQKGCNFSRAMLKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGADFRGASFMNMITTR 108
QnrB9   EF653270.1   ESQKGCNFSRAILKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGSDFRGASFMNMITTR 109
QnrB12  ABX72042.1   ESQKGCNFSRAILKDAIFKSCDLSMADFRNVSALGIEIRHCRAQGSDFRGASFMNMITTR 109
QnrB4   DQ303921.2   ESQKGCNFSRANLKDAIFKSCDLSMADFRNINALGIEIRHCRAQGSDFRGASFMNMITTR 109
                     ***:**** ****************** .*************:**************
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Figure 2. Amino acid sequence alignment of the *qnrB* genes from K8-5 and K2-78 detected in this study in comparison with all the *qnrB* variants that have been published so far. GenBank accession numbers for the published *qnrB* variants are shown. The ClustalW program was used for alignment.

```
QnrB    K8-5        TWFCSAYITNTNLSYANFSKVVLEKCELWENRWIGAQVLGATFSGSDLSGGEFSTFDWRA 117
QnrB1   DQ777878.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWIGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB16  CAP45902.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB3   DQ30392     TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB6   EF523819.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB17  CAP45903.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB14  ABX72044.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB15  ABX72227.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB2   AB281054.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 168
QnrB13  ABX72043.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 168
QnrB8   EF526508.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFSTFDWRA 168
QnrB11  EU136183.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB7   ABW03156.3  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFTTFDWRA 168
QnrB    K2-78       TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFTTFDWRA 117
QnrB5   DQ303919.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB10  DQ631414.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRA 180
QnrB19  ACB12691.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSTFDWRA 168
QnrB9   EF653270.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSSFDWRA 169
QnrB12  ABX72042.1  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSSFDWRA 169
QnrB4   DQ303921.2  TWFCSAYITNTNLSYANFSKVVLEKCELWENRWMGTQVLGATFSGSDLSGGEFSSFDWRA 169
                    **********************************:*:******************::*****


QnrB    K8-5        ANFTHCDLTNSELGDLDIRGVDLQGVKLDNY--------------- 148
QnrB1   DQ777878.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERL------ 220
QnrB16  CAP45902.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 226
QnrB3   DQ30392     ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 226
QnrB6   EF523819.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 226
QnrB17  CAP45903.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 226
QnrB14  ABX72044.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLTERLGIAVIG 226
QnrB15  ABX72227.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAIIG 226
QnrB2   AB281054.1  ANFTHCDLTNSELGDLDIRRVDLQGVKLDNYQASLLMERLGIAIIG 214
QnrB13  ABX72043.1  ANFTHCDLTNSELGDLDIRRVDLQGVKLDNYQASLLMERLGIAIIG 226
QnrB8   EF526508.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAIIG 214
QnrB11  EU136183.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAIIG 226
QnrB7   ABW03156.3  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAIIG 214
QnrB    K2-78       ANFTHCDLTNSELGDLDIRGVDLQGVKLD----------------- 146
QnrB5   DQ303919.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDSYQASLLMERLGIAIIG 226
QnrB10  DQ631414.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 226
QnrB19  ACB12691.1  ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERLGIAVIG 214
QnrB9   EF653270.1  ANFTHCDLTNSELGDLDVRGVDLQGVKLDSYQASLILERLGIAVMG 215
QnrB12  ABX72042.1  ANFTHCDLTNSELGDLDVRGVDLQGVKLDSYQASLILERLGIAVIG 215
QnrB4   DQ303921.2  ANVTHCDLTNSELGDLDIRGVDLQGVKLDSYQASLLLERLGIAVMG 215
                    **.***************:* *********
```

Figure 2 (cont). Amino acid sequence alignment of the *qnrB* genes from K8-5 and K2-78 detected in this study in comparison with all the *qnrB* variants that have been published so far. GenBank accession numbers for the published *qnr* variants are shown. The ClustalW program was used for alignment.

```
K8-5      ------------------------------------------------------------
QnrB1     MTPLLYKKTGTNMALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDR 60


K8-5      ---KGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 57
QnrB1     ESQKGCNFSRAMLKDAIFKSCDLSMADFRNSSALGIEIRHCRAQGADFRGASFMNMITTR 120
             ************************************************************


K8-5      TWFCSAYITNTNLSYANFSKVVLEKCELWENRWIGAQVLGATFSGSDLSGGEFSTFDWRA 117
QnrB1     TWFCSAYITNTNLSYANFSKVVLEKCELWENRWIGAQVLGATFSGSDLSGGEFSTFDWRA 180
          ************************************************************


K8-5      ANFTHCDLTNSELGDLDIRGVDLQGVKLDNY--------- 148
QnrB1     ANFTHCDLTNSELGDLDIRGVDLQGVKLDNYQASLLMERL 220
          ******************************
```

Figure 3. Amino acid sequence of the *qnrB* gene from K8-5 was 100% identical to that of the published *qnrB1*. GenBank accession number for *qnrB1* is shown. The ClustalW program was used for alignment.

```
K2-78     --------------------------------------------------KGCNFSRAM 9
QnrB7     MALALVGEKIDRNRFTGEKIENSTFFNCDFSGADLSGTEFIGCQFYDRESQKGCNFSRAM 60
                                                            *********


K2-78     LKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN 69
QnrB7     LKDAIFKSCDLSMADFRNASALGIEIRHCRAQGADFRGASFMNMITTRTWFCSAYITNTN 120
          ************************************************************


K2-78     LSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFTTFDWRAANFTHCDLTNSE 129
QnrB7     LSYANFSKVVLEKCELWENRWMGAQVLGATFSGSDLSGGEFTTFDWRAANFTHCDLTNSE 180
          ************************************************************


K2-78     LGDLDIRGVDLQGVKLD----------------- 146
QnrB7     LGDLDIRGVDLQGVKLDNYQASLLMERLGIAIIG 214
          ****************
```

Figure 4. Amino acid sequence of the *qnrB* gene from K2-78 was 100% identical to that of the published *qnrB7*. GenBank accession number for *qnrB7* is shown. The ClustalW program was used for alignment.

```
QnrS  K40-79    -------------------------------------------NCKFIEQGDIEGCHFD 16
QnrS  K39-31    -------------------------------------------NCKFIEQGDIEGCHFD 16
QnrS  K4-13     -------------------------------------------NCKFIEQGDIEGCHFD 16
QnrS  K40-47    --------------------------------------------CKFIEQGDIEGCHFD 15
QnrS  K38-15    -------------------------------------------NCKFIEQGDIEGCHFD 16
QnrS  K4-43     --------------------------------------------CKFIEQGDIEGCHFD 15
QnrS1 DQ485529.1 METYNHTYRHHNFSHKDLSDLTFTACTFIRSDFRRANLRDTTFVNCKFIEQGDIEGCHFD 60
QnrS2 DQ485530.1 METYRHTYRHHSFSHQDLSDITFTACTFIRCDFRRANLRDATFINCKFIEQGDIEGCHFD 60
                                                            ***************


QnrS  K40-79    VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 76
QnrS  K39-31    VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 76
QnrS  K4-13     VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 76
QnrS  K40-47    VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 75
QnrS  K38-15    VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 76
QnrS  K4-43     VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 75
QnrS1 DQ485529.1 VADLRDASFQQCQLAMANFSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMYFCSAFIS 120
QnrS2 DQ485530.1 VADLRDASFQQCQLAMANFSNANCYGIELRECDLKGANFSRANFANQVSNRMYFCSAFIT 120
                **************************:* **********:***:*************:


QnrS  K40-79    GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQ----- 131
QnrS  K39-31    GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQ----- 131
QnrS  K4-13     GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQ----- 131
QnrS  K40-47    GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQ----- 130
QnrS  K38-15    GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQG---- 132
QnrS  K4-43     GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQG---- 131
QnrS1 DQ485529.1 GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLSRGVFSEDVWGQFSLQGANLC 180
QnrS2 DQ485530.1 GCNLSYANMERVCLEKCELFENRWIGTHLAGASLKESDLSRGVFSEDVWGQFSLQGANLC 180
                **************************:*************************


QnrS  K40-79    ------------------------------------
QnrS  K39-31    ------------------------------------
QnrS  K4-13     ------------------------------------
QnrS  K40-47    ------------------------------------
QnrS  K38-15    ------------------------------------
QnrS  K4-43     ------------------------------------
QnrS1 DQ485529.1 HAELDGLDPRKVDTSGIKIAAWQQELILEALGIVVYPD 218
QnrS2 DQ485530.1 HAELDGLDPRKVDTSGIKIASWQQEQLLEALGIVVFPD 218
```

Figure 5. Amino acid sequence alignment of the *qnrS* genes from K38-15, K4-13, K4-43, K39-31, K40-47, and K40-79 detected in this study in comparison with the published *qnrS1* and *qnrS2* variants. Amino acid sequence of all the *qnrS* genes was 100% identical to that of the published *qnrS1*. GenBank accession numbers for *qnrS1* and *qnrS2* are shown. The ClustalW program was used for alignment.

Appendix C

Reagents and Solutions

Antibody solution 30.0 ml Buffer #1 Solution 3.00 µl AP-conjugate antibody (Vial 8, DIG DNA labeling and detection kit, Roche Applied Science, Germany)	**Basic buffer** 100 mM EDTA pH 7.5 6 mM Tris-HCl pH 7.6 1 M NaCl 0.5 % Brij 58
10X Blocking solution 50 g Blocking Reagent powder 500 ml Maleic acid buffer	**Blocking solution** 1 volume 10X Blocking solution 9 volume Maleic acid buffer
Color-substrate solution 40 µl NBT/BCIP (vial 9, DIG DNA labeling and detection kit, Roche Applied Science, Germany) 2 ml Detection buffer	**Denaturing buffer** 58.4 g of NaCl 20 g of NaOH 1 L ddH2O
Depurination buffer 20.8 mls concentrated HCl Brought to 1 liter with ddH2O	**Detection buffer** M Tris-HCl M NaCl
Lysis buffer (for 5 plugs) 10 ml basic buffer 0.02 g deoxycholate 0.05 g N-laurosylsarcosine, Na-salt 0.01 g lysozyme 1 µl RNase ONE (10U/µl)	**Hybridization solution** 25.0 ml 20X SSC 10.0 ml 1% Lauroyl Sarcosine 100.0 ml 20% SDS 10.0 ml 10% Blocking Reagent 55.0 ml ddH2O
2 % LMP-agarose 50 ml PIV buffer 1 g LMP-agarose (Bio-Rad, USA)	**ESP buffer:** 0.5 M EDTA (pH 9.0 to 9.5) 1 % Sarkosyl 50 µg/ml proteinase K
Maleic acid buffer 0.1M Maleic Acid 0.15 M NaCl	**Neutralizing buffer 5x** 79.25 g Na2HPO4 60.25 g NaH2PO4·H2O Brought to 1 liter with dd H2O
Neutralizing buffer 1x 1 volume 5x neutralizing buffer 4 volume dd H2O	**PIV buffer:** 1 M NaCl 10 mM Tris-HCl (pH 7.6)

Reagents and Solutions

Phosphate-buffered saline (PBS) solution 0.14 M NaCl 0.003 M KCl 0.008 M Na2H2PO4x2H2O 0.0015 M KH2PO4	**Prehybridization solution** 250 ml 20X SSC 10 ml 1 % N-lauroylsarcosine 2 ml 10 % SDS 100 ml 10X Blocking solution 638 ml ddH2O
Proteinase K-stock solution 100 mg Proteinase K 10 ml 50 mM Tris-CL pH 8.0 10 mM CaC	**20X SSC** 88.2 g Trisodium citrate (citric acid) 175.3 g NaCl Brought to 1 liter with ddH2O
S1-nuclease buffer 1 volume S1-nuclease buffer (10X) (Sigma-Aldrich, USA) 9 volume ddH2O	**10X TBE buffer** 108 g Tris base 55 g boric acid 9.3 g EDTA ddH2O to 1 liter
TE buffer 0.79 g Tris HCl 0.19 g EDTA ddH2O to 0.5 liter	**Washing buffer** 0.1 M Maleic acid buffer 0.15 M NaCl 0.3 % Tween 20 (v/v)

Bacterial Growth media

Brain Heart Infusion (BHI) broth BHI broth base (Oxoid Ltd, UK) ddH2O	**Muller Hinton (MH) agar** MH II agar base (Becton, Dickinson and Company, USA) ddH2O
Iso-seneitest agar (ISA) ISA agar base (Oxoid Ltd, UK) ddH2O	**Freeze broth** Brain Heart Infusion base (Oxoid Ltd, UK) ddH2O Glycerol
Luria-Bertani (LB) agar LB agar base (Becton, Dickinson and Company, USA) ddH2O **Luria-Bertani (LB) broth** LB broth base (Becton, Dickinson and Company, USA) ddH2O	**Green agar** Tryptose blood agar base (Oxoid Ltd, UK) Lactose LP0070 Bromtymolblue solution 0.2 % ddH2O
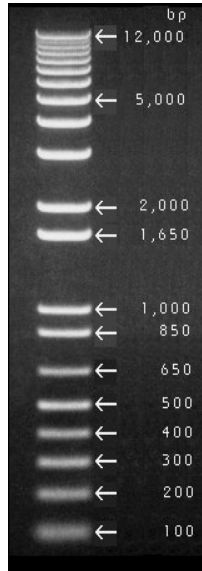
Figure 1. Gel electrophoresis of the 1 Kb Plus DNA Ladder
(http://tools.invitrogen.com/content/sfs/manuals/10787018.pdf)
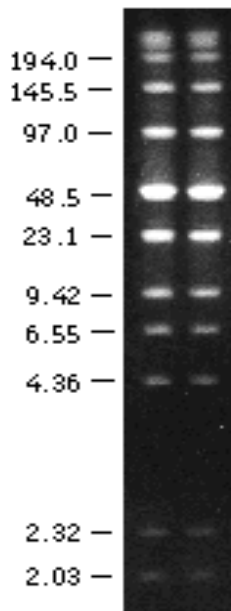

Figure 2. Pulsed field gel separation of Low Range PFG Markers using a CHEF apparatus.

(http://www.neb.com/nebecomm/products_intl/productN0350.asp)

Appendix D

Kit 1. Purification of PCR products according to the E.Z.N.A™ Cycle-Pure Kit Spin Protocol (Omega Bio-tek, USA):

1. 4-5 volumes of CP Buffer (90 µl) were added to 1 volume of PCR-mixture (20 µl) and mixed.
2. A HiBind® DNA spin column was placed in a 2 ml collection tube.
3. The sample was transferred to the column and centrifuged at 13000 rpm for 1 min.
4. The flow-through was discarded and the spin column was placed back into the same tube.
5. 0.75 ml Wash Buffer diluted with ethanol was added and the tube was centrifuged at 13000 rpm for 1 min.
6. The flow-through was discarded, and the washing was repeated once.
7. The flow-through was discarded and the column was placed in the same tube and the tube was centrifuged at 13000 rpm for 1min to dry the column matrix.
8. The column was placed in a clean eppendorf tube.
9. 30 µl Elution Buffer was added to the centre of the column-membrane and the tube was centrifuged at 13000 rpm for 1min.

Kit 2. Plasmid DNA extraction according to the E.Z.N.A™ Plasmid Miniprep Protocol I (Omega Bio-tek, USA):

1. Green agar plate was streaked and incubated over night at 37°C.
2. LB-broth medium (5 ml each) was inoculated with a single isolated colony from the agar plate and incubated over night at 37°C with vigorous shaking (about 300 rpm).
3. The bacterial cells were pelleted by centrifugation at 10,000 x g for 1 min at room temperature.

4. The supernatant was discarded and the bacterial ballet was resuspended by adding 250 μl of Solution I/RNase A solution, and vortexing.

5. 250 μl of Solution II were added and gently mixed by inverting and rotating the tube several times to obtain a clear lysate.

6. 350 μl of Solution III were added and mixed immediately by inverting several times until a flocculent white precipitate was formed.

7. The tube was centrifuged at 13000 x g for 10 min.

8. The cleared supernatant was added carefully into a HiBind® Miniprep spin column assembled in a provided 2 ml collection tube and centrifuged at 13000 x g for 1 min.

9. The flow-through was discarded and the spin column was placed back into the same tube.

10. 500 μl of Buffer HB was added and the tube was centrifuged at 13000 x g for 1 min.

11. The flow-through was discarded and 700 μl of DNA Wash Buffer diluted with absolute ethanol was added and the tube was centrifuged at 13000 x g for 1 min.

12. The flow-through was discarded and the column was placed in the same tube and the empty tube was centrifuged at 13000 x g for 2 min to dry the column matrix.

13. The column was placed in a clean eppendorf tube.

14. 50 μl Elution Buffer was added to the centre of the column-membrane and the tube was centrifuged at 13000 x g for 1min.

Kit 3. Extraction and purification DNA from agarose gel according to the QIAquick SpinHandbook for QIAquick Gel Extraction Kit (QIAGEN, Germany):

1. The DNA fragment was excised from the agarose gel with a clean sharp scalpel.

2. The gel slice was weighed and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg or approximately 100 μl)

3. The tubes were incubated at 50º C for 10 min.

4. A QIAquick spin column was placed in a provided 2 ml collection tube.

5. The sample was applied to the QIAquick spin column and centrifuged for 1 min.

6. The flow-through was discarded and QIAquick spin column was placed back in the same collection tube.

7. 0.5 ml of Buffer QG was added to the QIAquick spin column and centrifuged for 1 min.

8. The flow-through was discarded and QIAquick spin column was placed back in the same collection tube.

9. 0.75 ml of Buffer PE was added to the QIAquick spin column and centrifuged for 1 min.

10. The flow-through was discarded and the empty QIAquick spin column was placed back in the same collection tube and centrifuged for 1 min.

11. The empty QIAquick spin column was placed in a clean 1.5 ml microcentrifuge tube.

12. 30 μl Elution Buffer was added to the center of the QIAquick spin column membrane. Then the column was let stand for 1 min after that centrifuged for 1 min.

13. All centrifugation steps were carried out at 13000 rpm.

14. Ethanol 96% was added to Buffer PE before use.