Plasmid mediated quinolone resistance in Enterobacteriaceae

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(met een samenvatting in het Nederlands)

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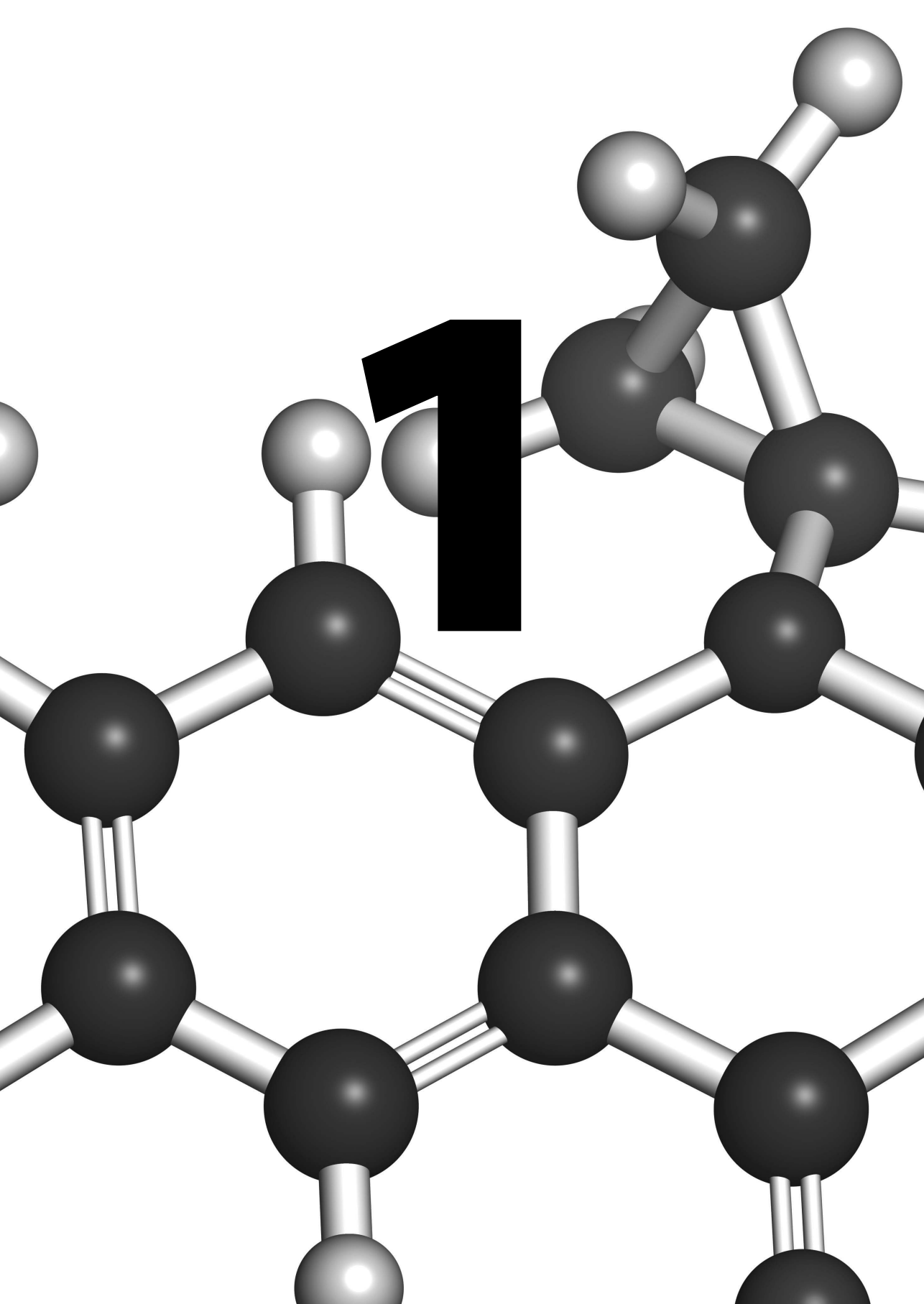

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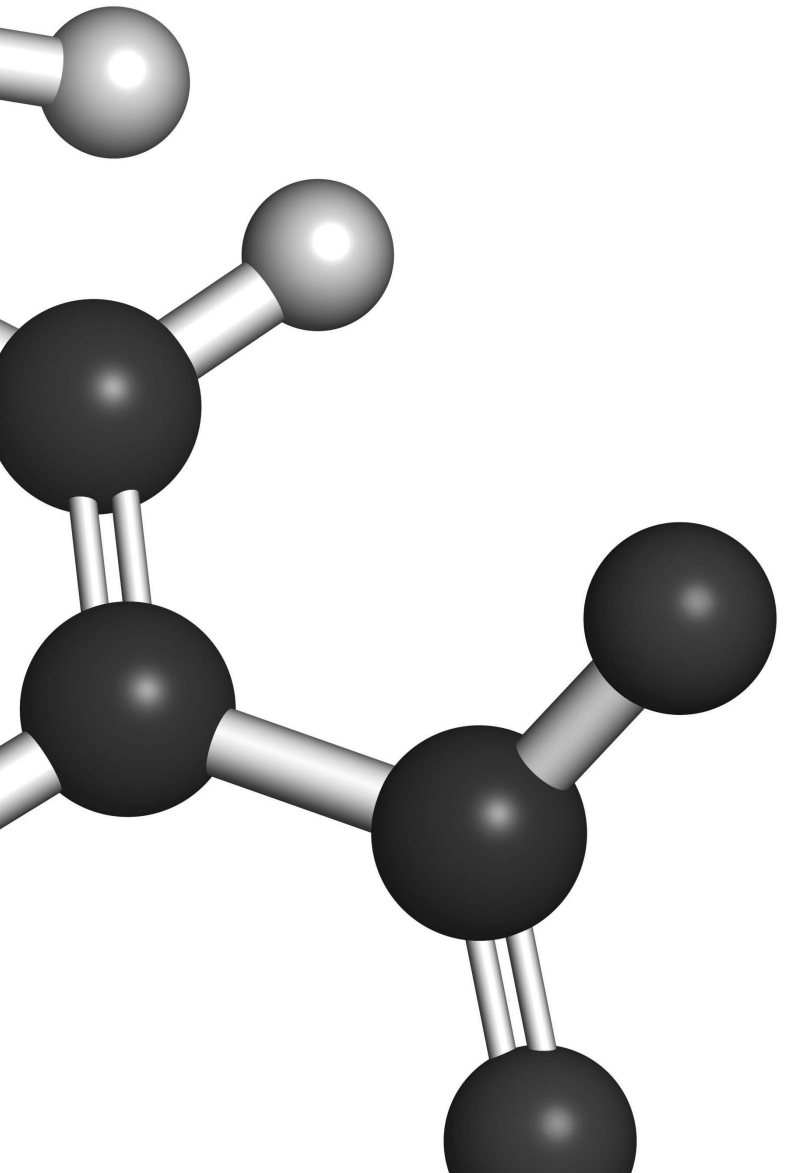
Chapter 1

General Introduction



Quinolones

Quinolones are synthetic drugs active against a broad range of bacteria. This class of antibiotics is important in human and veterinary medicine and used for treatment of various bacterial infections. The World Health Organization labeled quinolones as 'critically important antibiotics' for human medicine based on defined criteria concerning (I) the exclusivity of an antimicrobial agent to treat a serious human infectious disease and (II) the chance that the treated disease is caused by organisms that may be transmitted via nonhuman sources or antimicrobial resistance is gained via nonhuman sources [1].

History of quinolones

The introduction of quinolones started in 1962 with the discovery of the bactericidal effects of nalidixic acid, which was actually a byproduct of synthesis of chlorequine (an anti-malaria drug). Nalidixic acid was used for treatment of uncomplicated urinary tract infections caused by Gram-negative bacteria [2]. In the 1970s, other quinolones like oxolinic acid and flumequine, were synthesized and introduced. The more potent second generation quinolones were called fluoroquinolones, because of the addition of a fluor atom at position C6 of the quinolone nucleus. The first licensed fluoroquinolone was norfloxacin for urinary tract infections, soon followed by the introduction of ciprofloxacin. Ciprofloxacin was the first fluoroquinolone active outside the urinary tract and is globally used in humans for a variety of infections caused by Gram-negative or Gram-positive bacteria. Other new generation fluoroquinolones like levofloxacin, sparfloxacin and moxifloxacin were introduced for treatment of respiratory infections caused by Gram-positive bacteria. In the last two decades, the use of fluoroquinolones has grown and is considered as one of the most important classes of antibiotics in human medicine. In food-producing animals older generation quinolones (like oxolinic acid and flumequine) were licensed for use at the beginning of the 1970's and fluoroquinolones during the late 1980's and early 1990's [3]. Since then new fluoroquinolones came available on the market as veterinary medicines. Some of the most important fluoroquinolones for veterinary use are enrofloxacin, difloxacin, danofloxacin and marbofloxacin for treatment of respiratory, urinary tract or skin infections[4].

Usage of antibiotics (including (fluoro)quinolones) in The Netherlands

The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) of the European Medicine Agency (EMA) reported in 2012 that The Netherlands was one of the countries in Europe with the highest sales of antibiotics for use in food-producing animals in relation to the biomass produced [5]. In contrast, antibiotic usage in human medicine in The Netherlands was reported to be among the lowest in Europe [6]. This situation and the concerns about the high prevalence of resistant bacteria, like MRSA and ESBL-producers in food-producing animals triggered a political debate resulting in mandatory targets on reduction of antibiotic consumption in animals [7]. Consequently, governmental reduction targets and measures initiated by private parties involved in animal production and the Dutch Royal Veterinary Association resulted in a decrease in sales of antibiotics of 58% in five years (2009-2013) based on annual sales data of the Association of Veterinary Pharmacy in The Netherlands FIDIN (see Figure 1). This means that the policy objective for 2013 (a 50% reduction) is accomplished. To be able to reach this goal, The Netherlands Veterinary Medicine Authority (SDa) was established which launched a registration system for antibiotic usage in animals in 2011. This system is based on prescription per animal per farm expressed as the number of days an average animal is treated in that year on that particular farm ($DDDA_F/Y$). With the data of the registration system SDa is able to benchmark veterinarians and farmers targeting further reduction of antibiotic usage in animals[8, 9]

Figure 1. Antimicrobial veterinary medical products sales 1999-2013 in kg (thousands) [9]

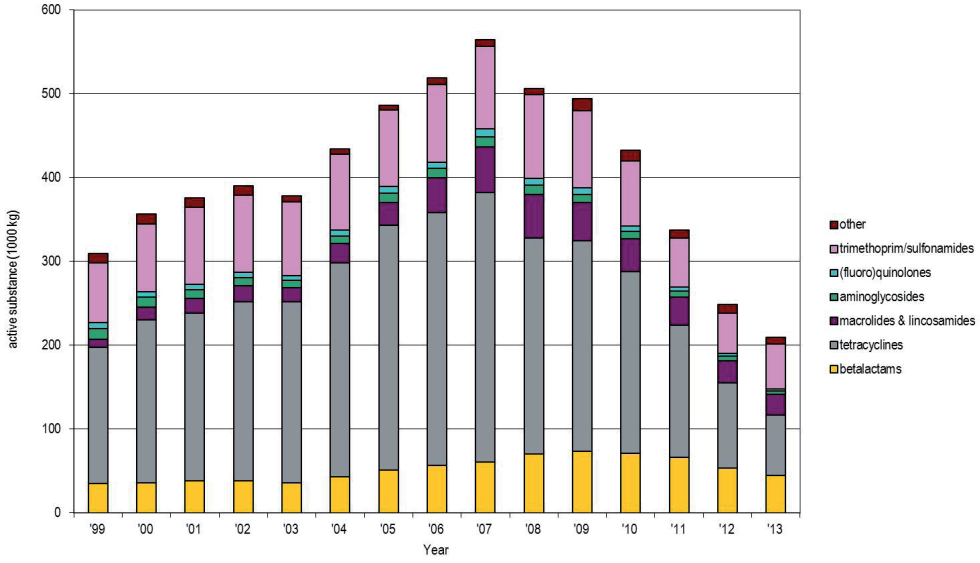


Figure 1 was printed with permission of The Netherlands Veterinary Medicines Authority (SDa)

The sales of (fluoro)quinolones (all quinolones) in The Netherlands decreased from nine tons (= 1.59% of total sales) in 2007 to three tons in 2012 (= 1.25% of the total sales). In the same period the sales of fluoroquinolones decreased to 0.8 tons in 2012 (= 0.33% of the total sales)[10]. However, one has to bear in mind that fluoroquinolones are very potent agents. Hence, conversion of the amount of fluoroquinolones sold (weight of active substance per year) to actual usage (animal defined daily dosages per year) demonstrates that large numbers of food-producing animals were exposed to these drugs. In 2013, a further reduction (50%) in sales of fluoroquinolones was accomplished, but the sales of quinolones slightly increased[11]. These data suggest an ongoing reduction in usage of fluoroquinolones in animals with a slight shift to the usage of quinolones as alternative therapy.

In The Netherlands, quinolones (mainly flumequin) are licensed for oral use in broilers, veal calves and pigs. Fluoroquinolones are licensed for oral and parenteral use in broilers, turkeys, veal calves, cattle and pigs (http://www.cbg-meb.nl/CBG/en/, last accessed on 02-07 2014). The amount of (fluoro)quinolones used differs considerably between animal species. The most recent data on the usage of quinolones and fluoroquinolones per animal species in 2013, as provided by SDa[9], are presented in Table 1.

The highest usage of quinolones was reported on broiler farms (1.29 $DDDA_F$/Y) and the highest usage of fluoroquinolones was reported on turkey farms (1.33 $DDDA_F$/Y). The usage of quinolones was also relatively high in young calves on white veal farms and rosé starter farms. Importantly, the usage of fluoroquinolones in calves decreased to a maximum usage of 0.02 $DDDA_F$/Y on rosé starter farms. In addition, fluoroquinolones were not used on cattle farms and pig farms.

Table 1. Usage of quinolones and fluoroquinolones in 'Defined Daily Dose Animal' per year ($DDDA_F$/Y) for different farm types in 2013[12].

Farm type	Average DDDAF/Y Quinolones (O)	Average DDDAF/Y Fluoroquinolones (O/P)
White veal farms	1.08	0.01/0.01
Rosé veal starter farms	0.33	0.00/0.02
Rosé veal fattening farms	0.01	0.00/0.01
Rosé combination farms	0.06	0.00/0.01
Dairy farms	0.00	0.00/0.00
Suckler cow farms	0.00	0.00/0.00
Rearing cattle	0.00	0.00/0.00
Beef bull farms	0.01	0.00/0.00
Sow and piglet farms	0.02	0.00/0.00
Pig fattening farms	0.01	0.00/0.00
Broilers	1.29	0.22/0.00
Turkeys	0.14	1.33/0.00

O = Oral administration and P = parenteral administration

Usage of (fluoro)quinolones and resistance

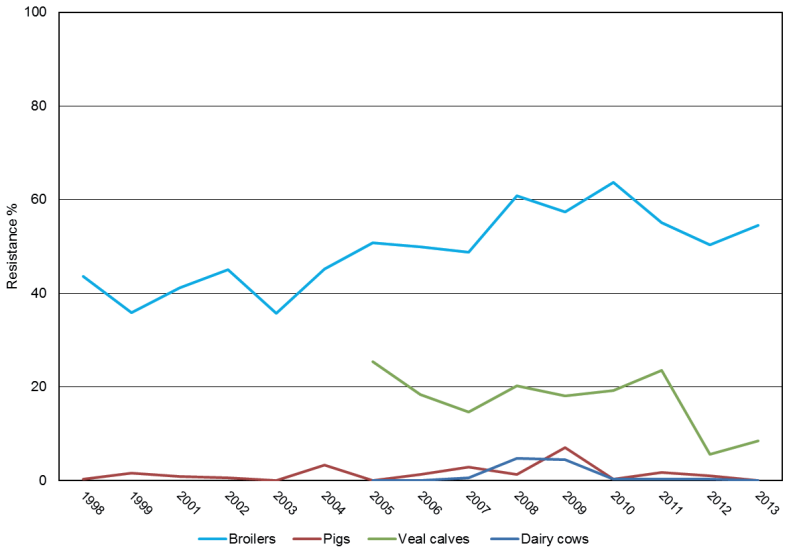
The introduction of fluoroquinolones for veterinary use in the late 80'ties was followed by fluoroquinolone resistance in bacteria of food-producing animals and spread of zoonotic bacteria to humans[13]. Actually, the risk of antibiotic usage in animals for the public health is clearly illustrated by a Dutch study in which the emergence of quinolone resistant campylobacters in animals and humans since the use of a fluoroquinolone (enrofloxacin) in the drinking water of broilers was described [14]. Several other studies have documented an increase of fluoroquinolone resistance in campylobacter after treatment of food-producing animals[15, 16]. For *Salmonella enterica* different European countries reported an increase of nalidixic acid resistance after introduction of enrofloxacin[17, 18].

Because of the potential risk of transmission of quinolone resistant bacteria from animals to humans, the usage of (fluoro)quinolones in animals in Europe has been under discussion for many years [3]. Fluoroquinolones are not indicated for uncomplicated gastrointestinal infections, but these antibiotics are important for treatment of complicated *Salmonella* infections in humans and in patients at risk. Possibly, the potential risk of treatment failure due to fluoroquinolone resistance in humans might lead to a total ban in usage of fluoroquinolones in food-producing animals in the future.

In The Netherlands, data on antimicrobial usage and resistance in animals are published in annual reports called 'MARAN'; Monitoring of Antimicrobial Resistance and Antimicrobials Usage in Animals in The Netherlands (http://www.wageningenur.nl/en/Research-Results/Projects-and-programmes/MARAN-Antibiotic-usage.htm). The monitoring system includes commensal *E. coli* as indicator organism of the Gram-negative intestinal flora according to EFSA protocols [19]. For the interpretation of minimum inhibitory concentrations (MIC) values resistant isolates are defined using epidemiological cut-off values (ECOFF) according to EUCAST (www.eucast.org). Especially for ciprofloxacin, the cut-off value for *E. coli* (ECOFF: > 0.06 mg/L) is much lower than the clinical breakpoint (R: > 1 mg/L). Non-wild type isolates with MICs above the ECOFF are listed resistant which is appropiate for monitoring purposes. As a consequence, the resistance levels for ciprofloxacin in animal *E. coli* isolates will be higher compared to resistance data of human *E. coli* isolates in which the clinical breakpoint of 1 mg/L is used.

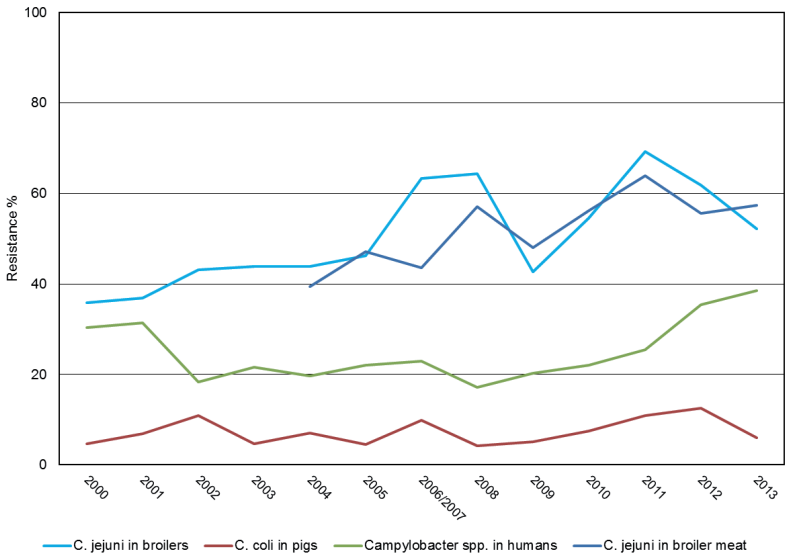
Resistance to fluoroquinolones in commensal *E. coli* was reported at a constant high level from 2002 to 2012 in broilers (35.8 – 63.7%) and at a lower level in veal calves (5.6 – 25.5%). In the same period, resistance to fluoroquinolones was infrequently present at low levels in *E. coli* in slaughter pigs (0 - 7.1%) and dairy cattle (0 - 4.7%) [10]. In 2013, the resistance rates for fluoroquinolones were still high in broilers (54.5%) and at a lower level in veal calves (8.5%). No fluoroquinolone resistant *E. coli* were identified in slaughter pigs and dairy cattle (Figure 2).

Figure 2. Trends in ciprofloxacin resistance (%) of commensal *E. coli* from broilers, pigs, veal calves and dairy cows in 1998 - 2013



The comparison of fluoroquinolone resistance levels of *Campylobacter* spp. from different animals and humans demonstrates high levels of fluoroquinolone resistance in *C. jejuni* obtained in broilers and broiler meat, but low levels resistance in *C. coli* obtained in slaughter pigs [10] (Figure 3). The differences in resistance grades between animal species clearly reflect the difference in usage of fluoroquinolones (see Table 1). In addition, fluoroquinolone resistance of campylobacters obtained in humans is still increasing. Nevertheless, the fluoroquinolone resistance level of human *C. jejuni* was slightly lower in 2013 compared to 2012[11].

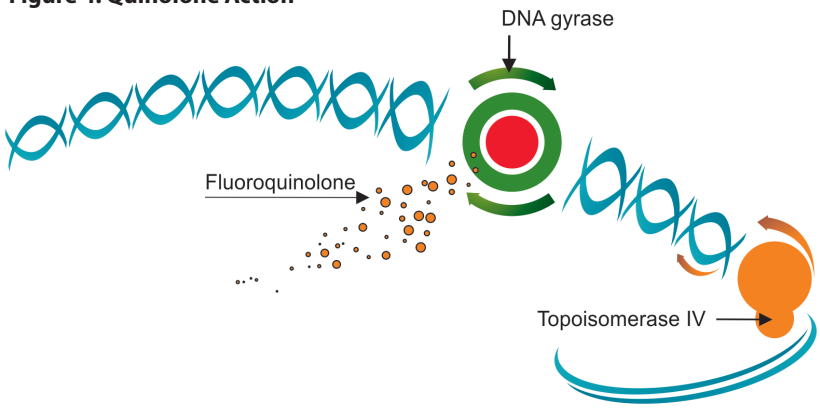
Figure 3. Trends in ciprofloxacin resistance(%) of *Campylobacter* spp. from broilers, broiler meat, pigs and humans in 2000 - 2013



(Fluoro)quinolone action

The targets for (fluoro)quinolones are two essential bacterial enzymes beloninging to the topoisomerase II enzymes; DNA gyrase and topoisomerase IV (Figure 4). Both homologous enzymes are involved in replication, transcription, recombination and repair of DNA[20]. More specifically, DNA gyrase induces the negative supercoiling of DNA and topoisomerase IV is involved in the segregation of replicated daughter chromosomes[2] . DNA gyrase consists of two pairs of subunits called GyrA and GyrB. Similarly, topoisomerase IV also consists of two pairs of subunits designated as ParC and ParE [20]. (Fluoro)quinolones trap topoisomerase enzymes by formation of drug-enzyme-DNA complexes (cleaved complexes) which interfere with the DNA replication and cause permanent double-stranded breaks lethal for the bacterial cell [21].

Figure 4: Quinolone Action[22]



Fluoroquinolones bind topoisomerase II enzymes (DNA gyrase and topoisomerase IV) interfering DNA replication

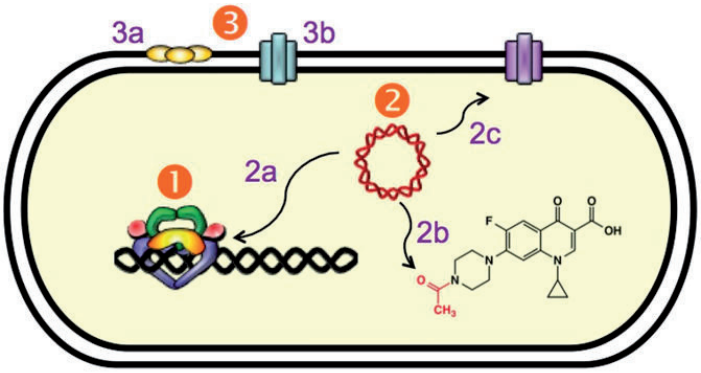
Quinolone resistance determinants

Chromosomally encoded resistance

Quinolone resistance is most frequently arised spontaneously by mutations in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). These mutations are clustered in the quinolone resistance determining region (QRDR). Mutations outside these regions are mostly not associated with quinolone resistance [4]. In Gram-negative bacteria DNA gyrase (*gyrA*) is considered the primary target of (fluoro)quinolones, whereas in some

Gram-positive bacteria topoisomerase IV (*parC*) seems to be more important for resistance[4]. Both in *E. coli* and *Salmonella*, mutations in *gyrA* appear most frequently at codons Ser83 and Asp87.The number of mutations in the QRDR confer a stepwise increase in the level of resistance to fluoroquinolones. One single point mutation in the QRDR of *gyrA* usually leads to high-level resistance to nalidixic acid and reduced susceptibility to fluoroquinolones. However, one or more additional mutations in *gyrA* and/or *parC* are needed for high-level resistance against fluoroquinolones [20] . Other resistance mechanisms caused by chromosomal mutations can also play a role in quinolone resistance like decreased outer membrane permeability or up regulation of efflux pumps [20] (Figure 5).

Figure 5: Mechanisms of quinolone resistance[2]



Mechanisms of (fluoro)quinolone resistance. (1) Target-mediated resistance. Mutations in gyrase and topoisomerase IV weaken quinolone–enzyme interactions. (2) Plasmid-mediated resistance. (2a) Qnr proteins (yellow) decrease topoisomerase–DNA binding and protect enzyme–DNA complexes from (fluoro)quinolones. (2b) Aac(6′)-Ib-cr is an aminoglycoside acetyltransferase that acetylates the free nitrogen on the C7 ring of ciprofloxacin and norfloxacin, decreasing their effectiveness. (2c) Plasmid-encoded efflux pumps decrease the concentration of (fluoro)quinolones in the cell. (3) Chromosome-mediated resistance. (3a) Underexpression of porins in Gram-negative species decreases drug uptake. (3b) Overexpression of chromosome-encoded efflux pumps decreases drug retention in the cell.

Plasmid Mediated Quinolone Resistance (PMQR)

Currently, three different types of resistance mechanisms are described associated with plasmid mediated quinolone resistance: (I) target protection proteins encoded by *qnr* genes, (II) specific efflux pumps encoded by *qepA* [23] and a multidrug pump *oqxAB* [24] and (III) an altered aminoglycoside acetyl transferase encoded by *aac(6′)-1b-cr* capable of reducing ciprofloxacin activity [25]. The presence of PMQR genes generally results in reduced

susceptibility to (fluoro)quinolones in Enterobacteriaceae below clinical breakpoints [26, 27]. In contrast to chromosomally encoded quinolone resistance, plasmid mediated quinolone resistance (PMQR) can spread horizontally between bacteria.

Qnr families

The most prevalent PMQR gene family in Enterobacteriaceae encode Qnr proteins which belong to the pentapeptide repeat family. Although, the exact mechanism is not completely understood, Qnr proteins are believed to protect the bacterial cell from quinolone action by binding directly to DNA gyrase and topoisomerase without affecting the enzyme activity [28, 29]. Currently, five different sub-families of *qnr* genes have been identified with an increasing number of variants: *qnrA(1-7), qnrS (1-9) qnrB(1-74), qnrC* and *qnrD (1-2)* (http://www.lahey.org/qnrStudies/, last accessed 15 May 2014). The first transferable quinolone resistance was reported in 1998 in a clinical multi-drug resistant *Klebsiella pneumoniae* isolate by Martinez-Martinez. This group studied a plasmid called pMG252 which unexpectedly increased the Minimal Inhibitory Concentrations (MICs) to quinolones. Subsequent cloning experiments revealed the presence of a 657-bp open reading frame which encoded for a Qnr (quinolone resistance) protein later renamed QnrA1[30]. In 2003, QnrS was identified in an outbreak strain of *Shigella flexneri* causing enterocolitis in Japan with 59% amino acid identity to QnrA1[31]. Another Qnr protein, designated QnrB, was reported in 2006 in clinical *Klebsiella pneumonia* isolates from India by Jacoby and colleagues [32] sharing 40% identity to QnrA1. In 2009, QnrC was identified in a clinical *Proteus mirabilis* isolate from China [33]. In the same year, QnrD was identified in different serovars of *Salmonella enterica* also from China [34]. The proteins shared 68% and 48% amino acid identity with QnrA1, respectively.

The origin of *qnr* genes

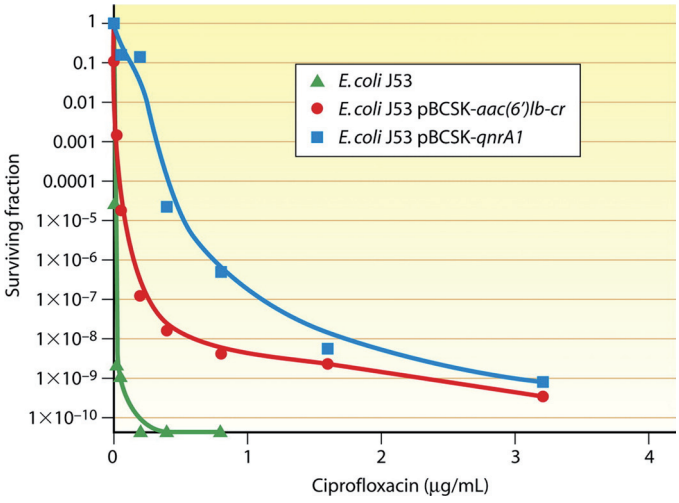
Several studies indicate that the aquatic environment is the original source of *qnr* genes. For example a *qnrA* gene, closely related to *qnrA1*, was identified on the chromosome of *Shewanella algae* [35]. In addition *Vibrio splendidus* was identified as a source for *qnrS*-like determinants [36]. More recently, *qnrB*-like genes were identified on the chromosome of *Citrobacter* spp [37]. Homologues of *qnr* genes have also been identified on the chromosomes of different *Vibrio* species [38].

All these bacterial species are commonly found in aquatic environments which indicates that *qnr* genes probably origin from chromosomes of aquatic bacteria.

Effect of PMQR on susceptibility to (fluoro)quinolones (MIC and MPC)

In general, PMQR-positive isolates display a unusual quinolone resistance phenotype conferring reduced susceptibility to both fluoroquinolones and nalidixic acid with a 8- to 32-fold increase in MICs of ciprofloxacin and 2- to 8-fold increase in MICs of nalidixic acid[27]. These properties can be useful for detection of PMQR determinants[26, 39]. This distinctive resistance phenotype can only be recognized in isolates without mutations in the QRDR. The presence of PMQR has an additional effect on other quinolone resistance mechanisms which can ultimately lead to fluoroquinolone resistant bacteria with MICs above the clinical breakpoint [40, 41]. Moreover, PMQR is known to facilitate the selection of chromosomal mutants by elevation of the Mutations Prevention Concentration (see Figure 6). The effects of PMQR on the MIC and MBC of fluoroquinolones elucidate the clinical importance of these resistance determinants.

Figure 6. MPC assay of *E. coli* J53 without any PMQR compared to *E. coli* J53 with *aac(6')-1b-cr* or *qnrA1*[27]



Genetic environment of PMQR genes

PMQR genes have been identified on different types of plasmids varying from large conjugative plasmids to small non-conjugative plasmids [27]. Plasmids carrying *qnrA1* belong to at least 3 different incompatibility (Inc) groups: FII, HI2 and A/C. The *qnrA1* gene is often bracketed by insertion sequence IS*CR1* as part of a *sul1*-type class 1 integron complex[27] . Variants of the *qnrB* gene have also been found on different conjugative plasmids (IncL/M and IncN) often associated with IS*CR1* [27]. More recently, *qnrB19* has been found on a novel transposon with IS*Escp1* in *E. coli* from France [42] and on a 40kb IncR plasmid brackened by IS26 elements in *E. coli* from The Netherlands [43]. Both *qnrA* and *qnrB* are frequently associated with other antibiotic resistance genes conferring resistance to aminoglycosides, tetracycline, sulfonamides, trimethoprim and β-lactam antibiotics[21]. In contrast, *qnrS* is less often associated with other resistance genes, not associated with IS*CR1* and mainly present on smaller plasmids (e.g. IncX2). Moreover, *qnrS* is not embedded in an integron structure, although it has been found upstream a Tn3-like transposon or upstream insertion sequence IS*Ec1* [21]. Other plasmid mediated quinolone resistance genes like *aac(6')-1b-cr* and *qepA* are frequently identified on large conjugative IncF plasmids [27]. Both *qnrC* (reported once in *Proteus mirabilis*) and *qnrD* have been identified on small non-conjugative plasmids predominantly in *Proteeae* tribe (*Proteus*, *Providencia* and *Morganella* genera). Such small plasmids can be mobilizable in presence of conjugative helper plasmids using their relaxase genes for transfer [44]. However, no relaxase genes were identified in these *qnrD*-carrying plasmids. In summary, PMQR genes are present on a variety of plasmids embedded in different mobile genetic elements.

Occurrence of PMQR genes in Enterobacteriaceae

Early reports on the occurrence of PMQR origined from the USA [45] and Asia [46] followed by reports from Africa[47], Europe[48], Australia [49] and South America [50]. This increasing number of reports from different continents was probably more the result of initial screening studies rather than indicating a futher spread of PMQR genes. The majority of the studies included human clinical isolates, often fluoroquinolone resistant or reduced susceptible to fluoroquinolones or resistant to 3rd generation cephalosporins[21]. Less frequently, isolates from animals, food, feed or the environment were studied [27, 51]. Additional reports on PMQR quickly appeared from several European countries (Germany[48], UK [52], and Denmark [53, 54]) indicating the dissemination of this new type of quinolone resistance throughout Europe.

Although, several European countries reported the presence of PMQR genes, a study on a European scale was missing at the start of this thesis . Furthermore, different inclusion criteria were used in different studies which makes comparison between countries rather difficult. In The Netherlands, the first PMQR (*qnrA1*) was described in 2006 in a Dutch *Enterobacter cloacae* complex outbreak strain [55]. Besides this coincidental finding no other information was available on the occurence of PMQR in The Netherlands.

Goal of the thesis

- To determine the occurrence and prevalence of PMQR genes in *Salmonella* from human and animal sources and in *E. coli* from animal sources in The Netherlands and other European countries.
- To characterize the genetic background of the PMQR genes and the plasmids they are located on.
- To characterize multidrug resistant PMQR positive Enterobacteriaceae imported in The Netherlands via people from Africa or via food from Southeast Asia.

Outline of this thesis

Chapter 2 is about PMQR in Enterobacteriaceae in The Netherlands and contains two small studies about the description of PMQR genes in *Salmonella enterica* (chapter 2a) and *E. coli* (chapter 2c) in The Netherlands. This chapter also covers a study on the characterization of PMQR carrying plasmids in *Salmonella enterica* obtained in The Netherlands (chapter 2b). Chapter 3 is about PMQR in Enterobacteriaceae in Europe and contains a study initiated by the EURL-AR about the prevalence of PMQR genes in *Salmonella enterica* and *E. coli* from thirteen different European countries (chapter 3a). As a result of this study, *qnrD* was identified for the first time in Europe in several *Salmonella* serovars. Consequently, a study was conducted to characterize *qnrD*-positive strains and plasmids which is included in chapter 3b. Finally, chapter 4 contains two studies describing the import of PMQR-positive, multidrug resistant Enterobacteriaceae. The first study (chapter 4a) describes the characterization of multi-drug resistant *Salmonella* imported by patients from the African continent and the second study (chapter 4b) describes the finding of multi-drug resistant Enterobacteriaceae obtained in imported fresh culinary herbs from Southeast Asia.

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2

Chapter 2

PMQR in Enterobacteriaceae in The Netherlands


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CHAPTER 2A

First report of *qnr* genes in *Salmonella* in The Netherlands

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Sir,

Currently a variety of *qnrA, B and S* genes are commonly isolated from clinically important Enterobacteriaceae[1]. Moreover *qnr* genes have been detected in non-typhi *Salmonella enterica* serotypes in Europe, the United States, Africa, Australia and Asia[2].
In a recent study the presence of *qnrA1* in The Netherlands was first detected in a multidrug-resistant *Enterobacter cloacae* carrying a conjugative R plasmid originating from a large outbreak in the University Medical Centre Utrecht (UMCU)[3]. In The Netherlands, data on the occurrence of *qnr* genes in other Enterobacteriaceae such as *Salmonella enterica* are lacking. The aim of our study was to detect and characterize *qnr* genes in a selection of Dutch *Salmonella* isolates.

The strain collection of the Central Institute for Animal Disease Control encompasses 15011 *Salmonella* isolates collected from humans (n = 8143), poultry (n = 2570), pigs (n = 992), cattle (n = 518) and other sources (n = 2788) in the period 1999 – 2006. Thirty-nine of these isolates showed a typical phenotype being low-level resistant to ciprofloxacin (MICs: 0.25 – 1 mg/L) but still susceptible to nalidixic acid (MICs: 8 – 16 mg/L). All isolates showing this typical phenotype were screened for the presence of *qnrA*, *qnrB* and *qnrS* genes by PCR using primers and conditions as previously described[4]. Transconjugants of *E. coli* J53 harboring the plasmids pMG 252 (*qnrA1*) or pMG298 (*qnrB1*) and *Salmonella enterica* serotype Bovismorbificans strain AM12888 (*qnrS*1) were used as positive controls. In addition all isolates were screened for the presence of *aac(6')-1b-cr* by PCR as described by Robicsek[5] using transconjugant 10-2 containing plasmid pHSH10-2 as a positive control. Gel electrophoresis revealed 3 *qnrB* and 31 *qnrS* positive isolates. Five isolates were PCR negative for all *qnr* genes. On these isolates a second PCR using degenerated *qnrB* primers as described by Cattoir[6] did not yield any specific products. No *qnrA* and *aac(6')-1b-cr* genes were detected in any of the isolates.

Sequence analysis of the 34 PCR products with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) revealed 2 variants of the *qnrB* gene (*qnrB2* and *qnrB5'*) in 2 different serovars and 1 variant of the *qnrS* gene (*qnrS1*) in 6 different serovars, predominately *S.* Corvallis (n = 25) (Table 1). Except for one *qnrB2* positive *S.* Bredeney isolated from a Dutch broiler chicken, all *qnr* positive isolates were of human origin. Additional sequence analysis of *gyrA*, *gyrB*, *parC* and *parE* PCR products revealed no mutations in the quinolone resistance-determining region (QRDR).

This is the first report of the occurrence of *qnrB* (*qnrB2* and *qnrB5'*) and *qnrS1* genes in The Netherlands and also the first report of the occurrence of *qnr* genes in *Salmonella enterica* strains isolated from humans as well as a poultry source from The Netherlands. Although human non typhoidal *S. enterica* predominantly have an animal origin, the source of the *qnr* genes in these isolates is uncertain. The occurrence of *qnr* genes in *Salmonella* isolated from German poultry has been reported earlier by Kehrenberg[7]. The presence of *qnr* genes in poultry is a reason for concern, because the use of fluoroquinoles in these animals may facilitate the horizontal transmission of *qnr* genes in the gastro-intestinal tract and thus increase the reservoir. In this study the *qnrS1* variant was predominantly present in *S.* Corvallis. *QnrS1* positive *S.* Corvallis isolates originating from Denmark and Thailand have recently been described[8]. PFGE analysis of the 25 *qnrS1* positive Dutch *S.* Corvallis isolates revealed 8 different subtypes in three major clusters (data not shown). Except for one cluster (2 isolates) the Dutch and the Danish PFGE patterns show a high similarity, indicating common sources for the *qnrS1* positive *S*. Corvallis isolates. The high detection rate of *qnr* genes in the strain selection demonstrates that isolates harbouring the *qnr* gene without any mutations in the QRDR region can very well be recognized by their phenotype. The variation in *qnr* genes and serotypes harbouring these genes observed in the present study indicate horizontal dissemination of genes between different serotypes of *Salmonella enterica* in The Netherlands. Although the impact of transferable quinolone resistance is not totally clear, the presence of *qnr* genes in *Salmonella* is considered a matter of concern. Diagnostic labs should therefore implement an optimal detection method of possible *qnr* phenotypes in their daily routine by testing an extra fluoroquinolone in addition to nalidixic acid. This may prevent possible treatment failure with quinolones.

Table 1. Results of sequence analysis of *qnr* genes in *Salmonellae*

Qnr gene (number of isolates)	GenBank accession number	Serovars
qnrB2 (n = 1)	AB281054	*S.* Bredeney
[1]*qnrB5'* (n = 2)	DQ303919	[2]*S.* Typhimurium ft 507, *S.* Typhimurium ft 510
qnrS1 (n = 31)	DQ485529	*S.* Anatum, S. Corvallis, S. Kentucky, *S.* Montevideo, S. Saintpaul, S. Stanley

[1]QnrB5' shows 99 % similarity with the originally detected qnrB5 gene, but differs at 4 sites; one mutation at Ser-210 (Ser → Asn: AGC → AAC) and 3 silent mutations at Arg-60 (CGC→ CGT), Ser-62 (AGT →AGC) and Leu-208 (TTA→TTG).
[2] Dutch phage typing system

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Transparency declarations

None to declare.

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2b

CHAPTER 2B

Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*

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Abstract

Little information is available on plasmids promoting the dissemination of the *qnr* genes. The aim of this study was to identify and characterize plasmids carrying *qnrS1, qnrB2* and *qnrB19* genes identified in *Salmonella* strains from The Netherlands. The identification of plasmids may help to follow the dissemination of these resistance genes in different countries and environments.

Plasmids from 33 *qnr*-positive *Salmonella* strains were transferred to *E. coli* and analyzed by restriction, Southern blot hybridization, PCR and sequencing of resistance determinants. They were also assigned to incompatibility groups by PCR-based replicon typing, including three additional PCR assays for the IncU, IncR and ColE groups. The collection included isolates from humans and one from chicken meat.

Five IncN plasmids carrying *qnrS1*, *qnrB2* and *qnrB19* genes were identified in *S.* Bredeney, *S.* Typhimurium PT507, *S.* Kentucky and *S.* Saintpaul. *qnrS1* genes were also located on three further plasmid types, belonging to the ColE (in *S.* Corvallis and *S.* Anatum), IncR (in *S.* Montevideo) and IncHI2 (in *S.* Stanley) groups.

Multiple events of mobilization, transposition, and replicon fusion generate the complexity observed in *qnr*-positive isolates that are emerging worldwide. Despite the fact that the occurrence of *qnr* genes in bacteria from animals is scarcely reported, these genes are associated with genetic elements and located on plasmids that are recurrent in animal isolates.

Introduction

Plasmid-mediated quinolone resistance is emerging worldwide in *Enterobacteriaceae*, including *Salmonella enterica* (*S.*). Salmonellosis is treated with fluoroquinolones only in elderly or immunocompromised patients, but these drugs are also used for treating patients with enteric fever, invasive disease, or long-term salmonellae carriage. Recent studies on *Salmonella* showed that plasmid-located *qnr* genes confer decreased susceptibility to fluoroquinolones (MIC >0.06 mg/L) and nalidixic acid (MIC 8–16 mg/L), without association with mutations in the topoisomerase genes.[1-4] Recently, we reported the first *qnrB* and *qnrS* genes in *Salmonella* isolates from patients and a broiler chicken in The Netherlands.[5] These genes were previously described in *Salmonella* from USA, Asia, Africa and Europe, but scarce information is available on the structure and circulation of plasmids carrying the different *qnr* gene variants.[3-9] Currently, there are two completely sequenced plasmids carrying the *qnrS1* gene: one is named pTPqnrS-1a, a 10 kb plasmid obtained from a multiresistant *Salmonella* Typhimurium DT193 in the UK;[3] the other is pK245, a 98 kb multireplicon plasmid identified in a clinical *Klebsiella pneumoniae* from Taiwan.[10] DNA sequences of these plasmids highlighted some peculiar features that can be helpful to trace them and also provide information on mechanisms responsible for the horizontal transfer of the *qnr* genes among different isolates. Plasmid pTPqnrS-1a exhibited 89% nucleotide sequence identity to the ColE-plasmid pEC278 isolated from a pathogenic *Escherichia coli* strain (GenBank accession number AY589571) and the region adjacent to the origin of replication (*oriV*) showed 99% identity to plasmid pINF5 from *Salmonella* Infantis isolated from chicken carcasses in Germany.[6] The pK245 plasmid structure was also complex, being composed of four main scaffolds: i) a region deriving from an IncF plasmid; ii) a region deriving from the IncQ plasmid RSF1010; iii) a region encoding the RepA replication initiator protein found in *Pantoea stewartii* plasmid pSW800 (70% similarity); iv) a region encoding the bla_{LAP-2} and *qnrS* genes and the *repB* gene of the *K. pneumoniae* pGSH500 plasmid (96% similarity).[10]

Plasmids of the IncU (p37) and IncQ (pGNB2) groups were associated with the *qnrS2* gene: in *Aeromonas punctata* from France and in plasmid DNA obtained from a waterwaste treatment plant in Germany, respectively.[11,12] Finally, the *qnrA1* gene associated with the bla_{VEB-1} gene emerged worldwide located on IncA/C2 plasmids,[13] while little information is available on plasmids carrying the *qnrB* variants.

The aim of this study was to identify and characterize plasmids harboring *qnrS1*, *qnrB2* and *qnrB19* genes identified in quinolone resistant *Salmonella* strains from The Netherlands, with the final objective to provide a set of specific PCR assays, useful for monitoring the dissemination of these resistance traits in different countries and environments.

Material and Methods

Strains

A total of 33 *qnr*-positive *Salmonella* strains were analyzed in this study. Most of the isolates were from patients from The Netherlands, one strain (137.25) was from chicken meat (Table 1). The presence of *qnr* genes in these strains was previously described.[5] *qnrS1*-positive salmonellae belong to serotypes Corvallis (25 isolates), Kentucky (*n* = 2), Anatum (*n* = 1), Montevideo (*n* = 1), Stanley (*n* = 1), and Saintpaul (*n* = 1) (Table 1). Comparison of pulsed-field gel electrophoresis (PFGE) patterns of *S.* Corvallis strains suggested that they were clonally related (>90% of similarity), with the exception of two recent isolates of 2006 (strains 162.58 and 163.43), showing unrelated PFGE profiles (<80% similarity). One *S.* Bredeney and one *S.* Typhimurium PT507 were positive for *qnrB2* and *qnrB19* (formerly referred to as *qnrB5'*)[5] genes, respectively. The *qnrB5'* gene name was updated by DNA sequencing of the amplicon generated by the primers QnrB10/19Fw and QnrB10/19Rv listed in Table 2 identified as *qnrB19*[14] by comparison to the GenBank database and Lahey Clinic website. The DNA sequence of the Quinolone Resistance Determining Region (QRDR) of the *gyrA* and *parC* genes was analyzed for all the strains.[15]

Antimicrobial susceptibility

The resistance patterns were determined by broth microdilution according to EUCAST guidelines (www.eucast.org) using microtiter trays (TREK Diagnostic Systems, UK). MIC-breakpoints used for susceptibility and resistance to ciprofloxacin were respectively ≤ 0.5 and > 1 mg/L and for resistance to nalidixic acid >16 mg/L as recommended by EUCAST.

Plasmid-mediated quinolone resistance transferability

Plasmid DNA was purified by the Qiagen Plasmid Midikit (Qiagen Inc., Milan, Italy). Purified plasmids were used to transform MAX Efficiency DH5 *E. coli* chemically competent cells (Invitrogen, Milan, Italy). DH5 was chosen as recipient because it is capable of being transformed efficiently with large plasmids.[16] However, this strain is resistant to nalidixic acid (MIC> 64mg/L) due to mutations in the *gyrA* gene but it is fully susceptible to ciprofloxacin at the MIC = 0.03 mg/L (Table 1). Consequently, transformants (T) were selected on LB agar plates containing 0.06 mg/L ciprofloxacin.
Conjugation experiments were performed at 25°C by liquid mating assay using a rifampicin-resistant *E. coli* CSH26 as recipient and selecting transconjugants (Tc) on LB agar

supplemented with 100 mg/L rifampicin and 0.06 mg/L ciprofloxacin.[17]
Undigested (Supplementary Figure 1) and *PvuII* restricted plasmids in the original and recipient strains were analyzed by Southern blot hybridization using the digoxigenin labeled *qnrS*, *qnrB* and repN amplicons as probes (PCR DIG probe synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany).[18,19] Hybridization and detection were performed according to the manufacturer's instructions.

Plasmid typing

Plasmids from parental and transformant/transconjugant strains were assigned to incompatibility groups by the PCR-based replicon typing (PBRT) performed on total DNA using previously described primers and conditions.[19] Total DNA was obtained by the Wizard Genomic DNA purification System (Promega, Madison, WI). Plasmids that were negative for the 18 replicons of the PBRT scheme were tested for three additional targets: the *oriV* of ColE-like plasmids (colE PCR), the *repA* gene of the pRA3 plasmid from *Aeromonas hydrophila* (IncU PCR), and the *repB* gene of the *K. pneumoniae qnrS1*-plasmid pK245. Since plasmid pK245 was not assigned to any known Inc group this assay was named IncR PCR (Table 2). Amplicons were sequenced for confirmation. The colE PCR was devised to amplify all the colE-like plasmids. A $colE_{Tp}$ amplification was devised to specifically detect the subset of ColE-positive plasmids showing a different *oriV* sequence (74% of homology with the other ColE-like variants), but 100% identical to that of the pTPqnrS-1a plasmid from *S.* Typhimurium DT193 (GenBank accession number AM746977). Primers are listed in table 2. $ColE_{Tp}$ positive strains were further analyzed by the qnrS-$colE_{Tp}$ PCR (Table 2) to confirm the co-linearity of the *qnrS1* gene with the $colE_{Tp}$ *oriV*, as described in the pTPqnrS-1a plasmid. Furthermore, the *qnrS1* and $colE_{Tp}$ *oriV* containing region of plasmid 138.31 (T) was cloned and fully sequenced by ligating the *PstI* digested plasmid into the pZero-2.1 kanamycin-resistant vector (Invitrogen, Milan, Italy). Ligation mixture was introduced by transformation into the MAX Efficiency *E. coli* DH5 chemically competent cells (Invitrogen, Milan, Italy). Transformants were selected on LB agar plates, containing 100 mg/L kanamycin and 0.06 mg/L ciprofloxacin. Recombinant plasmids were extracted by the Qiagen Plasmid Midikit (Qiagen Inc., Milan, Italy) and inserts were sequenced on both strands by standard and walking primers.
Plasmids assigned by PBRT to the IncHI2 group were further typed applying the previously described HI2-plasmid typing scheme, consisting of ten PCRs, devised on the IncHI2 R478 plasmid (Table 2).[20]

All plasmids were also screened for the presence of the bla_{LAP}, bla_{OXA}, bla_{TEM} , *aac(6')-Ib*-cr and *qepA* genes and for the presence of IS*2* flanking the *qnrS1* gene (Table 2). [21-24]

Results

Localization of qnr and beta-lactamase genes on Salmonella plasmids

Qnr-positive plasmids were successfully transferred by transformation from all the parental strains to the recipient *E. coli* DH5 strain (the 131.17 strain was chosen as representative for the *S.* Corvallis clonal strain), with the exception of *S.* Stanley strain 146.71 that did not produce transformants but it positively transferred the plasmid by conjugation (Table 1). PCR and DNA sequencing experiments confirmed the *qnr*-gene presence in all transformants/transconjugants obtained (Table 1). MICs for ciprofloxacin and nalidixic acid were measured for the parental and recipient strains and for the empty CSH26 and DH5 *E. coli* recipient strains (Table 1). Parental and recipient strains were PCR negative for the presence of the *aac(6')-Ib*-cr and *qepA* genes, conferring reduced susceptibility to fluoroquinolones. No mutations previously described to be associated with quinolone resistance were identified in the QRDR of the parent strains (data not shown).
Ampicillin resistance was associated with the presence of the bla_{TEM} gene in all strains, except strain 146.71(Tc) that was positive for the bla_{LAP} gene, identified by DNA sequencing of the amplicon as the bla_{LAP-2} gene variant.[25]

Typing of both *qnrB2*- and *qnrB19*- plasmids

Plasmids from both parental and recipient strains were tested for twenty-one replicons (listed in Table 2 and reference 18). Both *qnrB2* as *qnrB19* genes were located on IncN plasmids (100% identity to the *repA* gene of the R46 IncN reference plasmid, GenBank accession number AY046276). ColE plasmids were also identified in the parental strains but they were *qnr*-negative and were not transferred to the recipient strain (Table 1). IncN plasmids in the transformant strains were further analyzed by *PvuII* RFLP and the localization of the *qnr* gene was confirmed by Southern blot hybridization experiments (Figure 1).

Typing of *qnrS1*- plasmids

The *qnrS1* genes were located on four different plasmid types.
i) ColE-like plasmids of approximately 10 kb were identified in all *S.* Corvallis and *S.* Anatum strains. Several strains also showed co-resident I1, B/O or A/C type plasmids, which were

not transferred by transformation. DNA sequencing of the ColE-amplicons obtained from strains 138.31, 131.17 and 120.52 revealed that these plasmids had the origin of replication identical to that of the pTPqnrS-1a plasmid of *S.* Typhimurium DT193.[3] The presence of this type of *oriV* was then confirmed in all the *S.* Anatum and *S.* Corvallis strains by PCR assay specific for the $ColE_{Tp}$ *oriV* (Table 1). Furthermore, all these strains were also positive for the qnrS-$colE_{Tp}$ PCR indicating the localization of the *qnrS1* gene on pTPqnrS-1a-like plasmids. This observation was confirmed by cloning and fully sequencing the 4375 bp *PstI* fragment from plasmid 138.31(T). This region contained the *mobC* gene, the *oriV* and the *qnrS1* gene in an array identical to that previously described for the pTPqnrS-1a plasmid.

ii) the *qnrS1* gene was identified on IncN plasmids of approximately 50 kb in the *S.* Kentucky and *S.* Saintpaul strains. Replicons of the I1- and P-type were also detected in the *S.* Kentucky parental strain but they were not transferred to the recipient strain. A co-resident ColE plasmid was identified in the *S.* Saintpaul strain but it was *qnr*-negative and not transferred by transformation. IncN plasmids from the transformants were further analyzed by *PvuII* RFLP and the localization of *qnr* gene was confirmed by Southern blot hybridization (Figure 1).

iii) the *qnrS1* gene was also identified on an IncHI2 plasmid in *S.* Stanley strain 146.71 and its relative transconjugants (Table 1). Plasmid DNA from this strain could not be purified (the estimated minimal size for IncHI2 plasmids is >250 kb), but the transferred plasmid was further characterized applying the previously described HI2-typing scheme, discerning the two reference IncHI2 R478 and pAPEC-O1-R plasmids (Table 2).[20] This analysis identified the IncHI2 plasmid from *S.* Stanley as a pAPEC-O1-R-like plasmid. In fact, it lacked three genes that are present in the R478 but are missing in pAPEC-O1-R (*smr92, smr93* and *smr201*) and it was positive for the O1R_160 locus, which is disrupted by the Tn10 insertion in R478.[26] The *qnrS1*-$HI2_{pAPEC\text{-}O1\text{-}R}$ plasmid conferred resistance to ampicillin, aminoglycosides, sulphonamides, streptomycin, tetracycline and chloramphenicol and it was positive for the $bla_{LAP\text{-}2}$ gene.

iv) The *qnrS1* gene was located on an IncR plasmid of approximately 50 kb in the *S.* Montevideo strain. This plasmid conferred a multidrug resistant phenotype, including aminoglycosides, chloramphenicol and tetracycline resistance. The parental strain was also positive for a *qnr*-negative ColE plasmid.

All the *qnrS1* genes, regardless of location on the different plasmid scaffolds, were flanked by a truncated IS*2* element as previously described.[3,27]

Table 1. Characteristics of *qnr*-positive *Salmonella enterica* isolated in The Netherlands and their

Strain	Resistance pattern	Sample	Serotype	MIC CIP
137.25	SMX, TET, TMP, CIP_L	chicken	*S.* Bredeney	0.25
137.25(T)	SMX, TET, TMP, CIP_L			0.25
152.40	CIP_L	human	*S.* Typhimurium PT507	0. 5
152.40(T)	CIP_L			0.25
128.12	AMP, CIP_L	human	*S.* Kentucky	1.0
128.12(T)	AMP, CIP_L			0.5
168.27	AMP, STR, TET, CIP_L	human	*S.* Saintpaul	0.5
168.27(T)	AMP, STR, TET, CIP_L			0.5
174.70	AMP, STR, CIP_L	human	*S.* Kentucky	0.5
174.70(T)	AMP, STR, CIP_L			0.5
146.71	AMP, CHL, GEN, KAN, SMX, STR TET, CIPL	human	*S.* Stanley	1.0
146.71(Tc)	CHL, GEN, KAN, SMX, STR TET, CIPL			0.5
172.23	SMX, TMP, CIP_L	human	*S.* Montevideo	0.5
172.23(T)	SMX, TMP, CIP_L			0.5
138.31	SMX, CHL, TET, CIP_L	human	*S.* Anatum	1.0
138.31(T)	CIP_L			1.0
131.17	CIP_L	human	*S.* Corvallis	0.5
131.17(T)	CIP_L			0.5
73.75	TET, CIP_L	human	*S.* Corvallis	0.25
93.29	TET, CIP_L	human	*S.* Corvallis	0.5
102.46	SMX, TET, CIP_L	human	*S.* Corvallis	0.25
108.04	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
117.43	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
117.76	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
120.52	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
126.41	SMX, TET, CIP_L	human	*S.* Corvallis	0.25
130.08	CIP_L	human	*S.* Corvallis	0.5
141.44	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
143.23	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
143.42	TET, CIP_L	human	*S.* Corvallis	1
144.12	CIP_L	human	*S.* Corvallis	0.5
145.50	AMP, GEN, SMX, TET, CIP_L	human	*S.* Corvallis	0.5
145.62	AMP, GEN, SMX, TET, CIP_L	human	*S.* Corvallis	0.5
147.28	CIP_L	human	*S.* Corvallis	1
155.08	AMP, CIP_L	human	*S.* Corvallis	0.5
162.58	SMX, TET, CIP_L	human	*S.* Corvallis	0.25
163.43	SMX, TET, CIP_L	human	*S.* Corvallis	0.5
168.20	CIP_L	human	*S.* Corvallis	0.5
171.04	CIP_L	human	*S.* Corvallis	0.5
171.22	CIP_L	human	*S.* Corvallis	0.5
177.29	CIP_L	human	*S.* Corvallis	1
175.40	CIP_L	human	*S.* Corvallis	0.5
DH5	NAL	recipient		0.03
CSH26	-	recipient		0.03

Abbreviations: AMP: ampicillin, CHL: chloramphenicol; GEN: gentamicin; KAN: kanamycin, NAL: nalidixic acid, SMX: sulfamethoxazole,

relative transformants/transconjugants (T/Tc)

MIC NAL	qnr	Beta-lactamase	PBRT+IncR+ColE	qnr- $colE_{Tp\ PCR}$
16	*qnrB2*	neg	N, colE	neg
64	*qnrB2*	neg	N	neg
16	*qnrB19*	neg	N, colE	neg
64	*qnrB19*	neg	N	neg
16	*qnrS1*	TEM	N, I1, P	neg
64	*qnrS1*	TEM	N	neg
16	*qnrS1*	TEM	N, colE	neg
64	*qnrS1*	TEM	N	neg
16	*qnrS1*	TE*M*	N	neg
64	*qnrS1*	TEM	N	neg
16	*qnrS1*	LAP-2	HI2 pAPEC-O1-R, P, colE	neg
16	*qnrS1*	LAP-2	HI2pAPEC-O1-R	neg
8	*qnrS1*	neg	R, $colE_{Tp}$	neg
64	*qnrS1*	neg	R	neg
16	*qnrS1*	neg	A/C, $colE_{Tp}$	pos
64	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	B/O, $colE_{Tp}$	pos
64	*qnrS1*	neg	$colE_{Tp}$	pos
8	*qnrS1*	neg	$colE_{Tp}$	pos
16	q*nrS1*	neg	$colE_{Tp}$	pos
8	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$, I1	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
8	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$, I1	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
8	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	TEM	$colE_{Tp}$, I1	pos
16	*qnrS1*	TEM	$colE_{Tp}$, I1	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	TEM	$colE_{Tp}$, I1	pos
8	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp}$	pos
16	*qnrS1*	neg	$colE_{Tp,}$ I1	pos
64	neg	neg	neg	neg
<4	neg	neg	neg	neg

STR, streptomycin, TET: tetracycline; TMP: trimethoprim; CIP_L reduced susceptibility to ciprofloxacin.

Table 2. Primers used in this study

Primer name	Sequence	Nucleotide positions
oricolE FW[a]	5′- GTT CGT GCA TAC AGT CCA -3′	3797-3983
oricolE RV	5′- GGC GAA ACC CGA CAG GAC T -3′	
oricolE FW[a]	5′- GTT CGT GCA TAC AGT CCA -3′	3878-3983
oricolETp RV[b]	5′- GGT TTA CCG GTG TCA TTC C -3′	
qnrSc	5′- TAA ATT GGC ACC CTG TAG GC -3′	6628-3878
oricolETp RV[b]	5′- GGT TTA CCG GTG TCA TTC C -3′	
IncR FW	5′- TCG CTT CAT TCC TGC TTC AGC -3′	19367-19617
IncR RV	5′- GTG TGC TGT GGT TAT GCC TCA -3′	
IncU FW	5′- TCA CGA CAC AAG CGC AAG GG -3′	51-893
IncU RV	5′- TCA TGG TAC ATC TGG GCG C -3′	
QnrB10/19FW	5′- CGG GTT TGA CGC ATA AC -3′	2779-2038
QnrB10/19RV	5′- CAA ACG CAT CTC CCG GT -3′	
deltaresIS2FW	5′- TCA TAA TGC GAT ACA CCC GC -3′	5082-6628
qnrS[c]	5′- TAA ATT GGC ACC CTG TAG GC -3′	
R478 vs pAPEC-O1-R IncHI2 subtyping[d]		
10 FW	5′- AAT CGC CTG AAT CAG CTG G -3′	6655-7713
11 RV	5′- TTC TTT ACT ACA CCA GAG CC -3′	
17 FW	5′- AAC TCT TGA AAA TCG TGG -3′	18211-19101
18 RV	5′- CTT CAG GCT ATC GTT TCG -3′	
Ter FW	5′- ATG CAG GCT CAA GGA ATC GC -3′	80270-81163
Ter RV	5′- TTC ATC GAT CCA CGG TCT G -3′	
92 FW	5′- CTA TGT AAG CAA TGA TCC TC -3′	88861-89862
93 RV	5′- TAT AGA GAG CAC CGA AGG -3′	
TnsDA FW	5′- AAT CCT TGT TCA GCC GG -3′	119360-120825
TnsDA RV	5′- CAA AAG CCA GCC ATG CCC -3′	
136A FW	5′- TAC GAA AAT GAA TTG TGG C -3′	120906-121768
136A RV	5′- AAT TTA CAA TCT GCA GCC C -3′	
ArsB FW	5′- AGT GAA AGA CAG ACG AAG CG -3′	159735-160870
ArsB RV	5′- GGC AGA TAG TGT GGA ATG CG -3′	
201 FW	5′- TGT CAG GCT AAG TCA CTG G -3′	180398-181466
201 RV	5′- ATT ATA CGG TAG ATC C -3′	
207 FW	5′- TTT CCC AAA TAG GCG ACG C -3′	190238-191131
208 RV	5′- ATG TGA AAT TAC TAT ACC GG -3′	
239 FW	5′- TGG AAC GCG TGG TAT GTG G -3′	219372-220364

[a]The oricolE FW primer is used in combination with oricolE RV for the generic detection of ColE-like plasmids and in combination with oricolE$_{Tp}$ RV for the detection of the ColE$_{Tp}$ plasmids showing oriV identical to the pTPqnrS-1a plasmid.

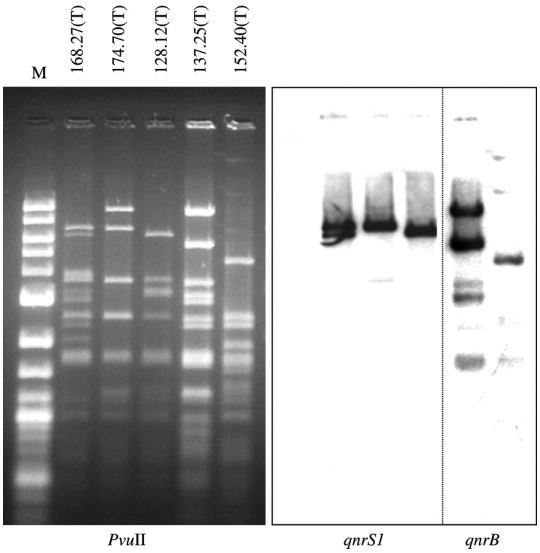
[b]The oricolE$_{Tp}$ RV is used in combination with qnrS RV for the localization of the qnrS1 gene on ColE$_{Tp}$ plasmids.

[c] The qnrS primer is described by Robicsek et al. 2006. [17]

[d] Primers and conditions used for the IncHI2 subtyping are described in García A et al. 2007. [19]

Accession No	PCR Target	Amplicon size (bp)
AM746977	ColE plasmids [Generic]	187
AM746977	ColETp like pTPqnrS-1a	106
AM746977	*qnrS1*-ColETp localization	2751
DQ449578	IncR plasmids	251
DQ401103	IncU plasmids	843
EU523120	*qnrB5*-like genes	742
AM746977	Truncated IS2 and *qnrS1*	1547
BX664015	HtdV protein	1059
BX664015	SMR0017 lipoprotein	891
BX664015	Tellurium resistance	894
BX664015	SMR0092-93 protein	1002
BX664015	Tn7 transposon	1466
BX664015	SMR0136 protein	863
BX664015	Arsenical pump membrane protein	1136
BX664015	SMR0201 protein	1069
BX664015	SMR0207-208 proteins	894
BX664015	SMR0239-240 proteins	993

Figure 1. Restriction analysis of plasmids obtained from IncN transformants



Restriction analysis of plasmids obtained from IncN transformants restricted by PvuII. Southern blot hybridization with digoxigenin labelled qnrS1 and qnrB probes. M: 1 Kb DNA Extension Ladder (Invitrogen, Milan, Italy).

Discussion

This study describes plasmids harboring *qnr* genes identified in eight different *Salmonella* serovars from The Netherlands. The sample included isolates from humans and one from chicken meat. IncN plasmids were the most recurrent plasmid type since the three *qnr* gene variants, *qnrS1*, *qnrB2* and *qnrB19* were located on this kind of plasmid in four different *Salmonella* serotypes, from both human and the animal source. Four of the five IncN plasmids showed a common scaffold and variable *PvuII* fragments corresponding to the variable region containing the *qnr* gene. It is interesting to note that *qnrS1*-positive IncN plasmids were also identified in *S.* Virchow isolated in UK in 2004-2005, causing an outbreak associated with imported cooked meat from Thailand[7] and the *qnrS* plasmid pINF5 from *S.* Infantis was hypothesized to have an IncN plasmid-like ancestor (pMUR050).[6] The association *qnr*-IncN is likely a fortuitous event of integration of the Qnr determinant into a very common plasmid species. IncN was the most frequently identified (26%) plasmid type in a collection of 58 multidrug resistant *S. enterica* strains from animals and food of animal origin isolated in Italy in 2000-2001.[19,28] Furthermore, in a study performed on a large collection of *E. coli* isolates from the USA, the prevalence of IncN plasmids was

10.9% and 16.1% in avian fecal and pathogenic *E. coli*, respectively, but interestingly *E. coli* strains from faeces of healthy humans and from human urinary tract infections, were all negative for IncN plasmids.[29] Therefore, it is plausible that IncN plasmids are common in zoonotic enterobacterial pathogens but rarer in bacteria from humans, suggesting that IncN *Salmonella* plasmids could have acquired the *qnr* gene by transposition events occurring in animals.

The *qnrS1* gene was also located on small ColE-like plasmids in *S.* Corvallis and *S.* Anatum strains. The Corvallis serotype carrying the *qnrS1* gene recently emerged in Denmark associated with the consumption of imported food products from Thailand. Twenty-three isolates showing related PFGE patterns were obtained from humans from Denmark and Thailand and from chicken, pork and beef, imported from Thailand.[30] The *S.* Corvallis PFGE patterns were also very similar to those described in that study (data not shown). It could be speculated that the ColE$_{Tp}$-*qnrS1* plasmid is present also in *S.* Corvallis from Denmark and Thailand and contributed to the worldwide spread of *qnrS1* gene in this clone. However, the association *S.* Corvallis-ColE$_{Tp}$-*qnrS1* is not exclusive, since the same plasmid was identified in *S.* Typhimurium and *S.* Virginia in UK, and in *S.* Anatum in this study. Recently, a small *qnrS1*-positive plasmid showing 99% homology with the pTPqnrS-1a plasmid was also identified in a *S.* Typhimurium isolated in Taiwan (GenBank accession number EU715253), suggesting that this plasmid is very common worldwide, but it seems to have a preference for the *Salmonella* species. More in general, ColE-like plasmids are largely present in *Enterobacteriaceae* and they are not self-conjugative, but they can be mobilized by co-resident conjugative plasmids, through the presence of the *mobABC* genes.[31] The simultaneous presence of ColE$_{Tp}$-*qnrS* plasmid with additional plasmids belonging to I1, B/O or A/C groups within the same parental strain, strongly suggests that the latter plasmids can participate in the mobilization of the small ColE plasmids, promoting their circulation in different *Salmonella* serotypes.

In this study, the *qnrS1* gene was, for the first time, located on an IncHI2 plasmid identified in a *S.* Stanley strain. The plasmid scaffold resembled the pAPEC-O1-R plasmid, previously described in avian pathogenic *E. coli* in the USA.[26] It is important to note that HI2$_{pAPEC-O1-R}$ plasmid variants were recently recognized in *S.* Virchow producing the extended-spectrum β-lactamase (ESBL) CTX-M-2 from poultry flocks, poultry meat, and humans in Belgium and French Guyana.[32] The chronology of isolation of those strains suggested that these bacteria were transmitted to humans via the food chain, specifically by poultry meat. The *bla*$_{CTX-M-2}$ gene was not present on the original pAPEC-O1-R plasmid[26] and it was not identified on

the *qnrS1*-HI2$_{pAPEC-O1-R}$ plasmid either. In conclusion, the HI2 plasmid variant associated with avian pathogenic *E. coli* in the USA has evolved by acquisition of ESBL or *qnr* genes in *Salmonella* in Europe.

The fourth plasmid type carrying the *qnrS1* gene was the IncR plasmid identified in the *S*. Montevideo strain. The replicase gene of this plasmid was the same as that of the previously described pK245 plasmid from *K. pneumoniae*, showing the association *qnrS1-bla*$_{LAP-2}$ genes. The IncR plasmid from *S*. Montevideo was not positive for the *bla*$_{LAP-2}$ gene, which was identified on the IncHI2 plasmid from the *S.* Stanley.

From our findings, a complex figure of variably assorted plasmid replicons and resistance determinants clearly appears. Common genetic traits are organized on different scaffolds in the various strains. Multiple events of mobilization, transposition, illegitimate recombination, replicon fusion and resolution can generate the apparent complexity observed in the different *qnr*-positive isolates that are emerging worldwide but these are in fact, mosaics of recurrent genetic traits. Despite the low prevalence of *qnr* genes in bacteria from animals in comparison to isolates from humans; these genes were located on plasmids frequently associated with *E. coli* and *Salmonella* isolates from animals and rarely occurring in humans. This aspect would merit a further investigation of the prevalence of *qnr* genes in faecal flora from animals as a potential unexpected source for these resistance genes.

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Transparency declarations

None to declare.

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2C

CHAPTER 2C

Characterization of *qnr*-positive *Escherichia coli* isolates from food producing animals in The Netherlands

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Sir,

Recently, we participated in a collaborative study on the occurrence of PMQR-genes in *Salmonella* and *Escherichia coli* conducted by the European Community Reference Laboratory for Antimicrobial Resistance (EURL-AR). As part of that study, we screened the MIC-database of 6139 *E. coli* isolates from food-producing animals for reduced susceptibility against ciprofloxacin (MIC: ≥0.06 mg/L) and nalidixic acid (MIC: 4-32 mg/l) indicative for the presence of plasmid mediated quinolone resistance (PMQR) genes.[1] Subsequently, 21 *E. coli* isolates were tested for the presence of PMQR-genes by PCR. As a result, one *qnrS1*-positive *E. coli* (no 66.12) was detected, obtained from a Dutch broiler chicken in 2009. This isolate also showed resistance to cefotaxime and ceftazidime indicative for the presence of extended spectrum beta-lactamase (ESBL) or AmpC-genes. In 2010, two other PMQR-positive *E. coli* isolates were isolated from a broiler chicken (no 74.21) and a veal calf (no 77.01) respectively. These three isolates were selected for molecular characterization being the first PMQR-positive *E. coli* isolates from our Dutch national surveillance program. Antibiotic resistance genes were detected by a commercial microarray (Identibac, AMR-ve 05, Alere Technologies). We used published primers to amplify the PMQR,[1] ESBL and AmpC-genes.[2] The subtypes were characterized by sequence analysis of the PCR-products. In *E. coli* 66.12 two different ESBL-genes ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$) and one AmpC-gene ($bla_{CMY\text{-}2}$) were detected next to *qnrS1* (Table 1). Surprisingly, no other resistance genes were found in this isolate. In *E. coli* 74.21 (*qnrB19*-positive) and *E. coli* 77.01 (*qnrS1*-positive) multiple additional resistance genes were detected (Table 1). Plasmid characterization was performed using PCR-based replicon typing as described earlier [3] with extra primer sets for IncR, IncU and ColE followed by electroporation of plasmids into DH10B cells (Invitrogen Life Technologies). PMQR-positive transformants were selected on Luria-Bertani (LB) agar + 0.125 mg/L ciprofloxacin and ESBL/ AmpC-positive transformants were selected on LB + 1 mg/L cefotaxime. Subsequently, S1 nuclease PFGE was performed on the parent strains and the corresponding transformants. The resistance profile of the transformants demonstrated the transfer of PMQR-and/or ESBL/AmpC-carrying plasmids (Table 1). Analysis of *E. coli* 66.12 revealed the co-existence of *qnrS1* and $bla_{SHV\text{-}12}$ on a 45 kb sized non-typeable plasmid, next to $bla_{CMY\text{-}2}$ on a 80 kb sized IncK plasmid and $bla_{CTX\text{-}M\text{-}1}$ on a 100 kb sized IncI1 plasmid. The IncI1 plasmid was further typed by plasmid multi locus sequence typing (pMLST) as sequence type 7 (ST7).[4] The coexistence of *qnrS1* and $bla_{SHV\text{-}12}$ has been reported on IncN plasmids in *Klebsiella* isolates from Italy.[5] Nevertheless, to our knowledge, we describe the first *E. coli* isolate

harbouring *qnrS1* and *bla* $_{SHV-12}$ on a single non-typeable 45 kb sized plasmid. The presence of *bla*$_{CMY-2}$ on an IncK plasmid and *bla* $_{CTX-M-1}$ on an IncI1 plasmid was previously identified in *E. coli* isolates from Dutch broiler chickens.[2] Moreover, IncI1 plasmids of ST-7 harbouring *bla* $_{CTX-M-1}$ are frequently detected amongst ESBL-producing *E. coli* from Dutch broiler chickens (Plasmid MLST databases: http://pubmlst.org/plasmid/). Yet, we report the first coexistence of *bla* $_{CTX-M-1,}$ *bla* $_{SHV-12}$, and *bla*$_{CMY-2}$ genes next to *qnrS1* in a *E. coli* isolated from animals. In the *E. coli* isolate from a veal calf (no 77.01), *qnrS1* was located on an IncX2 plasmid which has recently been described in *E. coli* from healthy animals in Nigeria.[6] In the *E. coli* isolate from a broiler chicken (no 74.21) *qnrB19* was also identified on an InX2 plasmid. The presence of *qnrB19* has been reported in *E. coli* isolated from animals on ColE [6], IncN[7] and IncR[8] plasmids, but not on IncX2.

Our results demonstrate the presence of *qnr*-genes on two different types of plasmids in *E. coli* isolated from animals. These findings indicate the emergence of PMQR-genes in the commensal flora of food-producing animals in The Netherlands. The remarkable finding of the co-existence of three different cephalosporinase genes on three different plasmids in a single *E. coli* isolate demonstrates the complexity of the plasmid mediated dissemination of beta-lactamase and PMQR genes in Enterobacteriaceae.

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Transparency of declarations

None to declare.

Table 1. Phenotypic and molecular characteristics of three PMQR-positive *E. coli* parent isolates

Isolate number	MIC CIP (mg/L)	MIC NAL (mg/L)	MIC CTX (mg/L)	MIC CAZ (mg/L)	PMQR genes	ESBL/ AmpC-genes
66.12-parent	0.5	8	>4	>16	*qnrS1*	bla_{SHV-12}, $bla_{CTX-M-1}$, bla_{CMY-2}
66.12-TF-CIP	0.25	8	>4	>16	*qnrS1*	bla_{SHV-12}
66.12-TF-FOT-a	≤0.008	≤4	>4	4	none	$bla_{CTX-M-1}$
66.12-TF-FOT-b	0.25	8	>4	>16	*qnrS1*	bla_{SHV-12}
66.12-TF-FOT-f	≤0.008	≤4	>4	>16	none	bla_{CMY-2}
74.21-parent	0.25	16	<=0.06	≤0.25	*qnrB19*	none
74.21-TF-CIP	0.12	≤4	0.12	0.5	*qnrB19*	none
77.01-parent	0.5	16	≤0.06	≤0.25	*qnrS1*	none
77.01-TF-CIP	0.25	8	0.12	0.5	*qnrS1*	none

[1] *= resistance genes detected with the microarray,*
CIP = ciprofloxacin,
NAL = nalidixic acid,
CTX = cefotaxime,
CAZ = ceftazidime,
N.D. = Not Determined

and transformants

Additional resistance/ integrase genes[1]	Replicon type	Plasmid sizes (kb)
none	N.D.	10, 45, 80, 100, 140
N.D.	non-typeable	45
N.D.	IncI1	100
N.D.	non-typeable	45
N.D.	IncK	80
t*et*(A), *aad*A1, *dfr*A1, bla_{TEM-1}, *sul1*, *int1*	N.D.	35, 60, 90, 150
N.D.	IncX2	35
tet(A), *aadA1*, *dfrA14*, bla_{TEM-1}, *sul3*, *cmlA*-like, *int1*	N.D.	40, 70, 120
N.D.	IncX2	40

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3

Chapter 3

PMQR in Enterobacteriaceae in Europe


3a

CHAPTER 3A

International Collaborative Study on the Occurrence of Plasmid Mediated Quinolone Resistance in *Salmonella* enterica and *Escherichia coli* isolated from Animals, Humans, Food and the Environment in Thirteen European countries

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Abstract

This study was initiated to collect retrospective information on the occurrence of plasmid mediated quinolone resistance (PMQR) in *Salmonella enterica* and *Escherichia coli* isolates in Europe and to identify the responsible genes.

Databases of national reference laboratories containing MIC-values of *Salmonella* and *E. coli* isolated between 1994 and 2009 in animals, humans, food and the environment from thirteen European countries were screened for isolates exhibiting a defined quinolone resistance phenotype being reduced susceptible to fluoroquinolones and nalidixic acid. PCR and sequence analysis were performed to identify the responsible PMQR-genes.

Screening of databases of 13 European countries resulted in a selection of 1,215 *Salmonella* and 333 *E. coli* isolates. PMQR-genes were identified in 59% of the *Salmonella* isolates and 15% of the *E. coli* isolates selected. In *Salmonella*, *qnrS1* (n = 125) and variants of *qnrB* (n = 138) were frequently identified, whereas *qnrA1* (n = 3) and *aac(6')-1b-cr* (n = 3) were rarely found. *qnrD* was detected in 22 *Salmonella* isolates obtained from humans and animals. In *E. coli*, *qnrS1* was identified in 19 isolates and *qnrB19* was found in one isolate. No *qnrC* or *qepA* genes were detected neither in *Salmonella* nor *E. coli*.

This study shows the occurrence and dissemination of PMQR-genes in *Salmonella* and *E. coli* in Europe with a defined quinolone resistance phenotype. We also report the first detection of *qnrD* in *Salmonella* collected in Europe.

Introduction

Fluoroquinolones are important antibiotics for treatment of infections in both humans and animals.[1] Resistance against fluoroquinolones can lead to treatment failure and is therefore considered a public health risk.[2, 3]

In Enterobacteriaceae, (fluoro)quinolone resistance is mainly caused by point mutations in the quinolone resistance determining region (QRDR) of gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) genes. Increase of the number of mutations leads to a stepwise increase of the level of resistance against fluoroquinolones such as ciprofloxacin. However, one point mutation in *gyrA* already results in resistance against nalidixic acid.[1]

In addition, efflux pumps and decreased permeability of the outer membrane can also contribute to the level of quinolone resistance.[1] In the last decade plasmid mediated quinolone resistance (PMQR) has been increasingly reported in Enterobacteriaceae worldwide, including several European countries.[4, 5] However, studies reporting the occurrence of PMQR-genes in Enterobacteriaceae on an European level are lacking.

To date, three different transferable (fluoro)quinolone resistance mechanisms have been described: (I) five different *qnr* families each with different numbers of alleles (*qnrA1-7*, *qnrS1-4*, *qnrB1-31*, *qnrC* and *qnrD*)[6] (http://www.lahey.org/qnrstudies); (II) a modified aminoglycoside acetyl transferase gene (*aac(6')-1b-cr*)[7] and (III) a specific quinolone efflux pump (*qepA*)[8] and multidrug resistance pumps like *oqxAB* [9]. In general, PMQR-positive isolates display a distinctive phenotypic quinolone resistance pattern, which is related to a specific affinity to fluoroquinolones, resulting in reduced susceptibility to these substances, but only a minor reduction in susceptibility to nalidixic acid.[10, 11] This phenotype is only exhibited by bacterial isolates without mutations in the quinolone resistance determining region (QRDR), but can nevertheless, be of interest in retrospective studies.

In 2008, the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR at the National Food Institute, Lyngby, Denmark) initiated a collaborative study including all the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) which routinely used MIC determinations for susceptibility testing. The aim of this study was to collect retrospective information on the emergence and occurrence of PMQR in *Salmonella* and *E. coli* isolates from different sources in Europe and to identify the responsible genes.

Methods

All NRLs on antimicrobial resistance in animals and food were invited by the EURL-AR to participate in a collaborative study on the occurrence of PMQR in *Salmonella* and *E. coli*. Furthermore, the National Veterinary Institute (NVI) in Norway and the Health Protection Agency Centre for Infections (HPA) in the UK were also invited to participate in this study. A questionnaire S1 [available as Supplementary data at *JAC* Online (htttp://jac.oxfordjournals.org./)] was included to collect information concerning sample identity, maintenance of strain collections, testing methods and types of databases. The NRL-AR laboratories were asked to retrospectively screen their MIC-databases for *E. coli* and *Salmonella* isolates exhibiting a defined PMQR phenotype being reduced susceptible to ciprofloxacin and nalidixic acid. Based on epidemiological cut-off values set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for ciprofloxacin and enrofloxacin, inclusion criteria were set for *Salmonella* (MICs: ≥ 0.125 mg/L, ≥ 0.25, respectively) and *E. coli* (MICs: ≥ 0.06 mg/L) combined with nalidixic acid MICs: 4 – 32 mg/L. The majority of the participants tested ciprofloxacin and nalidixic acid, only Poland (until 2007) and Norway tested for susceptibility to enrofloxacin instead.

All databases, except for the HPA, consisted of MIC-values obtained with broth micro dilution according to ISO-20776-1:2006. [12] HPA used a semi-quantitative agar dilution system testing susceptibility to antibiotics at breakpoint levels. For our purpose this system provided sufficient information on the quinolone resistance phenotype as it included testing a single concentration of nalidixic acid (16 mg/L) and two different ciprofloxacin concentrations (0.125 mg/L and 1 mg/L). Isolates exhibiting growth on 0.125 mg/L ciprofloxacin but no growth on 16 mg/L nalidixic acid were considered PMQR suspected and included in the selection.

Furthermore, participants were asked to send epidemiological data (such as source and year of isolation) concerning the selected isolates to the EURL-AR in order to build a database of the PMQR-suspected isolates. Databases containing disc diffusion results were excluded from the study, since valid information on inhibition zone diameters indicative of PMQR-positive isolates was lacking. Subsequently, all participants were asked to screen their selected isolates with PCR for all known PMQR-genes. PCR protocols based on published primers for each of the following genes: *qnrA* [13], *qnrB* [14], *qnrC* [15], *qnrD* [16], *qnrS* [13], *qepA* [17] and *aac(6')-1b-cr* [18] were distributed to all participants. For all PCR tests,

positive control strains were available at the EURL-AR. Laboratories were allowed to use their in house PCR methods. All laboratories, except for HPA used the proposed PCR protocols. The HPA used described primers for *qnrA*, *qnrB* and *qnrS* [19], but home-designed primers for *qnrC* (5'–TGAATTATTCCCATAAAACG-3' and 5'-TGTCTGGAATAACAATCACC-3'), *qnrD* (5'-CGGGGAATAGAGTTAAAAAT-3' and 5'-TATCGGTGAACAATAACACC -3' and *qepA* (5'-GTAGATCGTCAGCAGCAC-3'and 5'-TCTCTGGATCCTGGACAT-3').

Finally, participants were requested to sequence the amplicons to identify the detected resistance genes and their respective variants. For the identification of *qnrB* variants a second set of primers was used in order to analyze a larger amplicon.[20] To discriminate between *qnrB5* and *qnrB19* a third set of primers based on the linkage between of IS*Ecp1C* and *qnrB19* was used.[21] In case laboratories were not able to perform PCR and sequencing, they were invited to send their PMQR-suspected isolates either to the EURL-AR in Denmark or to the Central Veterinary Institute (CVI) in The Netherlands for further analysis.

Results

National Reference Laboratories from Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, Norway, Poland, Spain, The Netherlands and the United Kingdom participated in this study.

Retrospective screening of the MIC-data of 661,629 *Salmonella* isolates and 31,132 *E. coli* isolates resulted in a selection of 1,215 PMQR-suspected *Salmonella* and 333 PMQR-suspected *E. coli* isolates. Subsequently, 485 *Salmonella* and 133 *E. coli* were genetically characterized (Table 1). The remaining isolates were not analyzed, because they were either not stored, not viable or were retested for susceptibility and based on the new results excluded from the study. The isolates tested were obtained between 1994 and 2009 from humans, turkeys (and turkey meat), poultry (broilers, laying hens and broiler meat), pigs (and pork), cattle, sheep and reptiles, but also from food (undefined meat, food products and spices), animal feed and environmental samples.

For *Salmonella*, PCR testing revealed PMQR genes in 59% (288/485) isolates tested originating from eleven different European countries (Table 1). PMQR genes were identified in *Salmonella* isolates obtained from humans, turkeys, poultry, reptiles, pigs, sheep, food and the environment and in two isolates of unknown origin (Table 2). The earliest PMQR-positive *Salmonella* isolate detected was a *qnrS1*-positive *S*. Typhimurium isolate obtained in 2002 from a human in the UK.

The *qnrS1* gene was predominant in the selected *Salmonella* isolates and was detected in 26% of the isolates (125/485). This PMQR gene variant was found widespread among 25 different *Salmonella* serovars and was most frequently detected in human *S*. Corvallis isolates mainly from The Netherlands and in human *S*. Typhimurium isolates from the UK (Table 3). In addition, *qnrS1* was frequently identified in *S*. Saintpaul isolates obtained from turkeys in Denmark, Poland and Germany. *Salmonella* isolates obtained from poultry, pigs, sheep, reptiles and food were also *qnrS1*-positive. In two *qnrS1*-positive isolates from Italy the concurrent presence of the *aac(6')-1b-cr* gene was demonstrated.

Six variants of *qnrB* (*qnrB2*, *qnrB4*, *qnrB6*, *qnrB7*, *qnrB12* and *qnrB19*) were identified in 138 *qnrB*-positive isolates. Most *qnrB*-positive isolates were obtained from turkeys, but *qnrB* gene variants were also identified in *Salmonella* isolates from other sources such as humans, reptiles and poultry (Table 2). Within the *qnrB*-positive group, *qnrB2* was the most frequent variant found and particularly prevalent in *S*. Derby and *S*. London obtained from turkeys in Spain (Table 3). Furthermore, *qnrB19* was most often detected in *Salmonella* isolated from turkeys in Germany and Denmark, including *S*. Hadar and *S*. Newport isolates (Tables 2 and 3). Part of these isolates were previously reported as *qnrB5*-positive.[22] However, in the present study we used an extra set of primers[21], and based on sequence analysis of the amplicons these isolates were now designated *qnrB19*-positive. In addition, *qnrB19* was identified in a particular group of *Salmonella* isolated from reptiles in German zoos [23], included in this study. In one case, *aac(6')-1b-cr* was detected together with *qnrB6* in a *Salmonella* isolate from a turtle.

The *qnrD* genes were detected in twenty-two isolates from eight different serovars noted in three different countries (Table 3). Most *qnrD*-positive isolates were identified in laying hens from Spain. However, *qnrD*-genes were also detected in *Salmonella* isolates obtained in poultry, turkeys and food from Italy and in two human isolates from The Netherlands (Table 2).

Finally, *qnrA1* gene was identified in two *S.* Paratyphi B variant Java isolates from poultry in Belgium and The Netherlands and in one *S.* Typhimurium from turkeys in Germany (Table 3). None of the *Salmonella* isolates tested was found positive for *qnrC* or *qepA* genes.

A small proportion of the PMQR-positive *Salmonella* isolates (n = 10) demonstrated resistance against 3rd generation cephalosporins, indicative for the presence of extended-spectrum beta-lactamases (ESBL) or ampC-type beta-lactamases (data not shown).

In *E. coli,* 15% (20/133) of the selected isolates were PMQR-positive originating from four different countries (Table 1), but mostly from Poland. The isolates were obtained from food, poultry, turkeys, cattle and pigs (Table 4). *qnrS1* was detected in 19 isolates, whereas one isolate from a turkey was found *qnrB19*-positive. In this study, the earliest PMQR-positive *E. coli* detected were two isolates obtained in 2008 from pigs in Finland. None of the *E. coli* isolates tested was found positive for *qnrC, qnrD,* or *qepA* genes. Furthermore, a single *qnrS1*-positive *E. coli* collected from a Dutch broilers exhibited resistance against 3rd generation cephalosporins.

In *Salmonella*, PMQR-genes were identified in isolates with a wide range of MICs, but most PMQR genes were found in isolates with ciprofloxacin MICs of 0.25 – of 1 mg/L combined with nalidixic acid MICs of 8 – 32 mg/L. The PMQR-positive *E. coli* isolates exhibited a narrow range of ciprofloxacin (0.25 – 0.5 mg/L) and nalidixic acid MICs (4 – 16 mg/L).

Table 1. Number of *Salmonella enterica* and *E. coli* isolates included, selected and tested by

Country	Isolates included	Isolates selected	Isolates tested
Belgium	[a]2500/ [b]0	24/0	4/0
Czech Rep.	536/0	1/0	1/0
Denmark	24526/11345	68/182	54/36
Finland	787/5238	10/54	1/11
France	0/3750	0/45	0/45
Germany	33185/0	194/0	67/0
Ireland	1151/0	46/0	3/0
Italy	850/0	10/0	10/0
Norway	279/3762	2/15	1/4
Poland	2091/898	14/16	14/16
Spain	4048/0	109/0	109/0
The Netherlands	19886/6139	150/21	150/21
UK	571790/0	587/0	71/0
total	661629/ 31132	1215/ 333	485/133

a: number of Salmonella enterica isolates;
b: number of E. coli isolates;
c: aac(6')-1b-cr in combination with qnrB6;
d: aac(6')-1b-cr in combination with qnrS1

country and their respective PMQR genes detected

Isolates PMQR+	*qnrA*	*qnrB*	*qnrD*	*qnrS*	*aac(6')-1b-cr*
2/0	1/0	-	-	1/0	-
1/0	-	1/0	-	-	-
16/1	-	9/0	-	7/1	-
1/2	-	1/0	-	0/2	-
0/0	-	-	-	-	-
33/0	1/0	24/0	-	8/0	[c] 1/0
1/0	-	1/0	-	-	-
10/0	-	-	7/0	3/0	[d] 2/0
0/0	-	-	-	-	-
14/16	-	0/1	-	14/15	-
101/0	-	84/0	13/0	4/0	-
62/1	1/0	14/0	2/0	45/1	-
47/0	-	4/0	-	43/0	-
288/20	3/0	138/1	22/0	125/19	3/0

Table 2. Number (n) of identified PMQR genes in *Salmonella enterica* by country and source

country	environment	food	humans	pigs
Belgium	-	-	-	*qnrS1* (1)
Czech Republic	-	-	-	*qnrB2* (1)
Denmark	-	*qnrS1* (1)	-	-
Finland	-	-	-	-
Germany	-	*qnrB19* (2) *qnrS1* (2)	-	-
Ireland	*qnrB2* (1)		-	-
Italy	*qnrS1* + *aac(6')-1b-cr* (1)	*qnrD* (1) *qnrS1* + *aac(6')-1b-cr* (1)	-	-
Poland	-	-	-	*qnrS1* (2)
Spain	-	-	-	-
The Netherlands	-	*qnrS1* (2)	*qnrB2* (4) *qnrB7* (1) *qnrB12* (1) *qnrB19* (4) *qnrD* (2) *qnrS1* (43)	-
UK	-	-	*qnrB2* (2) *qnrB4* (1) *qnrB19* (1) *qnrS1* (43)	-

a: includes broilers, broiler meat and laying hens;
b. includes turkeys and turkey meat;
c: Salmonella obtained in turkey meat imported from Brazil

[a]poultry	reptiles	sheep	[b]turkeys	unknown origin
qnrA1 (1)	-	-	-	-
-	-	-	-	-
-	-	-	*qnrB19* (9) *qnrS1* (6)	-
-	-	-	[c]*qnrB19* (1)	-
qnrB19 (2)	*qnrB6* (1) *aac(6')-1b-cr* (1) *qnrB19* (9) *qnrS1* (1)	-	*qnrA1* (1) *qnrB2* (1) *qnrB19* (7) *qnrS1* (5)	*qnrB6* (2)
-	-	-	-	-
qnrD (3)		*qnrS1* (1)	*qnrD* (3)	-
qnrS1 (9) *qnrB2* (2) *qnrD* (13) *qnrS1* (4) *qnrA1* (1) *qnrB2* (1) *qnrB12* (1) *qnrB19* (2)	- - - -	- - - -	*qnrS1* (3) *qnrB2* (82) - -	- - - -
-	-	-	-	-

Table 3. Number (n) of identified PMQR-genes in *Salmonella enterica* by country and serovar

Serovar	Belgium	Czech Republic	Denmark	Finland	Germany
Agona	-	-	-	-	*qnrB2* (1)
Anatum	-	-	-	-	-
Braenderup	-	-	-	-	-
Bredeney	-	-	-	-	-
Cerro	-	-	-	-	-
Concord	-	*qnrB2* (1)	-	-	-
Corvallis	-	-	-	-	-
Dabou	-	-	-	-	-
Derby	-	-	-	-	-
Enteritidis	-	-	-	-	-
Give	-	-	-	*qnrB19* (1)	-
Goldcoast	*qnrS1* (1)	-	-	-	-
Hadar	-	-	*qnrB19* (6)	-	*qnrB19* (10)
Infantis	-	-	-	-	-
Kentucky	-	-	-	-	-
Kingston	-	-	*qnrS1* (1)	-	-
Litchfield	-	-	-	-	*qnrB6* + *aac(6')-1b-cr* (1)
London	-	-	-	-	-
Mbandaka	-	-	-	-	-
Meleagridis	-	-	-	-	-
Montevideo	-	-	-	-	*qnrB6* (2)
Newport	-	-	*qnrB19* (3)	-	-
Ohio	-	-	-	-	-
Paratyphi B var. Java	*qnrA1* (1)	-	-	-	-
Pomona	-	-	-	-	-
Rissen	-	-	-	-	-
Saintpaul	-	-	*qnrS1* (6)	-	*qnrS1* (7)
Senftenberg	-	-	-	-	-
Stanley	-	-	-	-	-
subsp. enterica 4,[5],12:i:-	-	-	-	-	-
Subspec. I rough	-	-	-	-	*qnrB19* (3)
Subspez. II	-	-	-	-	*qnrB19* (1)
Subspez. IV	-	-	-	-	*qnrB19* (2)
Typhimurium	-	-	-	-	*qnrA1* (1)
Uganda	-	-	-	-	*qnrB19* (1)
Urbana	-	-	-	-	*qnrB19* (3)
Virchow	-	-	-	-	-
Virginia	-	-	-	-	-
Wandsworth	-	-	-	-	-

Ireland	Italy	Poland	Spain	The Netherlands	UK
qnrB2 (1)	-	*qnrS1* (1)	-	-	*qnrS1* (1)
-	-	-	-	*qnrS1* (1)	-
-	-	-	*qnrD* (2)	*qnrD* (1)	-
-	-	-	-	*qnrB2* (1)	-
-	-	-	*qnrS1* (1)	-	-
-	-	-	-	*qnrB2* (3)	-
-	-	-	-	*qnrS1* (34)	*qnrS1* (6)
-	-	-	*qnrD* (1)	-	-
-	*qnrS1* (1)	-	*qnrB2* (56)	-	*qnrB19* (1)
-	-	*qnrS1* (2)	*qnrB2* (1) *qnrD* (3)	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	*qnrB2* (2)	*qnrD* (1)	-
-	-	*qnrS1* (1)	*qnrS1* (1)	*qnrS1* (1)	-
-	-	-	-	*qnrS1* (2)	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	*qnrB2* (22)	-	-
-	-	*qnrS1* (1)	-	*qnrB7* (1)	-
-	-	-	-	*qnrB19* (1)	-
-	*qnrD* (7)	-	*qnrB2* (3)	*qnrB12* (1) *qnrS1* (3)	*qnrS1* (1)
-	-	*qnrS1* (5)			
-	-	-	*qnrD* (5) *qnrS1* (1)	-	-
-	-	-	-	*qnrA1* (1), *qnrB19* (3)	*qnrS1* (1)
-	-	-	-	-	-
-	-	-	-	*qnrS1* (1)	-
-	-	*qnrS1* (4)	-	*qnrS1* (1)	*qnrS1* (1)
-	-	-	*qnrS1* (1)	*qnrB2* (1)	-
-	-	-	-	*qnrS1* (2)	-
-	*qnrS1* + *aac(6')-1b-cr* (1)	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	*qnrD* (2)	*qnrB12* (1), *qnrB19* (2)	*qnrB2* (1) *qnrB4* (1) qnr*S1* (31)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	*qnrB2* (1) *qnrS1* (1)
-	-	-	-	-	*qnrS1* (1)
-	*qnrS1* + *aac(6')-1b-cr* (1)	-	-	-	-

Table 4. Number (n) of PMQR-genes identified in *E. coli* by country and source

Country	cattle	food	pigs	poultry	turkeys
Denmark	-	*qnrS1* (1)	-	-	-
Finland	-	-	*qnrS1* (2)	-	-
Poland	*qnrS1* (1)	-	*qnrS1* (3)	*qnrS1 (7)*	*qnrB19* (1), *qnrS1* (4)
The Netherlands	-	-	-	*qnrS1 (1)*	-

Discussion

In general, the acquisition of PMQR-genes leads to decreased susceptibility to fluoroquinolones, which affects the mutation prevention concentration (MPC) and may accelerate selection of fluoroquinolone resistant mutants. [24, 25] Moreover, interactions between mutations in the QRDR and PMQR-genes can result in higher MICs for ciprofloxacin.[26] Hence, data on the occurrence of PMQR-genes are important for the surveillance of quinolone resistance in humans and animals. A limitation of our study was the potential exclusion of isolates in which both PMQR-genes and chromosomal mutations were present. The quinolone resistance phenotype of these isolates is not indicative for the presence of PMQR-genes. Isolates with mutations in the QRDR exhibit high level resistance against nalidixic acid (MIC ≥ 32 mg/L) in combination with reduced susceptibility to fluoroquinolones, independent of the presence of PMQR-genes. Therefore these phenotypes are not suitable for retrospective screening of MIC-databases for PMQR. As a result, our screening method has probably underestimated the prevalence of PMQR-genes in the total population, whilst coexistence of PMQR and mutations has been reported in Enterobacteriaceae from animals.[27, 28] Furthermore, the number of isolates included in this study depended on the number of isolates available in the strain collections, which varied considerably between countries (Table 1). Also, the number of isolates selected and tested, as well as the sources, varied between countries. The *Salmonella* strains in the collections originated from eleven countries and were isolated from humans, animals, food and the environment. The human clinical *Salmonella* isolates originated from the UK and The Netherlands only. The *E. coli* strains in the collections originated from six countries and were solely isolated from animals and food. Therefore, in depth epidemiological analysis of the results by country and source was not appropriate. The analysis was limited to a

description of the emergence and occurrence of PMQR and its characteristics instead of prevalences by source and country. Nevertheless, the strength of our study is the selection isolates with a defined quinolone resistance phenotype in a large strain collection resulting in a relatively high detection rate of PMQR-genes within the selection, especially in *Salmonella*.

We detected *qnrS1* in a large number of serovars particularly in human *S*. Corvallis and *S*. Typhimurium *Salmonella* isolates. Eight *qnrS1*-positive *Salmonella* isolates from the UK originated from people with a travel history to South-East Asia (data not shown). These findings confirm earlier reports of human *qnrS1*-positive *Salmonella* isolates from Denmark and the UK associated with foreign traveling or imported food from South-East Asia.[29, 30] The occurrence of *qnrS1* has recently been described in four *S*. Saintpaul isolates collected from turkey meat in Germany and Poland.[22] These four isolates were also included in our study and we identified 15 other *qnrS1*-positive *S*. Saintpaul isolates collected from turkeys, poultry, humans and food. Our findings demonstrate further dissemination of this serovar to other sources. To our knowledge, we report the first *qnrS1* in serovars Agona, Cerro, Derby, Goldcoast, Kingston, Mbandaka, Newport, Senftenberg, Ohio, Wandsworth and *S. enterica* serovar 4,[5],12:i- indicating horizontal transmission of this PMQR-genes within *S. enterica*.

Most of the *qnrB*-positive *Salmonella* isolates were of animal origin. However, this number was highly influenced by the proportion of animal isolates in the total selection and, in particular, by the number of *qnrB*-positive turkey isolates from Spain. Nevertheless, we identified *qnrB* in *Salmonella* of animal origin in seven different European countries.

To date, the only case of a *qnrB2*-positive *Salmonella* Bredeney isolate of animal origin was reported in a Dutch broiler chicken.[31] However, in the present study, we identified *qnrB2* genes in serovars Agona, Derby, Enteritidis, Hadar, London and Montevideo (Table 3), most obtained from turkeys in Spain. The high number of *qnrB2*-positive *Salmonella* from Spain found in this study suggests a potential source for transmission of these genes to humans in this country. Nevertheless, reports on the occurrence of human *qnrB2*-positive *Salmonella* isolates in Spain are scarce.[32, 33] In our study, the number of *qnrB2*-positive human isolates was also low.

The presence of *qnrB19* has been described in different *Salmonella* serovars of human origin, including *S*. Typhimurium isolates from The Netherlands and Italy.[21,34] We describe two *qnrB19*-positive *S*. Paratyphi B variant Java isolates obtained from chicken products and a human patient, all collected in The Netherlands in the same period (2007 – 2008) suggesting transfer of this gene between animals and humans. In addition, to our knowledge, we report the first *qnrB19*-positive serovars Derby, Meleagridis and Paratyphi B variant Java.

The first *qnrD* was described in *S*. Kentucky and *S*. Bovismorbificans isolates from humans in China.[16] To date, *qnrD* has also been reported in *Morganella morganii* and *Proteus mirabilis* from Italy[35] and in *M. morganii* isolate from Algeria.[36] However, to our knowledge, this is the first description of *qnrD* in *Salmonella* isolates from both humans and animals in Europe. Remarkably, the first *qnrD*-gene in our study was detected in a human isolate collected in 2003 from The Netherlands. Moreover, we identified *qnrD* in *Salmonella* from Spain collected in 2004-2008 from laying hens. These findings reveal that this novel described PMQR-gene was already present in Europe for several years, being able to spread over different European countries, before it was identified in China. Moreover, there seems to be a cluster of *qnrD*-positive *Salmonella* isolates in Italy and Spain which should be further verified by additional studies.

Although *qnrA1* is the first PMQR-gene described, this gene is rarely found in *Salmonella*.[37-39] To our best knowledge, we describe the first *qnrA1* in *Salmonella* isolates of animal origin in two *S*. Paratyphi B variant Java isolates collected from poultry products and in one *S*. Typhimurium isolate collected from turkey. Both *S*. Paratyphi B variant Java isolates were multidrug resistant, including resistance against cefotaxime. Moreover, the Dutch isolate was confirmed ESBL-positive harboring a $bla_{\text{CTX-M-9}}$ in addition to a *qnrA1* on an IncHI2 plasmid (data not shown). Even though *qnr* genes have often been associated with plasmid encoded extended beta-lactamase (ESBL) genes[11, 40], in our study low numbers of such isolates were found.

To date, European reports of PMQR-genes in *E. coli* isolates of animal origin are scarce.[41-43] In our study, sixteen of the isolates originated from different healthy animals in Poland suggesting a more frequent occurrence of PMQR-positive *E. coli* in this country. Almost all isolates were *qnrS1*-positive, but one isolate obtained from a turkey was found *qnrB19*-

positive. PFGE revealed low genetic similarity of those isolates indicating independent source and routes of infections (data not shown). To our knowledge, we report the first *qnr*-genes in *E. coli* isolates from animals in Finland (*qnrS1*) and Poland (*qnrS1* and *qnrB19*). These findings indicate that PMQR genes are infrequently present in the commensal *E. coli* in the gut flora of food producing animals in Europe with reduced susceptibility to fluoroquinolones.

Within the selection the occurrence of *Salmonella* isolates with *aac(6')-1b-cr* was low and in all cases coexistence with a *qnr*-gene was found as described in previous studies.[27, 28] In addition, the *aac(6')-1b-cr*-gene was not identified in any *E. coli* isolate. The low incidence of *aac(6')-1b-cr* in our study might be explained by the inclusion criteria defined for this study. Isolates with merely *aac(6')-1b-cr* exhibit a minor reduction in susceptibility to ciprofloxacin and therefore could have been missed in our selection.[10]
Despite of all the PMQR-genes identified, the resistance phenotype of a relative large proportion of the tested isolates remains unexplained: 41% of the *Salmonella* and 85% of the *E. coli* isolates tested negative for all known PMQR genes. This could be due to the presence of new undetected PMQR genes or other quinolone resistance mechanisms present or inadequate selection criteria. Since all isolates included exhibited nalidixic MICs ≥ 32 mg/L we did not look for mutations in the QRDR region. Therefore, additional analysis would be necessary to reveal other possible resistance mechanisms like efflux pumps involved.

Summarizing, our study confirms the occurrence of PMQR-positive *Salmonella* isolates of human and animal origin in Europe since 2002. Moreover, the detection of PMQR-genes in *Salmonella* obtained from animals in ten different countries is indicative of their wide spread occurrence in Europe. Within our selection, most PMQR-positive *Salmonella* isolates originated from Spain, The Netherlands, UK, Germany and Denmark and were predominantly obtained from humans, turkeys and poultry. Although these data are influenced by differences in the number of isolates per country and source, it suggests fowl is the main source for PMQR-determinants in Europe with predominant serovars Derby, London, Saintpaul and Hadar. Furthermore, part of the human PMQR-positive *Salmonella* in this study were associated with foreign travel, particularly *qnrS1*-positive *S.* Corvallis and *S.* Typhimurium isolates. In addition, we report the first *qnrD* in *Salmonella* isolated outside of Asia. Our study also demonstrates the presence of PMQR-genes in *E. coli* recently

isolated from animals in four European countries. Despite the fact that fluoroquinolones are used in food-producing animals since the end of the nineteen eighties, PMQR-genes occur relatively infrequently in animal isolates. This suggests that PMQR-positive isolates without additional mutations in the QRDR-region, are not selected during treatment of animals with fluoroquinolones. Apparently administration of fluoroquinolones in food-producing animals results in higher concentrations in the gastro-intestinal tract than the MICs of PMQR-positive isolates. Co-selection by usage of other antibiotics may occur once PMQR-genes are embedded on plasmids harbouring additional resistance genes. On the other hand, if PMQR-genes do accelerate the selection of fluoroquinolone resistant mutants during treatment of animals with fluoroquinolones, the focus of surveillance studies should also be aimed at detection of PMQR-genes in fluoroquinolone resistant isolates. Moreover, long-term surveillance is needed for future monitoring of the trends in the occurrence of PMQR-genes in Europe. Also, future studies are required focusing on plasmid analysis to understand more about the successful dissemination of PMQR-genes in Enterobacteriaceae.

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Transparency declarations

None to declare.

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3b

CHAPTER 3B

Characterization of plasmids harbouring *qnrD1* in *Salmonella enterica* isolates from Italy, Spain, and The Netherlands

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In preparation

Abstract

During an international study on the occurrence of plasmid mediated quinolone resistance (PMQR) in Europe *qnrD* (currently designated *qnrD1*) was detected in *Salmonella enterica* from Spain, Italy and The Netherlands. Since this was the first description of *qnrD1* in *Salmonella* isolates from Europe, the genetic environment of *qnrD1* was further characterized.

A total of 18 *qnrD1*-positive *Salmonella* of 8 different serovars (Braenderup (n=3), Dabou (n=1), Enteritidis (n=1), Hadar (n=1), Mbandaka (n=1), Montevideo (n=5), Ohio (n=5), Typhimurium (n=1) obtained from poultry (n=16) and humans (n=2) were studied. *XbaI*-PFGE was performed on total DNA of *S*. Braenderup, *S*. Ohio and *S*. Montevideo isolates to determine possible genetic relationships. *QnrD1*-carrying plasmids were transferred to *E. coli* DH10B by electroporation. Susceptibility (Minimum Inhibitory Concentration, MIC) of *qnrD1*-positive transformants to nalidixic acid, flumequine and eight fluoroquinolones was determined by broth microdilution. The genetic background of the *qnrD1* genes was studied in transformants by determining the size of the *qnrD1*-harbouring plasmids and performing PCR-based replicon typing (PBRT) combined with restriction fragment length polymorphism (RFLP) analysis. Finally, four different RFLP typed plasmid genomes were sequenced using an Illumina MiSeq platform.

The MICs of *qnrD1*-positive transformants for fluoroquinolones ranged from 0.125 - 0.5 mg/L. Compared to wild type DH10B cells MICs increased with a factor 4 to 64. All plasmids were relatively small with sizes ranging from 6 – 12 kb. Most plasmids (n = 15) were non-typeable with PBRT and exhibited the same RFLP type (type I). One PBRT non-typeable isolate revealed a different RFLP type (type II). Finally two ColE-like plasmids showed distinct RFLP patterns (type III and IV). Four different RFLP type plasmids were sequenced and designated pKVHS-001 to -004. Sequence analysis of the plasmids demonstrated the presence of an identical *qnrD1*-harbouring region in all four plasmids showing 99% identity to a *qnrD1*-harbouring plasmid pCGS49 from *Proteus mirabilis*. In three plasmids (pKVHS-001, pKVHS-002 and pKVHS-003) the *qnrD1*-harbouring region was flanked by IS1-elements. Remarkably, an extra pCGS49-like sequence was identified in pKVHS-002 harbouring a second *qnrD1*-gene and flanked by a third IS1-element. In pKVHS-004, the *qnrD1*-harbouring pCGS49-like region was flanked by direct repeats. Finally, *XbaI*-PFGE profiles revealed identical patterns for two *S*. Braenderup, three *S*. Ohio isolates and five *S*. Montevideo isolates.

The presence of *qnrD1* genes decreases the susceptibility to fluoroquinolones in *Salmonella enterica*. All *qnrD1* genes were identified on small plasmids in *Salmonella* from different serovars and sources, often with identical RFLP plasmid types. Analysis of the total sequence of these plasmids revealed a striking similarity in the genetic environment of *qnrD1* indicative for a common progenitor plasmid from *P. mirabilis*. *XbaI*-PFGE results indicate clonal dissemination of *qnrD1*-positive *Salmonella* serovars in Italy and Spain.

Introduction

To date, resistance to quinolones in Enterobacteriaceae is most frequently generated by target modification due to chromosomal mutations in the quinolone resistance determining region (QRDR) of DNA gyrase and topoisomerase IV. Nevertheless, in recent years different types of plasmid mediated quinolone resistance (PMQR) have arisen [1]. The first PMQR determinant was identified in a *Klebsiella pneumoniae* isolate in 1998 [2]. This human isolate from Alabama (US) possessed a gene designated *qnrA1* responsible for reduced susceptibility to fluoroquinolones located on plasmid pMG252. The Qnr protein is a pentapeptide repeat protein (PRP) binding to DNA gyrase and topoisomerase IV thus protecting the binding site for fluoroquinolones [3]. In the last decennium new variants of *qnr*-genes have been reported: *qnrB* in 2005, followed by *qnrS* in 2006 and both *qnrC* and *qnrD* in 2009 [1]. In the same period, other types of fluoroquinolone resistance mechanisms appeared including a modified acetyl transferase (*aac(6')-1b-cr*) [4] and a specific efflux pump (*qepA*) [5]. Currently, a large number of variants are described for *qnrA* (n=7), *qnrB* (n=72) and *qnrS* (n=9) (http://www.lahey.org/qnrStudies/) based on single or multiple DNA-mutations, but new variants of *qnrD* are still rare (n=2) and are absent for *qnrC*.

The first described *qnrC* gene was located on a 120 kb non-typeable plasmid pHS10 in *Proteus mirabilis* isolated from a urinary tract of a patient in Shanghai, China [6]. The first *qnrD* gene was found in two different *Salmonella enterica* serovars; Kentucky (n=1) and Bovismorbificans (n=3) isolated from patients in the Henan province in China[7]. Recently this gene was renamed *qnrD1*, since a related gene has been identified in *S*. Hadar designated *qnrD2*[8]. (http://www.lahey.org/qnrStudies/). In the study of Cavaco et al. [7] *qnrD1* was located on a small (4270 bp) non-typeable plasmid designated p20077057. To date, *qnrD*-genes have been reported in different bacterial species and sources comprising: *Escherichia coli* isolated from pigs, chickens, farmed fish, pigeon feces and dog feces all from China [9-11], a clinical isolate of *K. pneumonia* from China [11], clinical isolates of *Morganella morganii* in Italy and China [11, 12], clinical isolates of *Providencia rettgerii* from France [13], clinical isolates of *Proteus mirabilis* from Italy, Poland and Nigeria [12, 14, 15], *Proteus mirabilis* isolated from dogs [11] and chicken carcasses in China [16], environmental isolates of *Proteus vulgaris* from the Jiaozhou Bay in China [17], a clinical *P. vulgaris* isolate from Poland [14], a clinical *Pseudomonas aeruginosa* isolate from Nigeria [15] and unspecified Enterobacteriaceae from rooks in the Czech Republic and Poland [18].

In a recent publication *qnrD* was identified in 12 clinical *P. mirabilis* isolates from China in combination with *aac(6')-1b-cr* and bla_{KPC-2} on different plasmids [19].
Although, *qnrD* was first described in different serovars of *Salmonella enterica*, additional reports of *qnrD* in this species are still rare. Nevertheless, in a European study on the occurrence of PMQR in *E. coli* and *Salmonella enterica, qnrD1* was detected in several serovars of *Salmonella* from Spain, Italy and The Netherlands[20]. Because this was the first description of *qnrD* in *Salmonella enterica* isolates from Europe further study was conducted to reveal the genetic background of these genes. Therefore, full-length sequencing of identified *qnrD*-harbouring plasmids was performed.

Materials and Methods

Isolates used in the study

The study comprised 18 *qnrD1*-positive *Salmonella enterica* from Spain (n=10), Italy (n=6) and The Netherlands (n=2) including fifteen *Sa lmonella* isolates collected in 2003-2008 described in a previous study [20] and three *S*. Montevideo isolates from Italy collected in 2009-2011 (Table 1). The Spanish isolates originated from laying hens, and the Italian isolates from various poultry sources, while the two Dutch isolates were both obtained from human patients. The strain collection consisted of 8 different serovars (Braenderup (n=3), Dabou (n=1), Enteritidis (n=1), Hadar (n=1), Mbandaka (n=1), Montevideo (n=5), Ohio (n=5), Typhimurium (n=1).The majority (n = 15) of the isolates were identified during an international study on the prevalence of PMQR in *Salmonella* and *E. coli* based on a specific quinolone resistance phenotype (reduced susceptible to ciprofloxacin and susceptible to nalidixic acid) and PCR [20].

Susceptibility testing

Antimicrobial susceptibility was tested by broth micro-dilution with a custom made panel (Sensititre©, Trek Diagnostic Systems, UK) of thirteen antibiotics (ampicillin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, trimethoprim and tetracycline) according to ISO standards [21]. For interpretation epidemiological cut-off values were used as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://mic.eucast.org/Eucast2/).

Analysis of bacterial isolates and plasmids

Xbal-PFGE was performed on total DNA of *S*. Braenderup, *S*. Ohio and *S*. Montevideo to determine possible genetic relationships according to Pulsenet protocol (www.cdc.gov/pulsenet/). Next, dendrograms were constructed based on the restriction patterns using Bionumerics (Applied Maths, version 6.6) with Dice similarity coefficient and unweighted pair group method with arithmetic mean (UPGMA). Plasmid DNA of donor strains was purified using a modified miniprep method [22] and transferred to *E. coli* DH10B (Gibco Invitrogen, USA) by electroporation. Subsequently, *qnrD1*-positive transformants were selected on LB agar with 0.06 mg/L ciprofloxacin. The presence of *qnrD1* in the transformants was confirmed by PCR and sequencing of the amplicons using previously

described primers [7]. In addition, Minimum Inhibitory Concentrations (MICs) of wild-type *E. coli* DH10B and *qnrD1*-positive transformants were determined for quinolones (nalidixic acid and flumequine) and eight fluoroquinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, levofloxacin, moxifloxacin, norfloxacin and ofloxacin) by broth microdilution. Subsequently, the *qnrD1*-carrying plasmids were typed by PCR based replicon typing (PBRT) using a commercial kit (DIATHEVA, Italy). A home-designed RFLP based on a combined restriction of *Xba*I and *Mbo*II was applied for subtyping of the plasmids. Next, the size of the *qnrD1*-carrying plasmids was estimated for each RFLP type by agarose gel electrophorese after restriction of purified plasmid DNA using *Sma*I. In one case (RFLP type II), *Sma*I digestion failed and alternatively *Eco*RV was used for size estimation. Finally, sequencing of four plasmid genomes with different RFLP types was performed using 150 bp paired-end sequencing libraries (Nextera TAG-mentation sequencing kits [Epicentre, Madison USA]) on an Illumina MiSeq sequencer. High quality filtered reads were subsequently assembled using the SPAdes algorithm (version 3.0.0). BLAST searches were performed (d.d. 03-03-14) using the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov) blastn algorithm.

Results

Susceptibility

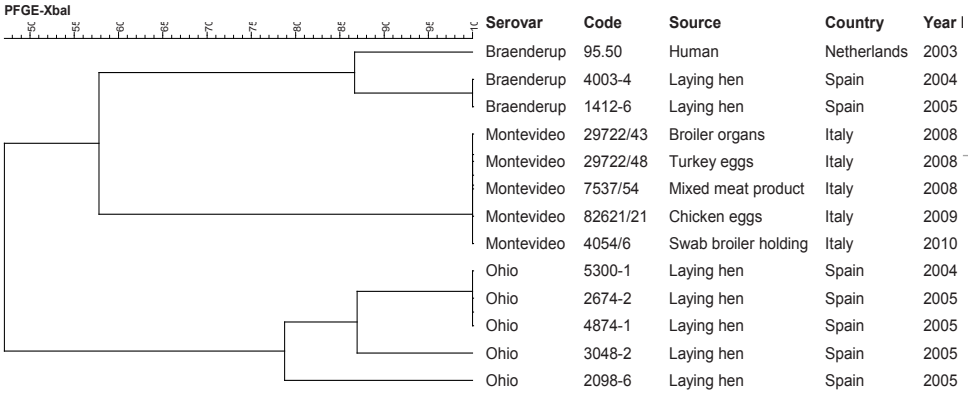
Except for the specific quinolone resistance phenotype (susceptible to nalidixic acid, but reduced susceptibility to ciprofloxacin) most *Salmonella* isolates were susceptible to all other antibiotics tested (data not shown). Nevertheless, one isolates was resistant to tetracycline, one isolate was resistant to ampicillin and another isolate was resistant to both ampicillin and cefotaxime due to the presence of bla_{TEM-52} (data not shown). Susceptibility testing of the *qnrD1*-positive transformants demonstrated exclusive transfer of resistance to quinolones without co-transfer of other microbial resistances. This indicates that no other resistant genes were present on the transferred *qnrD1*carrying plasmids. Compared to the wild type DH10B cells *qnrD1*-positive transformants revealed a 4-8 fold decrease in MICs to quinolones (nalidixic acid and flumequine) and 8-64 times reduction in susceptibility to fluoroquinolones (see Table 1).

Table 1. *In vitro* activity of quinolones against *qnrD1*-positive *Salmonella* isolates, wild-type *E. coli* DH10B and *E. coli* DH10B carrying *qnrD1*-genes

Antibiotic	E. coli DH10B (wild type)	qnrD+ E. coli DH10B transformants	
	MIC	MIC_{50} [a]	MIC increase factor[b]
Ciprofloxacin	0.004	0.125	32
Danofloxacin	0.008	0.25	32
Difloxacin	0.015	0.5	32
Enrofloxacin	0.004	0.25	64
Flumequine	0.5	4	8
Levofloxacin	0.008	0.125	16
Moxifloxacin	0.008	0.25	32
Nalidixic acid	2	8	4
Norfloxacin	0.03	0.25	8
Ofloxacin	0.015	0.25	16

a: MIC_{50} = minimum concentration of an antibiotic required to inhibit the growth of ≥ 50% of the isolates tested.
b: MIC increase factor = MIC increase of qnrD-positive transformants compared to DH10B cells

Figure 1. Dendrogram based on *Xba*I-PFGE restriction patterns of total DNA of *qnrD1*-harbouring *S*. Branderup (n = 3), *S*. Montevideo (n = 5) and *S*. Ohio (n = 5) isolates.



Serovar	Code	Source	Country	Year
Braenderup	95.50	Human	Netherlands	2003
Braenderup	4003-4	Laying hen	Spain	2004
Braenderup	1412-6	Laying hen	Spain	2005
Montevideo	29722/43	Broiler organs	Italy	2008
Montevideo	29722/48	Turkey eggs	Italy	2008
Montevideo	7537/54	Mixed meat product	Italy	2008
Montevideo	82621/21	Chicken eggs	Italy	2009
Montevideo	4054/6	Swab broiler holding	Italy	2010
Ohio	5300-1	Laying hen	Spain	2004
Ohio	2674-2	Laying hen	Spain	2005
Ohio	4874-1	Laying hen	Spain	2005
Ohio	3048-2	Laying hen	Spain	2005
Ohio	2098-6	Laying hen	Spain	2005

For similarity analysis, Dice coefficient was used and clustering was performed by using unweighted pair group method with arithmetic means (UPGMA) with 1% band position tolerance.

Analysis of isolates and plasmids

As shown in Figure 1, *Xba*I-PFGE profiles of three *S*. Braenderup isolates revealed identical patterns for two *S*. Braenderup isolates both from laying hens in Spain, but a distinctive pattern (< 90% similarity) for a human isolate from The Netherlands. Analysis of five *S*. Ohio isolates all from laying hens in Spain revealed a cluster of three isolates with identical patterns, but a fourth isolate and fifth isolate with different patterns. Finally, all five *S*. Montevideo isolates from different poultry sources in Italy showed identical restriction patterns with *Xba*I-PFGE.

In all isolates, *qnrD1*-carrying plasmids were transferred by electroporation into *E. coli* DH10B. Restriction analysis of purified plasmid DNA of the *qnrD1*-positive transformants demonstrated the presence of *qnrD1* genes on relative small plasmids with estimated sizes ranging from 6 – 12 kb. RFLP experiments revealed the presence of a predominant RFLP plasmid type (n = 15) designated as type I. The remaining three plasmids all showed unique RFLP types designated as type II, II and IV (see Table 2). Plasmids with RFLP type II and III were identified as ColE-like, but plasmids with RFLP type I and IV were non-typeable with PBRT.

Plasmid sequences

Plasmid pKVHS-001 was identified in *S*. Braenderup (code 95.50) isolated from a patient in The Netherlands in 2003 and belonged to the, predominant RFLP type I of this study. The total size of this non-typeable plasmid amounted 6887 bp. BLAST search (blastn) of the total DNA sequence demonstrated the presence of a 2678 bp sized fragment with 99% nucleotide identity to the *qnrD*- bearing plasmid pCGP248 from *P. mirabilis* (Genbank no JQ776503). The *qnrD1* gene present on pKVHS-001 showed 100% identity to the first described *qnrD* gene (Genbank no FJ228229) [7]. This *qnrD1*-bearing region was flanked by two identical 769 bp sized fragments with 99 % DNA identity to the insertion sequence (IS1) from *Shigella boydii* (Genbank no CP000036). Both IS-elements encoded a transposase-like protein called InsB. In addition, the plasmid harboured a fragment of 502 bp which showed 99% nucleotide identity to a fragment of plasmid pAm08CD9902 from *E. coli* (Genbank no GQ149344). Plasmid pKVHS-002 (RFLP type IV) was non-typeable with PBRT, but larger than pKVHS-001 with a total size of 11585 bp. This plasmid was identified in *S*. Typhimurium (code 14620) isolated from a laying hen in Spain in 2008. Alignment demonstrated that complete sequence of pKVHS-001 was identically present in pKVHS-002. Compared to pKVHS-001 plasmid pKVHS-002 contains an extra fragment of 1240 bp (positions 6201-7441). BLAST search (blastn) of this fragment did not reveal homology with other published sequences. Remarkably, another region of 3458 bp was identified (positions 8128 - 11585) an identical second pCGS49-like (99% identity to pCGS49) sequence was identified in pKVHS-002 harbouring a second *qnrD1*-gene and flanked by a third IS1-element. Plasmid pKVHS-003 (RFLP type III) was identified in *S*. Montevideo (code 29722/48) isolated from a turkey egg in Italy in 2008. This ColE-like plasmid is the largest in this study with a size of 9384 bp. This plasmid reveals a similar structure as pKVHS-001 and pKVHS-002. The first region of 4228 bp is identical to the other two plasmids with *qnrD1* flanked by two IS1-elements. The second region (5156 bp) contains a 2016 bp sized fragment with 99% identity to a ColE1-like plasmid (2495 bp) designated pR23 from *Enterobacter agglomerans* (Genbank no AF14880)

Plasmid pKVHS-004 (RFLP type II) was identified in *S*. Hadar (code 139.67) from a patient in The Netherlands in 2005. This plasmid, also typed as ColE-like, is the smallest plasmid in this study with 6277 kb. Comparable to the other three plasmids, pKVHS-004 also contains a region including the *qnrD1*-gene identical to pCGP248 from *P. mirabilis* (Genbank no JQ776503). However, unlike the other three plasmids, this *qnrD1*-bearing region is not flanked by IS1 elements, but flanked by two identical direct repeats (both 259 bp).

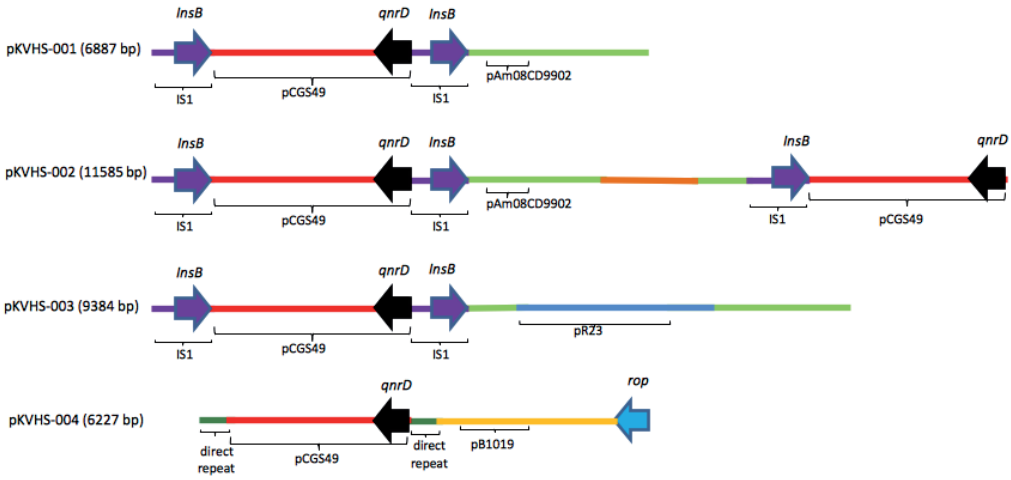
The second region (3024 bp) contains one fragment of 873 bp with 88% identity to a ColE-like plasmid pB1019 from *K. pneumoniae* (Genbank number JQ319775) and another fragment of 590 bp with 94% identity to plasmid pE66An from *E. coli* (Genbank number GQ149344). encoded a Rop protein (100% identity). This protein is involved in the regulation of DNA replication (ref).

Table 2. Data of *Salmonella enterica* from Europe and their *qnrD1*-carrying plasmids

Salmonella enterica					*qnrD1*-carrying plasmids	
Serovar	Code	Source	Country	Year	RFLP type	PBRT
Braenderup	95.50	wHuman	The Netherlands	2003	I[d]	n.t.
Hadar	139.67	Human	The Netherlands	2005	II[e]	ColE-like
Montevideo[a]	7537/54	Mixed meat product	Italy	2008	I	n.t.
Montevideo[a]	29722/48	Turkey eggs	Italy	2008	III[f]	ColE-like
Montevideo[a]	29722/43	Broiler organs	Italy	2008	I	n.t.
Montevideo[a]	82621/21	Chicken eggs	Italy	2009	I	n.t.
Montevideo[a]	4054/6	Swab broiler holding	Italy	2010	I	n.t.
Mbandaka	45538/10	Litter Broiler holding	Italy	2011	I	n.t.
Braenderup[b]	4003-4	Laying hen	Spain	2004	I	n.t.
Braenderup[b]	1412-6	Laying hen	Spain	2005	I	n.t.
Ohio[c]	5300-1	Laying hen	Spain	2004	I	n.t.
Ohio	2098-6	Laying hen	Spain	2005	I	n.t.
Ohio[c]	2674-2	Laying hen	Spain	2005	I	n.t.
Ohio	3048-2	Laying hen	Spain	2005	I	n.t.
Ohio[c]	4874-1	Laying hen	Spain	2005	I	n.t.
Dabou	806-4	Laying hen	Spain	2005	I	n.t.
Enteritidis	11149	Laying hen	Spain	2007	I	n.t.
Typhimurium	14620	Laying hen	Spain	2008	IV[g]	n.t.

Restriction Fragment Length Polymorphism (RFLP) types were determined with a combined restriction of XbaI and MboII.
n.t. = non-typeable with PCR Based Replicon Typing (PBRT). a = S. Montevideo isolates indistinguishable with XbaI-PFGE
b = S. Braederup isolates indistinguishable with XbaI-PFGE c = S. Ohio isolates indistinguishable with XbaI-PFGE
d = plasmid designated pKVHS-001 (Genbank number: KJ685894) e = plasmid designated pKVHS-004 (Genbank number: KJ685892)
f = plasmid designated pKVHS-003 (Genbank number: KJ685891) g = plasmid designated pKVHS-002 (Genbank number: KJ685893)

Figure 2. Map of four different *qnrD1*-bearing plasmids identified in *Salmonella enterica*



The most relevant ORFs are depicted in the map, identical colours indicate identical DNA sequences, IS1: Insertion Sequence including InsB identified in Shigella boydii (Genbank nummerCP00036), pCGS49: plasmid identified in P. mirabilis including qnrD1(Genbank nummer JQ776507), pAm08CD9902: plasmid identified in E. coli (Genbank no GQ149344), pR23: ColE-like plasmid identified in Enterobacter agglomerans (Genbank nummer AF14880), pB1019: ColE-like plasmid identified in Klebsiella pneumoniae (Genbank nummer JQ319775), pE66AN: plasmid identified in E. coli including a rop-like gene (Genbank GQ149344).

Discussion

In a previous study[20], *qnrD* (currently designated *qnrD1*) was identified in different serovars of *Salmonella enterica* from three different European countries. The majority of the isolates originated from poultry sources comprising laying hens from Spain and products of broilers and turkeys from Italy, and two *Salmonella* isolates were obtained from patients in The Netherlands. The Dutch patient who was infected with *S*. Braenderup had recently worked at an egg processing factory. *S*. Braenderup is occasionally isolated from different poultry sources in The Netherlands including laying hens [23]. Therefore, this patient might have been infected via direct contact with contaminated eggs. The other Dutch patient who was infected with *S*. Hadar had a travel history to Thailand. In this country, *S*. Hadar is a common serovar in food (frozen chicken meat and frozen shrimps) and humans[24] . More recently, *S*. Hadar was reported as one of the 25 most common serovars in humans in Thailand [25] . Hence, this patient might have been infected by consumption of contaminated food during holidays in Thailand. However, *S*. Hadar is a also a common

serovar in poultry from The Netherlands [23]. To our knowledge, besides the European study [20] there are no other reports of *qnrD1*-positive Enterobacteriaceae (including *Salmonella enterica*) obtained in European poultry. Noticeably, a new variant designated *qnrD2* was reported in *S*. Hadar from Switzerland[8] on a small (4268 bp) plasmid with 99% homology to p2007057. Conversely, the presence of *qnrD* in chickens from China has been reported in different Enterobacteriaceae species comprising *E. coli* [10], *C. freundii* [11] and *P.mirabilis* isolates [16].
In Europe, *qnrD* genes are exclusively reported in clinical human Enterobacteriaceae isolates from Poland, Italy and France comprising *P. mirabilis*, *P. vulgaris*, *M. morganii*, *and P.* rettgerii [12-14].

Our study demonstrates that *qnrD1* confers reduced susceptibility to a wide variety of fluoroquinolones, but to a lesser extent to nalidixic acid and flumequine, and that this feature is independent of plasmid type. These results are in line with previously reported data concerning the effect of *qnrD1* on susceptibility to quinolones [7] and comparable to the quinolone resistance phenotype conferred by other *qnr* genes [26]. Although, we did not screen for mutations in the QRDR, the relatively low nalidixic acid MICs (8 - 16 mg/L) for all *Salmonella* isolates involved in our study revealed the absence of functional mutations in the QRDR[27].

Similar to the first described *qnrD* gene in *Salmonella enterica* on plasmid p2007507[7], we identified *qnrD1* genes on relative small, mostly PBRT non-typeable plasmids. Plasmid p2007507 harboured five possible open reading frames (ORFs), but no replication proteins or repeat sequences. However, sequence analysis of the four plasmids included in our study revealed different structures compared to p2007507. Moreover, the similarity in the structure of the four plasmids is remarkable and different from any other published *qnrD1* plasmid sequence (see Figure 2). Firstly, all four plasmids analyzed contained an identical region harbouring the *qnrD1*-gene which is almost identical (99% DNA homology) to the complete sequence of plasmid pCGS49 identified in *P. mirabilis* from a pig liver in China [11]. Secondly, the *qnrD1*-harbouring region was either flanked by insertion sequences (in pKVHS-001, pKVHS-002 and pKVHS-003) or by direct repeats (in pKVHS-004). Thirdly, all four plasmids contained an extra region of variable size. Remarkably, pKVHS-002 harboured a second *qnrD1*-gene on an identical pCGS49-like region flanked by a third IS1-

element. The presences of a third IS-element suggest that this unusual plasmid structure was developed by successive recombination events. The presence of a second *qnrD1*-gene did not result in measurable higher MICs for the (fluoro)quinolones tested. Finally, in pKVHS-004, a complete gene involved in the regulation of DNA replication (designated as *rop*) was identified. This *rop*-gene seemed to be the only ORF with a known function on all four plasmids analyzed (results not shown).

The similarity in the genetic environment of the *qnrD1* genes in this study indicates the existence of a joint progenitor plasmid similar to plasmid pCGS49 identified in *P. mirabilis* [11]. This finding is in accordance with a previous publication in which bacterial species belonging to the genus *Proteae* are considered to be an essential source for *qnrD* genes [16]. Apparently, the evolution may have involved a progenitor *qnrD*-harbouring plasmid which fused with a second plasmid using the IS-elements or direct repeats as recombination regions resulting in the four fusion plasmids described in this study. However, the high degree of similarity between the PBRT non-typeable plasmids pKVHS-001 and pKVHS-002 (see Figure 2) indicates a more close relatedness between these two plasmids.

In general, small plasmids are not self-conjugative. Such plasmids lack regulation systems for the replication rate, copy numbers and maintenance in their bacterial host. Non-self-transferable plasmids can be mobilized by a helper plasmid. Still, such mobilizable plasmids must have a relaxase gene in order to facilitate transfer[28]. In none of the plasmids involved in our study we identified relaxase genes, so the mechanism for transfer of the plasmids described in our study remains unknown. Possibly, acquisition of *qnrD*-harbouring plasmids occurred via transformation followed by low diffusion (at least in Europe) via clonal dissemination of some *Salmonella* serovars.

Based on the sequences we can only speculate on the origin and the vehicles used for dissemination of the plasmids. Possibly, the progenitor plasmid was introduced in Europe via the food chain. After introduction, fusion plasmids could have evolved on different occasions. Although reports on *qnrD* genes are still rare in Europe, our study demonstrates a wide dissemination of *qnrD1* in different poultry sources from different countries. Nevertheless, the fact that *qnrD1* genes are scarcely found in *Salmonella* indicates the infrequency of *qnrD*-harbouring plasmids, which could be caused by the lack of other

resistance genes present on these plasmids making co-selection, in case of treatment with other antibiotics, impossible. However, one must keep in mind that this gene might be more frequently present in other Enterobacteriaceae.

Our study demonstrates the existence of four different *qnrD1*-harbouring plasmids in eight different *Salmonella* serovars from various sources in three different European countries. Although the number of isolates included in the study was low, *Xba*I-PFGE analyses indicates clonal spread of *qnrD1*-harbouring *S*. Braenderup and *S*. Ohio from laying hens in Spain and *S*. Montevideo from different poultry sources in Italy (Figure 1).

Acknowledgements

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4

Chapter 4

Import of multidrug resistant, PMQR-positive bacteria via food or travel


4a

Characterization of multidrug-resistant, *qnrB2*-positive and extended spectrum beta lactamase-producing, *Salmonella* Concord and *Salmonella* Senftenberg isolates

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Abstract

To characterize plasmids and resistance genes of multidrug-resistant *Salmonella* Senftenberg and *S*. Concord isolated from patients in The Netherlands.

The resistance genes of four multidrug-resistant (MDR) *Salmonella* isolates (three *S*. Concord and one *S*. Senftenberg) were identified by miniaturised microarray, PCR and sequencing. Plasmids were characterized by S1-PFGE and PCR Based Replicon Typing (PBRT). Linkage between plasmids and genes was determined by conjugation experiments and microarray analysis. The genetic relationship between the three *S*. Concord isolates was determined by XbaI-PFGE.

A large variety of resistance genes was detected, including *qnrB2* and the ß-lactamase genes bla_{TEM-1} and bla_{SHV-12} in all isolates; moreover all *S*. Concord isolates also harboured $bla_{CTX-M\ 15}$. *S*. Senftenberg harboured a large IncHI2 plasmid. Three *S*. Concord isolates harboured two large plasmids typed as IncHI2 and IncA/C.

We detected the first plasmid mediated multidrug-resistant *Salmonella* isolates in The Netherlands harbouring both *qnr* and extended spectrum beta-lactamase (ESBL) genes. In *S*. Senftenberg one large plasmid (IncHI2) and in *S*. Concord two large plasmids (IncHI2 and IncA/C) were responsible for the multidrug-resistance.

Introduction

Worldwide, *Salmonella* is one of the major causes of food-borne infections in humans. In the majority of the cases these infections are self-limiting. However, for patients at risk and for invasive or prolonged infections antibiotic treatment is indicated. Fluoroquinolones and third generation cephalosporins are drugs of choice for these cases. [1] Infections caused by multidrug-resistant (MDR) *Salmonella* will affect the available treatment options. This may result in treatment failure and the increase of complications. Although extended spectrum beta-lactamase (ESBL)-producing *S*. Concord isolates from Ethiopian adoption children have been reported earlier from different European countries including The Netherlands, [2-4] there is still scarce information about the genetic background of *S*. Concord isolates carrying both *qnr* and ESBL-genes. In addition, no information on the characterization of MDR *S*. Senftenberg isolates is available to date. The aim of the study was to characterize genes and plasmids of the first *qnr*-positive, ESBL-producing MDR *Salmonella* isolated from patients in The Netherlands.

Materials and Methods

Susceptibility tests and detection of resistance genes

In 2007, four *Salmonella* isolates expressing a remarkable type of multi-drug resistance were identified. The isolates were selected for further study, since all four strains showed resistance to third generation cephalosporins and exhibited an unusual quinolone resistance phenotype being low-level resistant against ciprofloxacin, but still susceptible to nalidixic acid. In addition, all isolates were multidrug resistant to most classes of antibiotics tested. Three *S*. Concord isolates (199.69, 206.54 and 210.52) originated from Ethiopian adoption children and a *S*. Senftenberg isolate (200.27) was obtained from a male adult patient who had recently traveled to Egypt. Susceptibility to antimicrobials was tested by broth microdilution according to ISO standards (ISO 20776-1: 2006) in microtiter trays with a custom-made dehydrated panel of antibiotics (Sensititre©, Trek Diagnostic Systems, UK). The results were interpreted using epidemiological cut-off values as recommended by EUCAST (www.eucast.org). The panel included the following antibiotics: ampicillin, cefotaxime, ceftazidime, tetracycline, sulfamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin and colistin.

To detect antimicrobial resistance genes a miniaturised microarray (AMR04, Identibac, Veterinary Laboratories Agency, UK) [5] was used followed by PCR for confirmation of the detection of plasmid mediated quinolone resistance genes [6-10] and of the beta-lactamase genes bla_{TEM} [11], bla_{CTX-M} [12] and bla_{SHV} (www.medvetnet.org/pdf/Reports/Appendix_2_Workpackage_9.doc). PCR products were purified by the QIAquick PCR product purification kit (Qiagen GmbH, Germany). Sequences were determined by using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA) on a 3100-Avant Genetic Analyzer (Applied Biosystems). Sequence data were analysed with the Sequencher 4.6 program. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for gene sequences homologous to the nucleotide sequences found.

Plasmid characterization and location of resistance genes

Transfer of resistance was tested by standard broth-mating experiments using a rifampicin-resistant, indole-negative *E. coli* K12 as recipient. Transconjugants were selected on MacConkey agar with 75 mg/L rifampicin and 1 mg/L cefotaxime. The plasmids in the donor strains and the transconjugants were analysed by PCR-based replicon typing

(PBRT).[13] IncHI2-positive plasmids were further typed using ten different PCRs, based on the sequences of the IncHI2 plasmids R478 and pAPEC-01-R.[14] The sizes of the plasmids were determined using PFGE of S1 nuclease digests of total DNA.[15] Linkage between plasmids and resistance genes was determined by miniaturized microarray analysis of the transconjugants. The location of $bla_{CTX\text{-}M\text{-}15}$ genes in the *S*. Concord isolates was determined by PFGE of I-CeuI and XbaI digests followed by Southern blot hybridization using a PCR-generated DIG-labeled CTX-M-15 probe. To determine the genetic relationship between the 3 S. Concord isolates, XbaI-PFGE was performed according to the PulseNet protocol (www.pulsenet.com).

Results

Susceptibility tests and detection of resistance genes

All *Salmonella* isolates were resistant against ampicillin, ceftazidime, cefotaxime, tetracycline, sulfamethoxazole, trimethoprim, chloramphenicol, gentamicin and streptomycin; the *S*. Concord isolates were also resistant against florfenicol and the *S*. Senftenberg isolate against kanamycin. Furthermore, all isolates were low-level resistant against ciprofloxacin (MIC: 0.12 - 0.5 mg/L), but still susceptible to nalidixic acid (MIC: 8 -16 mg/L). The resistance genes *qnrB2*, $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}12}$, *sul1*, *sul2*, *dfrA*19, *tetD*, *strA* and *strB* were detected in all isolates. Some resistance genes were solely detected in *S*. Concord, including $bla_{CTX\text{-}M\text{-}15}$, *floR* and *tetA*. The aminoglycoside resistance gene *aac(6')-1b* was only detected in *S*. Senftenberg. This classical variant of the gene was confirmed by sequencing the amplicon. In addition, the resistance genes *qnrC*, *qnrD* and *qepA* (not included in the microarray) were not detected by PCR in any of the four isolates.

Plasmid characterization and location of resistance genes

S. Senftenberg 200.27 harboured one 310 kb IncHI2 plasmid, which was transferred to *E. coli* K12 strain by conjugation. All 9 resistance genes identified in the donor strain were detected in transconjugant 200.27-T1 (Table 2). The IncHI2 plasmid of the *S*. Senftenberg isolate was characterized as a R478-like plasmid.

Both *S*. Concord 199.69 and *S*. Concord 206.54 harboured two plasmids identified by PBRT as IncHI2 (200 kb) and IncA/C (230 kb). Transconjugant 199.69-T1 harboured both plasmids including all 12 resistance genes identified in the donor strain. Transconjugants harbouring

IncA/C missed two resistance genes identified in the donor strain (bla_{SHV} and *tetD*). Transconjugants with solely IncHI2 were not obtained. *S*. Concord 210.52 also harboured IncHI2 and IncA/C plasmids, but with different sizes; 170 kb (IncA/C) and 290 kb (IncHI2). Conjugation experiments with *S*. Concord 210.52 resulted in transconjugants with either IncHI2 or IncA/C plasmids. Transconjugant 210.52-T1 harbouring an IncHI2 plasmid missed $bla_{CTX\text{-}M\text{-}15}$, *tetA* and *floR*, whereas transconjugant 210.52-T7 harbouring an IncA/C plasmid missed $bla_{SHV\text{-}12}$, *qnrB2*, *tetD* and *dfrA19* (Table 2). All IncHI2 plasmids in the 3 *S*. Concord isolates were characterized as R478-like plasmids. However all plasmids lacked three genes present in R478 (*arsB*, *smr136*, *tnsD*) and harboured the 01R_160 locus as in pAPEC-O1-R. Southern blot hybridization experiments demonstrated that the $bla_{CTX\text{-}M\text{-}15}$ gene was solely located on an IncA/C plasmid in all 3 *S*. Concord isolates (results not shown). Finally, XbaI-PFGE revealed a unique digestion pattern for all 3 *S*. Concord isolates indicative of the genetic variation of MDR *S*. Concord strains originating from Ethiopia (results not shown).

Table 1. Replicon types of plasmids and resistance genes detected in donor strains and transconjugants.

Isolates	IncHI2	IncA/C	intI1	TEM	SHV	CTX-M	qnrB	tetA	tetD	sul1	sul2	dfrA19	floR	strA	strB	aac-(6')-1b
200.27 (D)	+		+	+	+		+		+	+		+		+	+	+
200.27-T1	+		+	+	+		+		+	+		+		+	+	+
199.69 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
199.69-T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
199.69-T2		+	+	+		+	+	+		+	+	+	+	+	+	
206.54 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
206.54-T1		+	+	+		+	+	+		+	+	+	+	+	+	
210.52 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
210.52-T1	+		+	+	+		+		+	+	+	+		+	+	
210.52-T7		+		+		+		+		+	+		+	+	+	

Replicon types of plasmids and resistance genes detected in donor strains (D) and transconjugants (-T) of S. Senftenberg isolate (200.27) and S. Concord isolates (199.69, 206.54, 210.52).

Discussion

S. Senftenberg is a common serotype in The Netherlands; in the last decade, a total of 581 isolates (= 3%), originating from different sources, were tested for antibiotic susceptibility. Until 2007, all *S*. Senftenberg isolates were susceptible to third generation cephalosporins. On the contrary, *S*. Concord is a very rare serotype; in the last decade, only nine isolates of human origin (= < 0.01%) were tested. Moreover, eight of these nine isolates were resistant against third generation cephalosporins. However, until 2007, all *S*. Concord isolates were susceptible to ciprofloxacin. These figures show the rarity of *Salmonella* isolates that are resistant against both third generation cephalosporins and fluoroquinolones in The Netherlands. Although simultaneous increase of *qnr*-positive *Salmonella* [16] and of ESBL-producing *Salmonella* was reported in our national surveillance program since 2003 (www.cvi.wur.nl/NL/publicaties/rapporten/maranrapportage/), this is the first report of both *qnr* and ESBL-positive *Salmonella* isolated from human patients in The Netherlands.
The dissemination of *qnr*-genes in *Enterobacteriaceae* including *Salmonella* of human origin, is reported in increasing frequency. Recently, an IncHI2 plasmid associated with *qnrB2* and bla_{SHV-12} was identified in a human *S*. Bredeney isolate.[17] In our study *qnrB2* was detected on two different types of conjugative plasmids: IncHI2 and IncA/C. To our knowledge, this is the first description of a *qnrB2*-gene on an IncA/C plasmid in *Salmonella enterica*.

Fabre *et al*. [2] detected $bla_{CTX-M-15}$ genes in *S*. Concord isolates on chromosomal DNA, but also on an InHI2 plasmid and on a fusion plasmid of IncY and IncA/C2. However, in our study we detected $bla_{CTX-M-15}$ genes solely on InA/C plasmids (negative for IncY) and not on chromosomal DNA. In addition, we identified bla_{SHV-12} genes solely on IncHI2 plasmids. A study of Hendriksen *et al*. [4] included four Dutch *S*. Concord isolated from 2001 to 2006, which showed resistance to third generation cephalosporins, but were completely susceptible to ciprofloxacin. In this Danish study the coexistence of $bla_{CTX-M-15}$ and bla_{SHV-12} genes was reported on a single plasmid. Nonetheless, our study revealed the coexistence of these resistance genes on two different plasmids in all *S*. Concord isolates; $bla_{CTX-M-15}$ on IncA/C plasmids and bla_{SHV-12} on IncHI2 plasmids. The microarray revealed that the smaller IncA/C plasmid of transconjugant 210.52-T7 missed a class 1 integron (int1) and two resistance genes (*dfrA19* and *qnrB2*) compared to the plasmids of transconjugant 199.69-T2 and 206.54-T1. This indicates that a fragment harbouring a complex integron is lacking on the IncA/C plasmid of *S*. Concord 210.52.

Finally the IncHI2 plasmids of all *S*. Concord isolates were identically characterized as R478-like plasmids, all lacking the *arsB* gene. To our knowledge, this is the first description of such a R478-like plasmid.

The findings of this study provide additional information on the genetic background of ESBL-producing, *qnr*-positive *S*. Concord and *S*. Senftenberg isolates. The potential human health impact of infections with such MDR *Salmonella* emphasizes the necessity to monitor these resistance patterns in *Salmonella* carefully.

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Transparency declarations

None to declare.

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4b

CHAPTER 4B

Enterobacteriaceae resistant to third-generation cephalosporins and quinolones in fresh culinary herbs imported from Southeast Asia

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Abstract

Since multidrug resistant bacteria are frequently reported from Southeast Asia, our study focused on the occurrence of ESBL-producing Enterobacteriaceae in fresh imported herbs from Thailand, Vietnam and Malaysia.

Samples were collected from fresh culinary herbs imported from Southeast Asia in which ESBL-suspected isolates were obtained by selective culturing. Analysis included identification by MALDI-TOF mass spectrometry, susceptibility testing, XbaI-PFGE, microarray, PCR and sequencing of specific ESBL genes, PCR based replicon typing (PBRT) of plasmids and Southern blot hybridization. In addition, the quinolone resistance genotype was characterized by screening for plasmid mediated quinolone resistance (PMQR) genes and mutations in the quinolone resistance determining region (QRDR) of *gyrA* and *parC*.

The study encompassed fifty samples of ten batches of culinary herbs (5 samples per batch) comprising nine different herb variants. The herbs originated from Thailand (Water morning glory, Acacia and Betel leaf), Vietnam (Parsley, Asian pennywort, Houttuynia leaf and Mint) and Malaysia (Holy basil and Parsley). By selective culturing 21 cefotaxime resistant Enterobacteriaceae were retrieved. Array analysis revealed 18 isolates with ESBL genes and one isolate with solely non-ESBL beta-lactamase genes. Mutations in the *ampC* promoter region were determined in two isolates with PCR and sequencing. The isolates were identified as *Klebsiella pneumoniae* (n = 9), *Escherichia coli* (n = 6), *Enterobacter cloacae* complex (n = 5) and *Enterobacter* spp. (n = 1). All isolates tested were multidrug resistant. Variants of CTX-M enzymes were predominantly found followed by SHV enzymes. PMQR genes (including *aac(6')-1b-cr*, *qnrB* and *qnrS*) were also frequently detected. In almost all cases ESBL and quinolone resistance genes were located on the same plasmid.

Imported fresh culinary herbs from Southeast Asia are a potential source for contamination of food with multidrug resistant bacteria. Because these herbs are consumed without appropriate heating, transfer to human bacteria cannot be excluded.

Introduction

Currently, foodborne outbreaks are increasingly reported in fresh products from non-animal origin comprising fruit, vegetables and also herbs [1]. Several outbreaks have been reported originating from fresh culinary herbs, which include *Salmonella* serovar Thompson in cilantro [2] and *Shigella sonnei* and enterotoxigenic *Escherichia coli* (ETEC) in Parsley [3]. During growth herbs can be contaminated whilst fertilizing with manure or by contaminated irrigation systems. Besides, at harvest and distribution products can be contaminated with human faecal bacteria especially in countries with poor sanitary conditions [1]. After contamination, Enterobacteriaceae can persist and multiply on the surface of plants or even reach the interior of the plant via different pathways [4-6]. As a result, it becomes very difficult if not impossible to clean products adequately after contamination [7].

In Europe, the Rapid Alert System for Food and Feed (RASFF) reported in 2005 for the first time about the poor microbial quality of fresh culinary herbs notified in different European countries [8]. A majority of these notifications concerned fresh herbs imported from Thailand. Recurring notifications led to Commission Regulation (EC) No 669/2009 [9] in which guidelines for the quality control of imported feed and food of non-animal origin were specified. Recently, an amending regulation came into force designated Regulation (EC) No 1235/2012 [10] in which the check for the presence of *Salmonella* in Basil, Cilantro and Mint from Thailand is mentioned.

In the period 2008-2009 The Netherlands Food and Consumer Product Safety Authority (NVWA) identified a high prevalence of *Salmonella* (26%), but also found enteropathogenic *E. coli* and *Shigella* in fresh imported herbs from South-East Asia [11]. These results confirmed the poor microbial quality of the products and encouraged a more extensive monitoring. In 2011, screening of imported fresh culinary herbs for pathogenic bacteria was continued and expanded to the screening for ESBL/AmpC-positive Enterobacteriaceae. Multidrug resistant bacteria (including extended-spectrum β-lactamase (ESBL) producers) can be transmitted to humans via consumption of food. A recent Dutch study indicated transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain [12]. However, little information is available about the presence of resistant bacteria in food from non-animal origin like herbs [13].

Since multidrug resistant bacteria are frequently reported from Southeast Asia, our national study on the occurrence of ESBL-producing Enterobacteriaceae in food included fresh imported herbs from Thailand, Vietnam and Malaysia. These fresh herbs are often used uncooked as dressing or flavouring of food. As a consequence, consumption of contaminated fresh herbs might be a source for transmission of resistant strains or genes to consumers and possess a risk to human health.

Materials and Methods

In 2011 The Netherlands Food and Consumer Product Safety Authority (NVWA) collected fifty samples from ten different batches (five subsamples of approximately 100 grams per batch) of fresh culinary herbs imported from Southeast Asia upon arrival at different border inspection posts in The Netherlands. To detect ESBL/AmpC-positive Enterobacteriaceae 25 gram of each sample was selectively enriched in 225 ml Luria Bertani (LB) broth (Becton Dickinson, Germany) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich Germany). After 16 - 20h incubation at 37 °C from all samples 1 µl was inoculated on MacConkey (Becton Dickinson, Germany) supplemented with 1 mg/L cefotaxime as well as on Brilliance ESBL agar (Oxoid).

After 16 - 20h incubation at 37 °C isolates with growth typical for *E. coli* on MacConkey agar and typical for Enterobacteriaceae on Brilliance ESBL agar were pure cultured on blood agar plates and sent to the Central Veterinary Institute (CVI) in Lelystad for further analysis. All bacterial isolates were identified with MALDI-TOF mass spectroscopy (Biotyper MS, Bruker Daltonics GmbH, Germany) according to the instructions of the manufacturers. Antimicrobial susceptibility was tested by broth microdilution according to ISO standards [14] with a custom made panel of thirteen different dehydrated antibiotics (Sensititre©, Trek Diagnostic Systems, UK) including ampicillin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, trimethoprim and tetracycline.

For interpretation epidemiological cut-off values were used as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://mic.eucast.org). Screening for carbapenemase production was performed by disk diffusion tests with imipenem, meropenem and ertapenem using the EUCAST disk diffusion method (http://www.eucast.org/antimicrobial_susceptibility_testing/disk_diffusion_methodology/) and interpretive criteria. Subsequently, all putative ESBL/AmpC-positive isolates were tested with microarray (ATR0503, Alere Technologies, The Netherlands) to detect a broad spectrum of antibiotic resistance genes and gene-families. In addition, bacteria suspected of carbapenemase production were tested with another microarray (Check-Points, Check-MDR CT102) focussing on the presence of carbapenem resistance gene families. The presence of ESBL- and plasmid mediated quinolone resistance (PMQR) genes were confirmed with PCR and sequencing using previously described primers [15]. To amplify the full gene length of a new *qnrS* variant, in-house primers were used

(forward primer "qnrS-orf-Fw": 5'-TGTCTTGGCATATGTATAATGG and reversed primer qnrS-orf-Rv: 5'-GCAAGGTTGACAATATTATTCG). For characterization of quinolone resistance genotypes all isolates were screened for mutations in the quinolone resistance determining region (QRDR) of *gyrA* (all bacterial species) and *parC* (all *E. coli* and some *K. pneumoniae* isolates) using previously described primers [16]. These *parC* primers were originally designed for *E. coli*. As a consequence amplification of *parC* failed for part of the *K. pneumoniae* and *Enterobacter* isolates. For this reason, in-house primers were applied (forward primer "parC-F-2": 5'- ATGTACGTSATCATGGAC and reversed primer "parC-R-2": 5'-AGGATGTTCGGCAGACG) to amplify the QRDR of *parC* in *K. pneumoniae* isolates and in *Enterobacter* spp. isolates. Isolates without detectable ESBL/AmpC genes were screened for mutations in the promoter region of chromosomal *ampC* using previously described primers [17].

Purified plasmid DNA [18] was transferred to DH10B cells (Gibco Invitrogen, USA) by electroporation. Selection of ESBL-positive transformants was performed on LB agar with 1 mg/L cefotaxime and selection of PMQR-positive transformants on LB agar with 0.03 mg/L ciprofloxacin. The size of the plasmids was determined with S1-PFGE. If transformation experiments were not successful, conjugation experiments to rifampicin resistant *E. coli* K12 were performed by broth-mating. Transconjugants were selected on LB agar supplemented with 1 mg/L cefotaxime +100 mg/L rifampicin (Sigma-Aldrich) or selected on LB agar supplemented with 0.03 mg/L ciprofloxacin + 100 mg/L rifampicin. To identify the ESBL or PMQR-carrying plasmids PCR-based replicon typing (PBRT) was performed using a commercial kit (DIATHEVA, Italy). When transformation or conjugation failed, the location of the beta-lactamase or PMQR gene was determined by PFGE of S1- and I-CeuI digests of total bacterial DNA of the parent strain followed by Southern blot hybridisation. In case multiple isolates of the same bacterial species were obtained from the same batch of herbs , *XbaI*-PFGE was performed to reveal possible relatedness between bacterial isolates. The restriction patterns were analysed based on the number of band differences using published criteria [19].

Results

Samples

The studied products consisted of fifty samples originating from ten batches (five samples per batch) of fresh culinary herbs from Thailand (n=4), Vietnam (n=4) and Malaysia (n=2). Samples of Thai herbs were taken from two batches of Water morning glory (*Ipomoea aquatica*), one batch of Acacia leaf (*Acacia pennata*) and one batch of Betel leaf (*Piper betle*). Furthermore, from Vietnam one batch of Parsley (*Petroselinum crispum*), Asian pennywort, (*Centella asiatica*), Houttuynia leaf (*Houttuynia cordata*) and Mint (*Mentha cordifolia*) were sampled. Finally, from Malaysia one batch of Holy basil (*Ocimum sanctum*) and Parsley were sampled.

Bacterial isolates

Selective culturing resulted in twenty-one cefotaxime resistant Enterobacteriaceae isolates obtained from eight different batches of herbs. In two batches of herbs (Water morning glory from Thailand and Parsley from Malaysia) no cefotaxime resistant Enterobacteriaceae were found. *Klebsiella pneumoniae* (n=9) was most abundant and was obtained from Mint, Parsley, Asian pennywort and Acacia leaf. *Escherichia coli* (n=6) was identified in samples from Water morning glory, Betel leaf, Acacia leaf and Asian pennywort. Finally, isolates belonging to the *Enterobacter cloacae* complex (n=5) were isolated from Holy basil and Parsley and one *Enterobacter* spp. (n=1) from Houttuynia leaf.

Susceptibility testing and microarray analysis

All 21 isolates showed resistance to 3rd generation cephalosporins (cefotaxime MICs: ≥ 4 mg/L). Moreover, all isolates were multidrug resistant demonstrating resistance to four up to ten different antibiotic classes (see Table 1). Microarray analysis demonstrated the presence of multiple resistance genes comprising genes encoding resistance to beta-lactam antibiotics (bla_{TEM}, bla_{OXA}, bla_{CTX-M} and bla_{SHV}), quinolones (*qnrB* , *qnrS and aac(6')-1b*), tetracyclines (*tet*(A) , *tet*(B) , *tet*(E)), sulfonamides (*sul2*, *sul3*), trimethoprim (*dfrA1, dfrA13,dfrA15, dfrA19*), chloramphenicol (*catA1*, *cmlA1*-like), florfenicol (*floR*) and aminoglycosides (*aadA1*-like, *aadA2*-like, *aadA4*-like, *strA, strB*). All isolates tested susceptible to meropenem and imipenem with disk diffusion. However, four isolates (three *K. pneumoniae* and one *Enterobacter* spp.) showed relatively small inhibition zone diameters for ertapenem (≤ 27 mm). These four isolate tested negative for carbapenem

resistance genes with microarray. In *K. pneumoniae* isolates reduced susceptibility to ertapenem may be due to beta-lactamase activity. The mechanisms of resistance in the non-typeable *Enterobacter* isolate also remains unclear.

PCR and sequencing revealed eighteen isolates with ESBL genes, two isolates with specific mutations in the *ampC* promoter region (*ampC* mutation type 3 [20]), and one isolate with solely non-ESBL beta-lactamase genes (see Table 2). As a consequence, the mechanism for resistance to cephalosporins of this isolate remains unclear. Variants of bla_{CTX-M} ($bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$ and $bla_{CTX-M-40}$) were predominantly found (n = 13) followed by bla_{SHV-12} (n=4) and bla_{SHV-2a} (n=1). Furthermore, no plasmid mediated AmpC genes (e.g. bla_{CMY}) were detected. Notably, PMQR genes were frequently identified (n=18).

Coincidence of ESBL and PMQR genes

In almost all cases ESBL and quinolone resistance genes were located on the same plasmid (see Table 2) resulting in the presence of various combinations of resistance genes. Both $bla_{CTX-M-9}$ and *qnrB4* were detected on a 280 kb sized non-typeable plasmid in *K. pneumoniae* (Mint, Vietnam). Co-location of $bla_{CTX-M-14}$ with *qnrS1* was detected in *E. coli* (Betel leaf, Thailand) on an IncHI1 plasmid and in *K. pneumoniae* (Parsley, Vietnam) on an IncF plasmid. In *E. cloacae* complex (Parsley, Vietnam) $bla_{CTX-M-15}$ combined with both bla_{TEM-1b} and bla_{OXA-1} was detected on an IncHI2 plasmid with both *qnrB1* and *aac(6')-1b-cr*. The same combination of genes was found in *K. pneumoniae* (Asian pennywort, Vietnam) on a 135 kb sized non-typeable plasmid. Another cluster of genes ($bla_{CTX-M-15}$, bla_{TEM-1} and *qnrB1*) was found in *K. pneumoniae* (Parsley, Vietnam) on a 60 kb sized non-typeable plasmid. Furthermore, $bla_{CTX-M-15}$ was found chromosomally together with *qnrS1* in *E. coli* (Water morning glory, Thailand). In Holy basil from Malaysia $bla_{CTX-M-40}$ was detected in *E. cloacae* complex together with *qnrS1* on a non-typeable 230 kb sized plasmid. Remarkably, in four *K. pneumoniae* isolates obtained from the same batch of Acacia leaf (Thailand) bla_{SHV-12} was detected with three different combinations of PMQR genes. In one *K. pneumoniae* isolate bla_{SHV-12} was co-located with *qnrS1* on an IncF plasmid, a second isolate harboured both bla_{SHV-12} next to a new *qnrS* variant designated *qnrS9* (GenBank accession no. KF732714) on an IncR plasmid. Finally, in two other *K. pneumoniae* isolates *qnrB4* was detected on a 95 kb sized non-typeable plasmid together with bla_{SHV-12} and *qnrS9* on 60 kb sized IncR plasmid.

Characterization of quinolone resistance

The quinolone resistance genotype was characterized by both screening for PMQR genes and mutations in the QRDR region of *gyrA* and *parC*. Multiple mutations in the QRDR were identified in two *E. coli* isolates: OT-ESBL-626 (*gyrA*: S83L, *parC*: S80I) and OT-ESBL-636 (*gyrA:* S83L, D87N, *parC*: S80I) and in one *Enterobacter* spp. ESBL-OT-634 (*gyrA*: S83L, *parC*: S80I). The rest of the isolates revealed no mutations in the QRDR.

***XbaI*-PFGE**

XbaI-PFGE was performed on a subset of fifteen isolates (data not shown). As a result three *E. cloacae* complex isolates obtained in Holy Basil from Malaysia demonstrated identical PFGE profiles. This was also the case for two *E. cloacae* complex isolates obtained in Parsley from Vietnam. Finally, in Acacia leaf from Thailand two identical *K. pneumoniae* isolates both carrying *qnrS9* and *qnrB4* also revealed identical restriction patterns. In addition, two closely related *K. pneumoniae* isolates solely carrying either *qnrS1* or *qnrS9* were obtained from the same batch of herbs. The remaining isolates all revealed distinctive PFGE profiles.

Table 1.
General Information of bacterial isolates obtained from herbs in Southeast Asia and their

Isolate number	Bacterial species	Product	Batch number	Country
OT-ESBL-0582-1	*E. cloacae* complex	Holy basil	77419462	Malaysia
OT-ESBL-0582-2	*E. cloacae* complex	Holy basil	77419462	Malaysia
OT-ESBL-0582-4	*E. cloacae* complex	Holy basil	77419462	Malaysia
OT-ESBL-0611	*E. coli*	Water morning glory	77609261	Thailand
OT-ESBL-0627	*K. pneumoniae*	Mint	77608893	Vietnam
OT-ESBL-0628	*E. cloacae* complex	Parsley	77608745	Vietnam
OT-ESBL-0631	*E. cloacae* complex	Parsley	77608745	Vietnam
OT-ESBL-0629	*K. pneumoniae*	Parsley	77608745	Vietnam
OT-ESBL-0630	*K. pneumoniae*	Parsley	77608745	Vietnam
OT-ESBL-0626	*E. coli*	Asian pennywort	77608907	Vietnam
OT-ESBL-0632	*K. pneumoniae*	Asian pennywort	77608907	Vietnam
OT-ESBL-0633	*K. pneumoniae*	Asian pennywort	77608907	Vietnam
OT-ESBL-0634	*Enterobacter* spp.	Houttuynia leaf	77608923	Vietnam
OT-ESBL-0635	*E. coli*	Betel leaf	77609474	Thailand
OT-ESBL-0636	*E. coli*	Betel leaf	77609474	Thailand
OT-ESBL-0637	*E. coli*	Acacia leaf	77609482	Thailand
OT-ESBL-0638	*E. coli*	Acacia leaf	77609482	Thailand
OT-ESBL-0639	*K. pneumoniae*	Acacia leaf	77609482	Thailand
OT-ESBL-0640	*K. pneumoniae*	Acacia leaf	77609482	Thailand
OT-ESBL-0641	*K. pneumoniae*	Acacia leaf	77609482	Thailand
OT-ESBL-0642	*K. pneumoniae*	Acacia leaf	77609482	Thailand

[1] *AMP = Ampicillin,*
CEF = Cefotaxime,
CAZ = Ceftazidime,
CIP = Ciprofloxacin,
NAL = Nalidixic acid,
CHL = Chloramphenicol ,
FFN = Florfenicol,
GEN= Gentamicin,
KAN = Kanamycin,
STR = Streptomycin,
SMX, Sulfamethoxazole,
TMP= Trimethoprim,
TET = Tetracycline.

antimicrobial resistance profile

Antibiotic resistance profile[1]
AMP, CEF, CAZ, CIP, CHL, FFN, GEN, STR, SMX, TMP, TET, COL
AMP, CEF, CAZ, CIP, CHL, FFN, GEN, STR, SMX, TMP, TET, COL
AMP, CEF, CAZ, CIP, CHL, FFN, GEN, STR, SMX, TMP, TET, COL
AMP, CEF, CAZ, CIP, GEN, SMX, TMP, TET
AMP, CEF, CAZ, CIP, CHL, SMX, TMP
AMP, CEF, CAZ, CIP, NAL, CHL, GEN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, GEN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, STR, SMX, TMP, TET
AMP, CEF, CIP, CHL, GEN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CHL, SMX, TET
AMP, CEF, CAZ, CIP, CHL, GEN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, STR, SMX, TMP, TET, COL
AMP, CEF, CIP, CHL, FFN, GEN, STR, SMX
AMP, CEF, CIP, NAL, CHL, FFN, GEN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, KAN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CHL, FFN, GEN, KAN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, KAN, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, KAN, STR, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, KAN, SMX, TMP, TET
AMP, CEF, CAZ, CIP, NAL, CHL, FFN, GEN, KAN, SMX, TMP, TET

Table 2. Molecular characterization of bacterial isolates obtained from herbs imported from

Isolate number	Bacterial species	Beta-lactamase genes	PMQR genes
OT-ESBL-0582-1 [A]	*E. cloacae* complex	$bla_{CTX\text{-}M\text{-}40}$	*qnrS1*
OT-ESBL-0582-2 [A]	*E. cloacae* complex	$bla_{CTX\text{-}M\text{-}40}$	*qnrS1*
OT-ESBL-0582-4 [A]	*E. cloacae* complex	$bla_{CTX\text{-}M\text{-}40}$	*qnrS1*
OT-ESBL-0611	*E. coli*	$bla_{CTX\text{-}M\text{-}15}$	*qnrS1*
OT-ESBL-0627	*K. pneumoniae*	$bla_{CTX\text{-}M\text{-}9}$	*qnrB4*
OT-ESBL-0628 [B]	*E. cloacae* complex	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, $bla_{OXA\text{-}1}$	*qnrB1, aac(6')-1b-cr*
OT-ESBL-0631 [B]	*E. cloacae* complex	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, $bla_{OXA\text{-}1}$	*qnrB1, aac(6')-1b-cr*
OT-ESBL-0629	*K. pneumoniae*	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, $bla_{OXA\text{-}1}$	*qnrB1*
OT-ESBL-0630	*K. pneumoniae*	$bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}1b}$, $bla_{SHV\text{-}1}$	*qnrS1*
OT-ESBL-0626	*E. coli*	$bla_{CTX\text{-}M\text{-}15}$	*none*
OT-ESBL-0632	*K. pneumoniae*	$bla_{SHV\text{-}2a}$	*none*
OT-ESBL-0633	*K. pneumoniae*	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, $bla_{OXA\text{-}1}$	*qnrB1, aac(6')-1b-cr*
OT-ESBL-0634	*Enterobacter* spp.	$bla_{TEM\text{-}1}$	*qnrB26, aac(6')-1b-cr*
OT-ESBL-0635	*E. coli*	$bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}1b}$	*qnrS1*
OT-ESBL-0636	*E. coli*	$bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}1b}$	*qnrS1*
OT-ESBL-0637	*E. coli*	$bla_{TEM\text{-}1b}$ (ampC type 3)[5]	*qnrS1*
OT-ESBL-0638	*E. coli*	$bla_{TEM\text{-}1b}$ (ampC type 3)	*none*
OT-ESBL-0639	*K. pneumoniae*	$bla_{SHV\text{-}12}$	*qnrS9*
OT-ESBL-0640	*K. pneumoniae*	$bla_{SHV\text{-}12}$	*qnrS1*
OT-ESBL-0641 [C]	*K. pneumoniae*	$bla_{SHV\text{-}12}$	*qnrS9, qnrB4*
OT-ESBL-0642 [C]	*K. pneumoniae*	$bla_{SHV\text{-}12}$	*qnrS9, qnrB4*

[1] Inc.nt = none typeable incompatibility group,

[2] N.D. = not determined,

[3] both $bla_{CTX\text{-}M\text{-}15}$ and *qnrS1* were chromosomally located,

[4] N.A. = not applicable,

[5] chromosomal mutation in the *ampC* promotor region,

[6] both $bla_{SHV\text{-}12}$ and *qnrS9* were located on IncR, but *qnrB4* was located on a separate none typeable plasmid,

[A,B,C] restriction patterns of *Enterobacter cloaca* complex isolate numbers marked with A and B and *K. pneumoniae* isolate numbers marked with C were undistinguishable by *XbaI*-PFGE.

Southeast Asia

Location of ESBL and PMQR genes	Size of plasmids	Additional resistance genes detected with micro-array
Inc.nt[1]	230 kb	*floR, aadA2*-like, *strA, strB, sul3, dfrA13, tet*(B)
Inc.nt	N.D.[2]	*floR, aadA2*-like, *strA, strB, sul3, dfrA13, tet*(B)
Inc.nt	N.D.	*floR, aadA2*-like, *strA, strB, sul3, dfrA13, tet*(B)
chromosome[3]	N.A.[4]	*sul3, dfrA15, tet*(B)
Inc.nt	280 kb	*catA1, aadA4*-like, *sul2, dfrA1*
IncHI2	320 kb	*catA1, aadA1*-like, *strB, sul3, dfrA15, tet*(B)
IncHI2	320 kb	*catA1, aadA1*-like, *strB, sul3, dfrA15, tet*(B)
Inc.nt	60 kb	*strB, sul3, dfrA15, tet*(B)
IncF	280 kb	*strB, sul2, dfrA1, tet(B), tet*(E)
IncF	140 kb	*florR, dfrA19, tet*(C)
Inc.nt	240 kb	*catA1, sul3, tet*(E)
Inc.nt	135 kb	*catA1, strB, sul3, dfrA15, tet*(E)
Inc.nt	95 kb	*floR, strB, sul2, sul3, tet*(B)
IncHI1	240 kb	*floR, aadA1*-like
IncHI1	240 kb	*cmlA1*-like, *floR, aadA1*-like, *dfrA13, tet(A), tet*(B)
Inc.nt	80 kb	*floR, aadA1*-like *dfrA13, tet*(A), *tet*(B)
N.A.	N.A.	*cmlA1*-like, *floR, aadA1*-like *dfrA13, tet*(A), *tet*(B)
IncR	60 kb	*floR, sul2, sul3, dfrA13, tet*(B)
IncF	190 kb	*floR, aadA2*-like, *sul2, sul3, dfrA13, tet*(B), *tet*(E)
IncR, (Inc.nt)[6]	60 kb	*floR, sul2, sul3, dfrA13, tet*(B)
IncR, (Inc.nt)	60 kb	*floR, sul2, sul3, dfrA13, tet*(B)

Discussion

Our study focussed on the detection of cefotaxime resistant Enterobacteriaceae from herbs. In addition to cefotaxime resistance, the obtained isolates were non-wild-type susceptible (NWT) to a broad panel of antibiotics. Moreover, most of the isolates were clinically resistant to critically important antimicrobials as designated by the World Health Organisation [21] including third generation cephalosporins, quinolones and aminoglycosides.

The multidrug-resistance character of the isolates may reflect a high selection pressure through imprudent usage of antimicrobial in humans and animals in Southeast Asia [22]. The fast emergence of antimicrobial resistance in this area poses a global threat to public health. In an attempt to narrow this problem the WHO Regional Office for Southeast Asia recently developed a regional strategy on prevention and containment of antibiotic resistance [23]. However, national programs on antimicrobial stewardship in human and veterinary medicine have not been implemented yet in this area. To date, antimicrobial resistance is commonly present in commensal and pathogenic bacteria and spreads throughout Southeast Asia [24]. In Thailand, a high prevalence of ESBL-producing Enterobacteriaceae is reported in hospitalized patients as well as in healthy individuals [25, 26]. In addition, several studies reveal the presence of multidrug-resistant non-typhoidal *Salmonella enterica* on Thai food products [27, 28]. Similarly, studies from Vietnam [29, 30] and Malaysia [31-33] indicate a growing problem of antimicrobial resistance in bacteria in humans, animals and food.

In Thai herbs $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were identified in *E. coli* and $bla_{\text{SHV-12}}$ in *K. pneumoniae*. These ESBL-genes are described to occur in human *E. coli* and *K. pneumoniae* isolates in Thai hospitals [25]. Isolates obtained from various Vietnamese herbs belonged to different bacterial species harbouring different ESBL-genes; with $bla_{\text{CTX-M-15}}$ in *E. coli*; $bla_{\text{SHV-2a,}}$ $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ in *K. pneumoniae* and $bla_{\text{CTX-M-15}}$ in *E. cloacae* complex, genes that are frequently described in bacteria from humans. Finally, in Holy Basil from Malaysia *E. cloacae* complex were obtained carrying $bla_{\text{CTX-M-40}}$ on non-typeable plasmids. Although, no reports could be found on the presence of this ESBL-gene in Malaysia, the presence of $bla_{\text{CTX-M-40}}$ was reported in *E. coli* causing health care-associated infection in Thailand [34].
This study demonstrates the presence of bacterial isolates on herbs harbouring plasmids carrying multiple genes conferring resistance to beta-lactam antibiotics, quinolones,

aminoglycosides, tetracyclines, sulfonamides, trimethoprim and fenicols. Genes encoding resistance to aminoglycosides (*aadA1-like, aadA2-like, aadA4-like, strA, strB*), trimethoprim (*dfrA1, dfrA13, dfrA15, dfrA19*), sulfonamides (*sul2* and *sul3*) tetracyclines (*tet*(A) , *tet*(B) , *tet*(E)), and fenicols (*catA1*, *cmlA1*-like, *floR*) were frequently present (in seven out of eight batches of herbs with ESBL-producing bacteria).

Analysis of XbaI-PFGE restriction profiles revealed the presence of four bacterial isolates which were indistinguishable to other isolates within the collection. Identical profiles were observed for three *E. cloaca* complex isolates each retrieved from a different sample of one batch of Holy Basil, two *E. cloaca* complex isolates each from a different sample of one batch of Parsley and two *K. pneumoniae* isolates each from a different sample of one batch of Acacia leaf (see Table 2). As these isolates showed the same PFGE profile and originated from the same batch of herbs, it is very likely that the isolates are genetically identical. The fact that identical isolates were identified in multiple samples from the same batch of herbs indicates that the amount of ESBL-producing bacteria in those batches was relatively high.

Furthermore, a relatively high number of isolates harboured one or more PMQR genes. This finding confirms previous reports on the high prevalence of PMQR genes in Southeast Asia[35-37]. Although, the concurrence of ESBL and PMQR genes has been described repeatedly[38], our findings regarding the frequency and the variation in combinations of these two types of resistance genes are noticeable. We found co-location of these genes in a high proportion of the isolates on different types of plasmids including IncHI1, IncHI2, IncR, IncF and non-typeable plasmids (Table 2). To our best knowledge, this is the first description of $bla_{\text{CTX-M-14}}$ and *qnrS1* on an IncHI1 plasmid. Moreover, we identified this plasmid in two different *E. coli* isolates with unique PFGE patterns (data not shown) in the same batch of herbs (Betel leaf, Thailand), which suggests plasmid transfer. However, a Italian study [39] reported the coincidence of $bla_{\text{CTX-M-1}}$ on IncHI1 plasmids in *E. coli* with *qnrS1* or *qnrB19* on separate plasmids (IncX1 or IncN). Also, IncHI2 plasmids are known carriers of multiple antibiotic resistance genes in Enterobacteriaceae[40]. In our study we identified this large plasmid in multidrug resistant *E. cloacae* isolates harbouring $bla_{\text{TEM-1b}}$, $bla_{\text{oxa-1}}$, $bla_{\text{CTX-M-15}}$, *qnrB1* and *aac(6')-1b-cr*. This cluster of genes on IncHI2 is described to occur widespread and was also identified in *E. coli* from wild birds in The Netherlands [41], and on IncF in the uropathogeneic *E. coli* O25b:H4-B2-ST131[42]. However, in our *E. cloacae* complex isolates from herbs the additional resistance genes (*catA1*, *aadA1*, *strB*, *sul3*, *dfrA15* and *tet*(B)),

differed from the fully sequenced IncF plasmids designated pEK499 and pEK516 carrying one or more of the following resistance genes; *catB4*, *aadA5*, *sul1*, *dfrA7* and *tet*(A) [43]. Furthermore, our finding of *K. pneumoniae* with ESBL carrying IncF plasmids coinciding with PMQR genes has been reported in *K. pneumoniae* isolates with IncF plasmids carrying $bla_{CTX\text{-}M\text{-}14}$ or $bla_{SHV\text{-}12}$ sometimes associated with PMQR genes [44, 45]. Although *qnrS1* was previously identified on an IncR plasmid in a human *Salmonella* serovar Montevideo isolate [46] we report *qnrS1* as well as a new variant designated *qnrS9* coinciding with $bla_{SHV\text{-}12}$ on IncR plasmids in *K. pneumoniae* isolates obtained in Betel leaf from Thailand.
Nineteen of the twenty-one isolates included in the study showed non-wild type (NWT) susceptibility to ciprofloxacin using epidemiological cut-off (ECOFF) values according to EUCAST. Only three of the nineteen NWT isolates possessed mutations in the QRDR region and conferred resistance to both nalidixic acid (MIC > 64 mg/L) and ciprofloxacin (MIC > 8 mg/L). In addition, two of these mutants also harboured one or more PMQR genes. The NWT isolates without mutations in the QRDR all possessed one or more PMQR genes (n=16) exhibited variable NWT susceptibilities to ciprofloxacin (MIC range: 0.5 – 4 mg/L) and nalidixic acid (MIC range: 8 – 64 mg/L). These results indicate that clinical resistance to ciprofloxacin (R: ≥ 1 mg/L) can be expressed without mutations in the QRDR region. However, in these isolates the role of other resistance mechanisms like efflux pumps cannot be excluded.

Even though we examined a relatively small collection of herbs, in eight out of ten batches (80%) one or more samples were positive for ESBL-producing bacteria. This indicates a high contamination rate of fresh herbs from Southeast Asia. The direct source of this bacterial contamination remains unknown. However, these bacteria are not specifically related to the herbs, but probably originate from animal or human sources. Enterobacteriaceae like *E. coli*, *E. cloacae* and *K. pneumoniae* are faecal bacteria which normally reside in the intestines of animals and humans. It is likely that herbs are contaminated with faecal bacteria from animals during the fertilizing process. Another possibility is contamination with faecal human bacteria during harvest, distribution and packaging of the products due to insufficient hygiene during processing [1]. Since we did not quantify the bacteria present, we can only speculate on the actual bacterial loads on the surface of the plants. Still, Asian herbs are often eaten raw especially in European countries; therefore transfer of such

bacteria to humans cannot be excluded.

In summary, imported fresh culinary herbs from Southeast Asia are a potential source for contamination of food with multidrug-resistant bacteria. Bacteria frequently harboured both ESBL and multiple PMQR genes located on a single plasmid. Because these herbs are consumed without appropriate heating, transfer to humans cannot be excluded.

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Chapter 5

General Discussion

Introduction

(Fluoro)quinolones are considered important antibiotics in human and veterinary medicine used for treatment of various bacterial infections. Soon after the introduction of (fluoro) quinolones in humans and animals, (fluoro)quinolone resistance emerged. In general, antibiotic resistance including resistance to fluoroquinolones is considered to be a risk for human health, because of potential treatment failure. In The Netherlands, recent governmental measurements have been implemented targeting on further reduction of usage of antibiotics in food-producing animals. Beside an aim to reduce the total amount of antibiotics used in animals, the measures especially focus on classes of antibiotics defined as critically important for human health which includes fluoroquinolones and third/fourth-generation cephalosporins.

Quinolones are lethal for growing bacterial cells, because these drugs block essential steps in DNA replication and transcription by disturbing the functionality of topoisomerase II enzymes; DNA gyrase and topoisomerase IV. Resistance against (fluoro)quinolones is mainly caused by chromosomal mutations in the quinolone resistance determining region (QRDR) of topoisomerase genes. In Gram-negative bacteria, *gyrA* is considered to be the main target for quinolones. Other resistance mechanisms caused by chromosomal mutations can also play a role in quinolone resistance like decreased outer membrane permeability or upregulation of efflux pumps. More recently, plasmid mediated quinolone resistance (PMQR) mechanisms were identified in Enterobacteriaceae.

Currently, three different types of resistance mechanisms are described associated with PMQR: (I) target protection proteins encoded by *qnr* genes, (II) specific efflux pumps encoded by *qepA* and a multidrug pump *oqxAB* and (III) an altered aminoglycoside acetyl transferase encoded by *aac(6')-1b-cr* capable of reducing ciprofloxacin activity. PMQR in Enterobacteriaceae generally results in reduced susceptibility to (fluoro)quinolones to levels that are still below clinical breakpoints. However, PMQR genes can interact with other quinolone resistance mechanisms and might facilitate the acquisition of high level quinolone resistance. In contrast to chromosomally encoded quinolone resistance, plasmid mediated quinolone resistance can spread horizontally between bacteria. As a consequence, PMQR can spread rapidly in Enterobacteriaceae in humans and animals, especially when (fluoro)quinolones are used. Although, the clinical relevance of PMQR is not totally clear, it is emerging and complicates the fight against fluoroquinolone resistance.

Goal of the thesis

Before the start of this thesis, data on the occurrence and prevalence of PMQR in Enterobacteriaceae from humans and animals in The Netherlands and Europe were limited. Therefore, studies were designed (I): to determine the occurrence and prevalence of PMQR genes in *Salmonella* and *E. coli* from The Netherlands and other European countries, (II): to characterize the genetic background of the PMQR genes and the plasmids they are located on, (III): to characterize multidrug resistant PMQR positive Enterobacteriaceae imported in The Netherlands via people from Africa or via food from Southeast Asia.

The occurrence and prevalence of PMQR genes in *Salmonella enterica* and *E. coli* from The Netherlands and other European countries

PMQR in *Salmonella enterica* in The Netherlands

The earliest identified PMQR-positive *Salmonella* isolate in The Netherlands was a *qnrS1*-positive S. Corvallis isolate obtained from a human patient in 2003 (**chapter 2a** of this thesis). In this study, *S*. Corvallis was predominantly identified next to seven other PMQR-positive *Salmonella* serovars (Anatum, Bredeney, Kentucky, Montevideo, Saintpaul, Stanley and Typhimurium). To date, *S*. Corvallis is still the most prevalent PMQR-positive *Salmonella* serovar in The Netherlands. Infections with *qnrS1*-positive *S*. Corvallis isolates in Denmark and UK have previously been associated with the consumption of imported poultry meat from Thailand[1, 2]. Similarity in PFGE profiles of *S*. Corvallis isolates from Denmark and The Netherlands (unpublished results) suggest that human infections with *S*. Corvallis in The Netherlands could also be associated with imported poultry meat from Thailand. However, the proportion of foodborne infections with PMQR-positive *Salmonella* in The Netherlands due to consumption of imported poultry meat is unknown.

The selection method used in the national study described in **chapter 2a**, resulted in a high rate of PMQR-positive isolates within the subset. Nevertheless, the estimated overall prevalence of PMQR in the total strain collection in the study period (1999 – 2006) was low (0.2%). To screen for PMQR in *Salmonella* from more recent years, defined inclusion criteria (ciprofloxacin MICs: 0.25 -1 mg/L in combination with nalidixic acid MICs: 8 – 32 mg/L) were applied for the period 2007 - 2013. As a result a continuous low prevalence of PMQR-suspected isolates (<2%) was observed over the years with a tendency to increase from 0.56% in 2007 to 1.68% in 2013. The selection resulted in more than forty different serovars that included predominantly Corvallis, but also several Typhimurium, Stanley, Saintpaul and Montevideo isolates mainly observed in fowls (*Gallus gallus*) but also in turkey and veal calves. The growing variety in PMQR-positive serovars indicates a further horizontal spread of PMQR genes within the *Salmonella* family. Furthermore, the proportion of PMQR-suspected isolates from animal origin (living animals and meat) showed a slight tendency to increase over the years from 0.05% in 2007 to 0.47% in 2013 (unpublished data). Furthermore, a retrospective study in reduced ciprofloxacin susceptible *S*. Typhi and *S*. Paratyphi A isolates obtained from Dutch travellers to Southeast Asia did not reveal any plasmid mediated quinolone resistance[3].

The available data in this thesis indicate that human PMQR-positive *Salmonella* infections in The Netherlands can be associated with consumption of contaminated poultry products imported from Southeast Asia or travel to countries in this area (**chapter 3a**), but can also be linked to travel activities to Africa or the arrival of adoptees from this continent (**chapter 4a**). Nevertheless, national routes of contamination, like consumption of contaminated Dutch poultry meat or direct contact with animals, cannot be excluded. Still, it is known that a high proportion of human infections with S*almonella enterica* are caused by consumption of insufficient heated products of animals origin. For this reason, the main source of infections with PMQR-positive *Salmonella* is most probably also of animal origin. Currently, the prevalence of PMQR in *Salmonella* from Dutch food-producing animals is still low, but shows a tendency to increase. In the future, this might lead to more 'national' cases of foodborne infections with PMQR-positive *Salmonella*.

PMQR in *Salmonella enterica* in Europe

Although the first *qnrS1* in *Salmonella enterica* was described in *Salmonella* Infantis obtained from a European chicken carcass in 2005[4], reports on the occurrence of PMQR-positive *Salmonella* in animals from Europe are scarce. In The Netherlands, the first PMQR-positive *Salmonella* was a *qnrS1*-positive *S*. Bredeney obtained from poultry products in 2005 (**chapter 2a**). In the European study (**chapter 3a**), *qnrS1*, *qnrB2* and *qnrB19* were predominantly identified, especially in isolates from fowls (including broilers, laying hens and broiler meat) and turkeys (including turkey meat) from Spain, Poland, Germany and Denmark. In addition, the rare *qnrD1* gene was identified in *Salmonella* serovars from three different European countries, mainly from poultry sources. These results confirm previous findings of the occurrence of PMQR-positive *Salmonella* isolates in poultry (meat) from in- or outside of Europe [1, 2, 5].

Compared to the national study (**chapter 2a**), the percentage of PMQR-suspected *Salmonella* isolates in the European study (**chapter 3a**) was similar (0.2%), but the proportion confirmed PMQR-positive isolates within the selected subset was considerably lower (59% versus 87%). This indicates that the prevalence of PMQR-positive *Salmonella* isolates of the European database was overestimated by the less stringent inclusion criteria. Comparison of the estimated prevalence by country and source was not appropriate because of large differences between countries in the number of isolates and their sources. Nevertheless, the European study revealed a variety of PMQR genes

identified in different *Salmonella* serovars from eleven European countries predominantly comprising *qnrS1* and variants of *qnrB* (*qnrB2* and *qnrB19*) next to *qnrD1*, *qnrA1* and *aac(6')-1b-cr*. This is in concordance with previous findings where *qnrS1* is reported as the most frequent PMQR type in *Salmonella enterica*[6]. Furthermore, **chapter 3a** demonstrates the dissemination of PMQR genes in a variety of *Salmonella* serovars from different sources in Europe.

Similar to the national study (**chapter 2a**), the results of the European study (**chapter 3a**) suggest an impact of the consumption of contaminated poultry products imported on the occurrence of PMQR-positive *Salmonella* in humans. Among the human isolates, *qnrS1*-positive *S*. Corvallis from The Netherlands and *qnrS1*-positive *S*. Typhimurium isolates from the UK were predominantly identified. Both serovars have been associated with imported poultry products from Southeast Asia[1, 2, 5]. Other human PMQR-positive serovars in the European study (Virchow, Montevideo) have also been associated with imported poultry products in UK and Finland from the same area[2, 7]. A clear example of import of PMQR-positive *Salmonella* on poultry meat was the finding of a *qnrB19*-positive *S*. Give in Finland isolated from turkey meat imported from Brazil (**chapter 3a, table 3**). Different studies suggest that *qnrB19* is commonly present in South America [8, 9]. Possibly, the first *qnrB19* genes have entered Europe via contaminated poultry meat from Brazil or other South American countries. Interestingly, **chapter 3a** also describes the first detection of *qnrD1* in *Salmonella* serovars from Europe in poultry sources from Spain and Italy, but also in human patients from The Netherlands. The *qnrD1*-positive *Salmonella* in Dutch patients were probably also associated with poultry. One Dutch patient was most likely infected with *S*. Braenderup whilst working at an egg processing factory. The other Dutch patient infected with *S*. Hadar had a travel history to Thailand. This patient might have been infected by consumption of contaminated food during holidays in Thailand. *S*. Hadar is a common serovar in food and humans in Thailand [10], but is also commonly identified in poultry in The Netherlands [11]. Therefore, the infection with a *qnrD1*-positive *S*. Hadar might also be due to consumption of contaminated Dutch food.

In general, these findings demonstrate the global spread of PMQR-genes via the food chain. Furthermore, the European study in **chapter 3a** demonstrated the occurrence of PMQR-positive *Salmonella* in fowls, turkeys (and sometimes pigs) from Belgium, Czech Republic, Germany, Denmark, Poland, Italy and Spain. These data prove that PMQR genes

are already wide spread in food-producing animals in Europe. As a consequence, human infections with PMQR-positive *Salmonella* can be caused by consumption of poultry or pig products from in- or outside of Europe.

PMQR in *E. coli* in The Netherlands

The first PMQR (*qnrA1*) found in The Netherlands was reported in an outbreak strain of *E. cloacae* complex (Paauw, 2005). More recently, different PMQR genes were identified in clinical ESBL-producing *E. coli* ST131, *E. cloacae* and *K. pneumoniae* isolates. In *E. coli* ST 131, *aac(6')-1b-cr* was predominantly coinciding with $bla_{CTX\text{-}M\text{-}15}$, but *qnrA* and *qnrB* were frequently present in *K. pneumoniae* coinciding with different ESBL-genes [12]. The genetic environment of $bla_{CTX\text{-}M\text{-}15}$ in *E. coli* O25:H4-ST131 has been described comprehensively and is often found to coexist with *aac(6')-1b-cr* on incF plasmids[13]. For this reason, the first ESBL-producing *E. coli* ST131 in The Netherlands, found in blood stream infections in the Rotterdam area in 2008[14], might also have carried *aac(6')-1b-cr* next to $bla_{CTX\text{-}M\text{-}15}$. The reports mentioned above, all focused on clinical isolates in humans and little is known about the presence of PMQR in commensal *E. coli* from food-producing animals in The Netherlands. Therefore, this thesis only discusses the occurrence and prevalence of PMQR in commensal *E. coli* in animals.

The earliest PMQR-positive *E. coli* in animals in The Netherlands was a *qnrS1*-positive *E. coli* obtained from a broiler in 2009 followed by two PMQR-positive *E. coli* isolates in 2010 obtained from a veal calf (*qnrS1*) and a broiler chicken (*qnrB19*), respectively (**chapter 2c**). In addition, Hordijk and colleagues described a *qnrB19*-positive *E. coli* from a Dutch veal calf in 2010[15]. These reports indicate the emergence of PMQR in commensal *E. coli* from food-producing animals in The Netherlands started after 2008. Furthermore, PMQR genes were identified in ESBL-producing *E. coli* from wild birds[16] and in Enterobacteriaceae (not *E. coli*) in companion animals[17].

Using the defined inclusion criteria, the estimated prevalence of PMQR in *E. coli* from food-producing animals in The Netherlands remained low (< 1%) in the period 2010 – 2013. Isolates showing a PMQR-suspected phenotype were observed in broilers, turkeys and veal calves but not in pigs and milk cows (unpublished data). The fact that PMQR genes have so far not been identified in *E. coli* from food-producing animals in The Netherlands with the lowest usage of (fluoro)quinolones (pigs and cows) suggest an association between the

occurrence of PMQR and the usage of fluoroquinolones in food-producing animals. Finally, the existing data suggest that PMQR in The Netherlands emerged later in commensal *E. coli* from animals than in *Salmonella* from animals and humans. The reason for this is unknown. However, national data on the occurrence of PMQR in commensal *E. coli* from humans are lacking. Thus, additional information might give new insights in the introduction and spread of PMQR genes in Enterobacteriaceae in The Netherlands.

PMQR in *E. coli* in Europe

The first report of plasmid mediated quinolone resistance in Europe was described in a human clinical nalidixic acid resistant *E. coli* isolate from a French patient in 2003[18]. In the last decade several other study groups reported the occurrence of PMQR in clinical *E. coli*, often associated with ESBL-production[19, 20]. In contrast, reports on PMQR in *E. coli* from animals in Europe are scarce. The first PMQR-positive *E. coli* isolate from animals in Europe was a *qnrS1*-positive *E. coli* poultry isolate from Italy in 2009[21] followed by a report from UK of *qnrS1*-positive *E. coli* in cattle[22].

In the European study described in **chapter 3a**, the rate of confirmed PMQR-positive *E. coli* isolates within the subset of PMQR-suspected isolates was much lower compared to *Salmonella* (15% versus 59%). Apparently, the chosen inclusion criteria were less suitable for the identification of PMQR in *E. coli*. The reason for this remains unclear, but unconfirmed resistance mechanisms such as efflux pumps or loss of membrane permeability might have been more frequently present in these *E. coli* compared to *Salmonella*. Most of the PMQR-positive *E. coli* isolates exhibited MICs within a narrow range for ciprofloxacin and nalidixic acid. Nevertheless, the presence of PMQR genes was confirmed in twenty *E. coli* isolates of animal origin mainly from Poland, but also from Finland, Denmark and The Netherlands (**chapter 3a, table 1**). Although the number of PMQR-positive *E. coli* isolates was low it indicated the emergence of PMQR in commensal *E. coli* from food-producing animals in different European countries. Similar to *Salmonella*, the majority of the *E. coli* isolates harboured *qnrS1* and were mainly identified in fowls (*Gallus gallus*) and turkeys (**chapter 3a, table 4**).

More recent reports on the prevalence of PMQR-positive *E. coli* derived from veal calves[15], horses[23], pigs[24] and broilers[25] demonstrate the further spread of PMQR-genes in commensal *E. coli* in food-producing animals in Europe. In addition, PMQR genes have also been identified in zoo animals[26] and wild birds[16, 27, 28] demonstrating the wide dissemination of PMQR genes in commensal *E. coli*. The fact that wild birds carry PMQR-positive *E. coli* in their digestive tract suggest that birds can act as reservoir for resistance genes without direct antibiotic pressure.

Thoughts about the inclusion criteria

The inclusion criteria used in both the national and the European study were based on the selection of bacterial isolates demonstrating a distinct quinolone resistance phenotype being reduced susceptible to ciprofloxacin and nalidixic acid. These criteria were merely suitable for selection of isolates with PMQR genes without additional chromosomal mutations in the quinolone resistance determining region (QRDR). One mutation in *gyrA* confers high resistance against nalidixic acid, and as a consequence, will mask the typical quinolone resistance phenotype. For this reason, isolates with both PMQR genes and mutations in the QRDR were not selected in both studies. This inevitably led to an underestimation of the true PMQR prevalence**.** Numerous studies have described the occurrence of PMQR genes in fluoroquinolone resistant bacteria, but the estimated prevalence of PMQR in these studies varied considerably [6]. Therefore, the actual degree of underestimation in our studies remains unknown. Still, the inclusion criteria proved to be suitable for retrospective studies in which high rates of PMQR-positive in *Salmonella* and *E. coli* isolates were selected from a large database. Finally, the impact of PMQR in the development of quinolone resistance is still unknown and will be discussed later in this section.

II The genetic background of the PMQR genes and their plasmids

Genetic environment of PMQR genes

Resistance genes can disseminate by clonal spread of bacteria or by horizontal spread of plasmids transferred via conjugation from one bacteria to another. Transduction or transformation might also play a role in the spread of resistance genes.

Plasmids consist of extra-chromosomal, circular double-stranded DNA . They are often self-replicating by carrying systems responsible for the autonomous replication, the copy number in the cell and the stability of the plasmid during cell division[29]. Most of the larger plasmids are transferred by conjugation. Smaller plasmids often don't have a conjugative system (Tra system) and need helper plasmids for mobilization[30]. The region within a plasmid which is responsible for the replication of a plasmid is called a replicon. Plasmids with the same replication system are not able to multiply stably in the same cell. This phenomenon is called plasmid incompatibility. For many years now, a PCR based replicon-typing system (PBRT) has been used throughout the world for classification of the major plasmid families in Enterobacteriaceae[31]. Based on this PBRT system, a commercial kit (Diatheva, Fano, Italy) came available with an extended number of primer sets comprising 8 multiplex PCRs for the amplification of 25 replicons: HI1, HI2, I1, I2, X1, X2, L/M, N, FIA, FIB, FIC, FII, FIIS, FIIK, W, Y, P, A/C, T, K, U, R, B/O, HIB-M and FIB-M. For subtyping within plasmid families, schemes for multi locus sequence typing of plasmids (pMLST) were developed for the most frequently detected incompatibility groups: IncI1, IncF, IncHI1, IncHI2 and IncN: http://pubmlst.org/plasmid/). However, the PBRT scheme has its limitations and does not classify new replicon types or small plasmids without a replication system.
An alternative classification system was designed using degenerated primers targeting different relaxase gene families enabling the typing of plasmids not previously assigned to any Inc group[30]. Plasmids often harbour antibiotic resistance genes which makes them important vehicles in the horizontal spread of resistance by transfer to other bacteria via conjugation. In addition, resistance genes are often flanked by insertion sequences (IS-elements),present on integrons and/or part of transposon-like structures. This enables genes to transfer from one plasmid to another plasmid or incorporate in the chromosome of bacteria. This large variety in vehicles for spread makes the dissemination of resistance hard to tackle.

PMQR genes have been identified on different types of plasmids varying from large conjugative plasmids to small non-conjugative plasmids [32]. In **chapter 2b** of this thesis, the genetic environment of *qnrB2*, *qnrB19* and *qnrS1* is described in different *Salmonella* serovars from The Netherlands. In **chapter 2c**, the presence of *qnrS1* and *qnrB19* is described on small plasmids in *E. coli* from food-producing animals. Furthermore, **chapter 3b** describes *qnrD1* on small non-conjugative plasmids in *Salmonella enterica*. In addition, **chapter 4a** focusses on the genetic background of *qnrB2* in multidrug resistant *S*. Concord and *S*. Senftenberg isolates imported to The Netherlands via patients from Africa. Finally, **chapter 4b** showed the results of the molecular characterization of PMQR-positive, ESBL-producing, multidrug resistant Enterobacteriaceae obtained from culinary herbs imported from Southeast Asia. This section mainly focusses on the genetic environment of the most prevalent PMQR genes: *qnrS1*, *qnrB2*, *qnrB19* and *aac(6')-1b-cr* genes. In addition, it discusses the finding of *qnrD1* on small plasmids in different *Salmonella* serovars. Consequently, this section does not contain detailed information about *qnrA*, *qnrC* and *qepA*.

As described in **chapter 3a**, among the PMQR genes identified, *qnrB2* and *qnrB19* are commonly present in PMQR-positive *Salmonella enterica* both from humans and food-producing animals. As a consequence, the genetic background of both genes has been analyzed in different *Salmonella* serovars. The first characterization of the genetic environment of *qnrB* was described in *S*. Keurmassar where a *qnrB2* gene was identified downstream IS*CR1* and surrounded by two *sul1*-type class 1 integrons[33]. In **chapter 2b**, *qnrB2* gene is described on an IncN plasmid in *S*. Bredeney obtained from broiler meat in The Netherlands. In a follow-up study, the genetic environment of *qnrB2* on this plasmid was determined[34]. Similar as in *S*. Keurmassar, the *qnrB2* gene in *S*. Bredeney was identified downstream an IS*CR1* element flanked by two *sul1*-type integrons. However, the upstream integron in *S*. Bredeney carried a *dfrA25* gene instead of *aadA2*. Further downstream *qnrB2*, a second insertion element IS6100 was identified. A study in which IncN plasmids were commonly present in avian *E. coli*, but absent in human isolates[35] strengthens the hypothesis that *S*. Bredeney acquired *qnrB2* in avian bacterial flora. Remarkably, the genetic environment of *qnrB2* on IncHI2 in *S*. Bredeney from poultry in Spain was published simultaneously[36]. The different genetic environment of the two *qnrB2* genes suggest independent events. As described in **chapter 4a**, *qnrB2* was identified in multidrug resistant *S*. Concord and *S*. Senftenberg isolates.

In *S*. Senftenberg, *qnrB2* was identified on an 310 kb sized IncHI2 plasmid coinciding with multiple other resistance genes. In the same study, three multidrug resistant *S*. Concord isolates were analyzed. All three isolates harboured IncHI2 and IncA/C plasmids. Interestingly, *qnrB2* was identified either on large IncA/C plasmids or IncHI2 plasmids carrying multiple other resistance genes including $bla_{CTX\text{-}M\text{-}15}$ and/ or $bla_{SHV\text{-}12}$. These findings demonstrate that *qnrB2* itself is mobile and frequently coincides with multiple other resistance genes on large plasmids in different *Salmonella* serovars from humans and animals. Bacterial isolates with such multi-resistance plasmids can benefit in settings where different classes of antibiotics are used simultaneously or within a short range of time (such as farms or hospitals).

QnrB19 has been described in different human *Salmonella* serovars, but also in *E. coli* from food-producing animals (**chapter 3a**) on different plasmids. In this thesis, *qnrB19* has been identified on an IncN plasmid in a human *S*. Typhimurium isolate in The Netherlands associated with IS*Escp1* [37] (**chapter 2b**) and on a small none-typeable plasmid in *E. coli* obtained in a Dutch broiler chicken (**chapter 2c)**. In another study, *qnrB19* was identified on a small fully sequenced ColE-like plasmid in a Dutch human S. Typhimurium isolate carrying genomic island 1[38]. Remarkably, *qnrB19* was also identified on ColE-like plasmids in *Salmonella* in reptiles from a German zoo[39]. Previously, *qnrB19* was found in *S*. Typhimurium inserted in a Tn3 transposon on an IncL/M plasmid[40] and associated with IS*CR1* in *E. coli*[41]. In addition, Hordijk and colleagues identified *qnrB19* on a 40kb IncR plasmid flanked by IS26 elements in *E. coli* from a Dutch veal calve[15]. Interestingly, in a recent German study, *qnrB19* was identified in *E. coli* from a horse on an IncN plasmid also flanked by IS26 elements[42]. The variation in plasmids carrying *qnrB19* flanked by different insertion sequences indicates that this gene has spread to different plasmid families using a variety of IS elements for mobilization. As a result, the host-range for horizontal spread to other bacteria was extended. The variation in the genetic environment could explain why the dissemination of this PMQR gene seems to be more successful than others.

Unlike *qnrB*, *qnrS* is mainly present on smaller plasmids, often without other resistance genes[43]. This difference might be related to the plasmid size[44]. Although *qnrS1* is seldom embedded in an integron structure[32] different genetic environments have been described. For instance, associated with a Tn3-like structure in *S*. Infantis[4] or upstream insertion sequence IS*Ecl2* in *E. cloacae*[45]. A small ColE-like plasmid carrying the *qnrS1*

gene (designated pTPqnrS-1) in *S.* Typhimurium DT193, was fully sequenced lacking a Tn3-like structure[46]. In this thesis (**chapter 2a** and **chapter 3a**), *qnrS1* was the most prevalent identified PMQR gene in *Salmonella enterica* which is in concordance with previously findings[6]. In **chapter 2b**, *qnrS1* was identified in different *Salmonella* serovars on various plasmids: IncN (Kentucky, Saintpaul), IncHI2 (Stanley), IncR (Montevideo) and ColE (Corvallis, Anatum). Additional sequencing of the genetic environment on ColE plasmids revealed that the region with *qnrS1*, *oriV* (the origin of replication) and *MobC* was identical to pTPqnrS-1 in both *S.* Corvallis and *S.* Anatum which indicates a strong relationship between these plasmids. These small ColE plasmids are not self-conjugative, but need helper plasmids for transfer. The finding of additional conjugative plasmids (IncI, IncB/O and IncA/C) coinciding in the *Salmonella* strains with *qnrS1*-carrying ColE plasmids suggests the participation in mobilization of ColE plasmids (**chapter 2b**). This mechanism might be an explanation for the successful dissemination of such small plasmids. Still, the reason why *qnrS1* is more prevalent in *Salmonella enterica* than *qnrA* or *qnrB* remains unknown. Moreover, in this thesis, *qnrS1* was also the most prevalent PMQR gene identified in *E. coli* in food-producing animals (**chapter 2c** and **chapter 3a**). Nevertheless, the number of PMQR-positive isolates identified are small, so interpretation should be done with caution. Finally, the predominance and the variation in *qnrS1*-carrying plasmid families proves the wide dissemination of this PMQR-gene in humans and animals.

Similar to the first described *qnrD* gene in *Salmonella enterica* on plasmid p2007507[47], *qnrD1* was identified on relative small, PBRT non-typeable or ColE-like plasmids (**chapter 4b**). Sequence analysis of the plasmids demonstrated a region almost identical to a *qnrD1*-harbouring plasmid pCGS49 from *Proteus mirabilis*. This *qnrD1*-harbouring region was either flanked by IS1-elements or direct repeats. Furthermore, an extra, variable sized, region was present in all plasmids suggesting different fusion events with other bacterial plasmid DNA. In none of the plasmids involved in the study, relaxase genes were identified, so the mechanism for transfer of these plasmids was not revealed . Possibly, acquisition occurred via transformation followed by low diffusion (at least in Europe) via clonal dissemination of some *Salmonella* serovars predominantly in poultry.

III Import of PMQR positive, multidrug resistant Enterobacteriaceae via people or food

Antibiotic resistance bacteria can spread rapidly over great distances via travel of people or transport of animals, but also by the worldwide trade in food and feed. As a consequence, multidrug resistant bacteria can be introduced into countries with relatively low levels of multidrug resistance in their population. In this thesis two examples of import of multidrug resistant Enterobacteriaceae are discussed: (I) PMQR-positive, multidrug resistant *Salmonella* enterica obtained from humans associated to the African continent, (II) PMQR-positive, multidrug resistant Enterobacteriaceae identified on culinary herbs from Southeast Asia. Nevertheless, import of resistant bacteria from other parts of the world must not be excluded.

PMQR multidrug resistant *Salmonella* imported from the African continent

As described in **chapter 4a** of this thesis, four remarkable multidrug resistant *Salmonella* isolates were observed in our national surveillance program in 2007, comprising three *S*. Concord isolates and one *S*. Senftenberg isolate. The *S*. Concord isolates were obtained from Ethiopian orphans and the *S*. Senftenberg originated from a patient with a travel history to Egypt. These isolates were resistant to almost all antibiotic classes tested and harboured PMQR genes (*qnrB2*) next to extended spectrum beta-lactamase genes ($bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$) and a wide range of other resistance genes on large plasmids. Although, the association between PMQR and ESBL is frequently reported [48, 49], this was the first time *Salmonella* isolates were identified in The Netherlands harbouring both PMQR and ESBL genes. *S*. Concord is a very rare serovar in The Netherlands. In the period 1999 – 2010, only nine isolates (< 0.01%) of human origin were tested. Most of these isolates were resistant to a wide range of antibiotics, including third-generation cephalosporins. A Danish study described the emergence of multidrug resistant *S*. Concord isolates from Ethiopian adoptees in Europe including four Dutch isolates from the period 2001-2006[50].

These isolates were resistant to 3rd generation cephalosporins, but completely susceptible to ciprofloxacin. More cases of infections with multidrug resistant *S*. Concord isolates associated with Ethiopian adoptees were described in the last decade from several other European countries [51-53]. Moreover, multidrug resistant *S*. Concord isolates were identified as a major cause of salmonellosis in children in Ethiopia[54] which confirms the origin of the previous described infections of the Ethiopian orphans in Europe. After 2007, only one

other multidrug resistant *S*. Concord isolate was observed. In contrast to *S*. Concord, *S*. Senftenberg is commonly observed in The Netherlands obtained from animal feed, but also from humans and animals, but almost all of these isolates are susceptible to most antibiotics tested. Nevertheless, a multidrug resistant *S*. Senftenberg was identified in a Dutch patient with a travel history to Egypt. Another more recent study reported the finding of two extremely drug resistant *S*. Senftenberg isolates obtained from patient in Zambia[55]. Interestingly, both isolates harboured IncHI2 plasmids (and one isolate also harboured IncA/C) and carried multiple resistance genes including PMQR and ESBL genes. However, the isolates from Zambia carried other ESBL- and PMQR genes (*bla* $_{\text{CTX-M-15}}$ and *aac(6')-1b-cr*), compared to the Dutch isolate. Although reports are scarce, these cases demonstrate multidrug resistant *S*. Senftenberg isolates reside in different parts of the African continent. Finally, these findings emphasize that a continuous surveillance of antibiotic resistance is needed to screen for possible new introductions of multidrug resistant *Salmonella enterica* especially in people with a travel history to areas where multidrug resistant bacteria are more prevalent, like Africa[56] or Southeast Asia[57].

PMQR multidrug resistant Enterobacteriaceae imported from Southeast Asia

Next to import via people, multidrug resistant bacteria can also be imported via food. The majority of foodborne infections originate from food of animal origin, but occasionally also from food of non-animal origin comprising fruit, vegetables and herbs[58]. Outbreaks have been described originating from fresh culinary herbs as cilantro[59] and Parsley[60]. In **chapter 4b** of this thesis, the import of multidrug resistant Enterobacteriaceae is described on herbs from Southeast Asia. In a pilot study, ten different batches of culinary herbs from Thailand, Vietnam and Malaysia were screened for the presence of cefotaxime resistant Enterobacteriaceae. As a result, remarkable multidrug resistant Enterobacteriaceae were identified in eight batches. These isolates comprised of multidrug resistant *K. pneumoniae*, *E. coli* and *E. cloacae* complex isolates. Molecular characterization revealed plasmids carrying multiple resistance genes, including ESBL- and PMQR-genes (**chapter 4b, table 2**). The multidrug-resistance of the isolates identified on the herbs reflects the high selection pressure through imprudent use of antimicrobials in humans and animals in Southeast Asia[61]. To date, multidrug resistance is commonly present in commensal and pathogenic bacteria throughout Southeast Asia[62]. Unfortunately, information on the quantities of imported fresh culinary herbs in The Netherlands are not available. Moreover, the bacterial loads on the herbs were not determined. For these reasons, it is impossible to estimate

the actual risk of getting infected with multidrug resistant bacteria from consumption of contaminated fresh culinary herbs. Nevertheless, fresh culinary herbs are a potential source for multidrug resistant bacteria. Since, these herbs are often consumed without heating, transfer to humans cannot be excluded. Finally, results in this thesis demonstrate that PMQR-positive, multidrug resistant bacteria can be imported from Southeast Asia via food of animal origin (**chapter 2a**, **chapter 3a**) and, probably less frequently, also via food of non-animal origin (**chapter 4b**).

Risk to human health

Resistance to quinolones and fluoroquinolones has been reported increasingly among bacterial isolates from humans and animals, most probably as a consequence of the large scale use of this group of antibiotics[43]. Although, the most important mechanism for quinolone resistance are chromosomal mutations, causing target modifications (in DNA gyrase and topoisomerase IV), plasmid mediated quinolone resistance (PMQR) has emerged and is currently reported worldwide in commensal and pathogenic bacteria in different environments. The successful spread of PMQR genes on plasmids in human and veterinary bacteria suggests an evolutionary benefit for bacteria carrying these genes. The fact that PMQR determinants do not confer clinical resistance to (fluoro)quinolones, could imply that this type of resistance does not have any clinical relevance. However, PMQR determinants have an additional effect on other quinolone resistance mechanisms which can ultimately lead to fluoroquinolone resistant bacteria with Minimal Inhibitory Concentrations (MICs) above the clinical breakpoint [63, 64]. Furthermore, PMQR-genes are thought to facilitate the selection of higher-level quinolone resistant mutants[32]. For these reasons, the clinical relevance of these resistance genes, especially *qnr*-genes, has been explored by testing the effect of PMQR-genes on the activity of fluoroquinolones, both *in vitro* and *in vivo*.

As stated previously, PMQR is thought to facilitate the selection of chromosomal mutants by elevation of the Mutations Prevention Concentration (MPC)[32]. The MPC is the lowest drug concentration that prevents the growth of single step mutants present in a large bacterial population. The mutant selection window (the antibiotic concentration range found between MIC and MPC) is where single-step mutants can be developed. Hence, when the quinolone concentration remains above the MPC, single-step mutants are unlikely to occur. Conversely, widening of the mutant selection window will enhance the

selection of single-step mutants. The influence of *qnrA1* on the development of quinolone resistance was evaluated in *E. coli* and *K. pneumoniae* determining the MPCs[65]. It was concluded that mutations were easily selected in the presence of *qnrA1*. Similar effects of different PMQR genes on MIC and MPC were determined in *S.* Typhimurium[63]. In contrast, a low selection of single-step mutants in *qnr*-carrying isolates was reported in *E. coli* due to target protection of Qnr proteins[66]. This hypothesis is in concordance with recent findings in *E. coli* from different sources, where the resistant rates for ciprofloxacin were significantly lower in *qnr*-positive *E. coli* isolates than in *qnr*-negative isolates. Unlike *qnr*, most *oqxAB*, *aac(6')-1b-cr* and *qepA* carrying isolates were ciprofloxacin resistant[67]. Nevertheless, several other groups have stated that PMQR most probably facilitates the selection of mutants. A recent study looked at the *in vitro* effect of *qnrA1*, *qnrB1* and *qnrS1* and mutations in *gyrA* and *parC* in isogenic *E. coli* isolates on the activity of fluoroquinolones[68]. As a result, the presence of *qnr* increased the mutant prevention concentrations in isolates without chromosomal mutations, but also in isolates with additional mutations in *gyrA* and *parC*. This indicates that *qnr*-genes play a role in the acquisition of clinical resistance to fluoroquinolones. Moreover, in a study with clinical isolates of *E. coli*, *E. cloacae* and *K. pneumoniae* covering a 9-year period in a Korean hospital, the increasing frequency of ciprofloxacin was associated with an increasing prevalence of PMQR genes[69]. However, the assumed association could be due to a simultaneous independent increase of the different quinolone resistance mechanisms. Finally, there seems a conflict in thoughts on the influence of PMQR on the acquisition of fluoroquinolone resistance, especially regarding *qnr*: (a) PMQR widen the mutant selection window and thereby facilitate the selection of single-step mutants or (b) Qnr proteins protect the QRDR region of DNA gyrase and topoisomerase IV and thereby block the interaction with the quinolones resulting in a lower number of single-step mutants. Possibly, both mechanisms are simultaneously active.

The effect of PMQR genes on quinolone treatment was also investigated *in vivo*. In an murine pneumoniae model, the presence of *qnrA1*, *qnrB1* and *qnrS1* in *E. coli* reduced the efficacy of ciprofloxacin and levofloxacin[70]. Similar effect were measured with ciprofloxacin in the presence of *qnrA1*, *qnrB19* and *qnrS1* in murine models of urinary tract infections with *E. coli*[71,72]. In *E. coli* isolates from animals, time-kill experiments with enrofloxacin revealed that the bactericidal activity for all *qnr*-positive isolates was less dependent on the concentration compared to *gyrA* mutants[73]. These studies clearly indicate that

fluoroquinolone treatment of infections with PMQR-carrying isolates can ultimately lead to treatment failure. Still, reports on treatment failure when using fluoroquinolones as therapy for infections with PMQR-positive bacteria are lacking. Furthermore, clinical information about infections with PMQR-carrying isolates are very limited. In two studies focussing on blood stream infections with *qnr*-positive Enterobacteriaceae, there was no significant difference in the mortality rate of *qnr*-positive and *qnr*-negative groups[74, 75]. Although, cases of poor fluoroquinolone treatment results of infections with reduced fluoroquinolone susceptible *Salmonella* have been documented[76], the clinical impact of PMQR on the fluoroquinolone treatment of salmonellosis is unknown. Nonetheless, the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recently revised their breakpoints for *Salmonella*[77, 78]. CLSI lowered the susceptible breakpoints of ciprofloxacin (≤ 0.06 mg/L), levofloxacin (≤0.125 mg/L)and ofloxacin (0.125 mg/L) for all *Salmonella enterica* serovars (including Typhi and Paratyhi). EUCAST lowered the breakpoints for ciprofloxacin to ≤ 0.5 mg/L for all Enterobacteriaceae with a comment that *Salmonella* species with MICs > 0.06 mg/L might respond poorly to ciprofloxacin treatment. As a results of the revised breakpoints, reduced susceptible *Salmonella* isolates will be, more appropriately, classified as intermediate resistant to fluoroquinolones. This will prevent possible treatment failure of infections caused by *Salmonella enterica* exhibiting reduced susceptibility to fluoroquinolones, to chromosomal mutations or PMQR.

Final thoughts and conclusions

It is important to continuously monitor the prevalence of PMQR in Enterobacteriaceae in animals and humans next to other fluoroquinolone resistance mechanisms, because the emergence of PMQR complicates the development and spread of fluoroquinolone resistance. Moreover, PMQR is clinically relevant for a number of reasons; (I) it contributes to higher MICs for (fluoro)quinolones, also in presence of other resistance mechanisms; (II) it facilitates the acquisition of total quinolone resistance by widening the mutation prevention window; (III) it is, in contrast to other fluoroquinolone resistance mechanisms, horizontally transferable.

This thesis provides information on the occurrence of PMQR determinants in *E. coli* and *Salmonella enterica* in food-producing animals in The Netherlands and other European countries. Despite the fact that (fluoro)quinolones have been used extensively in food-producing animals, the estimated prevalence of PMQR-positive bacteria in the gut flora of these animals is low. Apparently, usage of fluoroquinolones does not strongly select for PMQR in Enterobacteriaceae. Nevertheless, the highest prevalence of PMQR was identified in animals with the highest usage of fluoroquinolones: fowls and turkeys. This suggests there is an association between usage of (fluoro)quinolones and the prevalence of PMQR-positive Enterobacteriaceae in the gut flora of these animals. Due to limitations in the studies, the true prevalence of PMQR was underestimated. Solely, bacterial isolates exhibiting reduced susceptibility to ciprofloxacin and nalidixic acid were screened for the presence of PMQR determinants. Considering the role of PMQR in the acquisition of fluoroquinolone resistance, PMQR determinants should also be expected in the fluoroquinolone resistant population. Future screening studies should focus on the prevalence of PMQR in commensal *E. coli* obtained from different animals as collected in the national surveillance program, regardless of their MICs for nalidixic acid and ciprofloxacin. This will deliver new valuable data on the prevalence of PMQR in reduced fluoroquinolone susceptible populations as well as in fluoroquinolone resistant populations.

The recent reduction in usage of 3rd generation cephalosporins in food-producing broilers has resulted in an unexpected fast reduction of the number of ESBL-producing commensal *E. coli*. Similarly, the reduction of fluoroquinolones usage in animals (50% in the last year) could lead to a decrease of PMQR-positive isolates.

However, what the short-term effect will be on the prevalence of PMQR-positive isolates is uncertain. As stated earlier, the selective effect of fluoroquinolones on PMQR is less evident. Furthermore, PMQR genes are often present on large conjugative plasmids carrying multiple resistance genes. Hence, PMQR can be co-selected by usage of other antibiotic classes. Maybe, the current simultaneous decrease in usage of cephalosporins, (fluoro)quinolones and other antibiotics in food-producing animals will result in a decrease in the number of PMQR-positive isolates. On the other hand, many plasmids carry addiction systems which makes it almost impossible for bacteria to loose plasmids. Conversely, the success of small non-conjugative plasmids carrying solely PMQR genes is poorly understood.

To determine the long-term effect of reduction in antimicrobial usage in food-producing animals on antimicrobial resistance, continuous phenotypic testing of bacteria in the gut flora of these animals will be necessary. However, monitoring of resistance based on whole genome sequencing (WGS) will become an important tool in the near future[79]. Moreover, new bioinformatics tools will make it possible to use WGS on a large scale to simultaneously determine the presence of resistance genes, integrons and plasmids and the genotype of the bacteria. This information can be used for monitoring of antimicrobial resistance, but also be used for outbreak studies or larger epidemiological studies.
In this thesis, the described PMQR-positive *Salmonella* infections in human patients were often associated with consumption of contaminated broiler meat imported from Southeast Asia or with a travel history to Southeast Asia or North Africa. The high prevalence of PMQR in these areas reflects the imprudent usage of fluoroquinolones in humans and animals. These cases demonstrate that antimicrobial resistance is global problem. Regulations to prevent the further spread of antimicrobial resistance should be implemented on a global scale as acknowledged by the World Health Organization (WHO). Nevertheless, the current reduction in usage of antimicrobials in food-producing animals in The Netherlands is an important example for the rest of the world.

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Summary

Fluoroquinolones are potent antibiotics active against a broad range of bacteria and are used widely in both human and veterinary medicine. Soon after the introduction of fluoroquinolones, resistance emerged and increased over the years in commensal and pathogenic bacteria in humans and animals due to extensive usage. Resistance to fluoroquinolones is considered to be a risk for human health, because of potential treatment failure.

In The Netherlands, recent governmental measurements have been implemented by the Dutch livestock farming sector in close collaboration with Netherlands Veterinary Medicines Authority (SDa) and veterinarians targeting on the further reduction antibiotics usage in food-producing animals with special attention to classes of antibiotics labelled as critically important for human health. This includes fluoroquinolones and third- and fourth generation cephalosporins. As a result, the total sales of antibiotics for animal use decreased with 58% in five years (2009-2013). The sales of (fluoro)quinolones for animal usage decreased from nine tons in 2007 to three tons in 2012. In 2013, a further reduction (50%) in sales of (fluoro)quinolones was accomplished. Nevertheless, resistance to fluoroquinolones is still high in commensal *E. coli* and *Campylobacter* spp. in some animal species, especially poultry.

Resistance to (fluoro)quinolones is mainly caused by chromosomal mutations in the quinolone resistance determining region (QRDR) of topoisomerase genes. Topoisomerase enzymes are involved in DNA replication. Other resistance mechanisms caused by chromosomal mutations can also play a role in quinolone resistance like decreased outer membrane permeability or up regulation of efflux pumps. Since 1998, different types of plasmid mediated quinolone resistance (PMQR) consecutively emerged encoded by different resistant genes: *qnrA*, *qnrS*, *qnrB*, *qnrC*, *qnrD*, *aac(6')-1b-cr*, *oqxAB* and *qepA*. PMQR in Enterobacteriaceae generally results in reduced susceptibility to (fluoro)quinolones to levels that are still below clinical breakpoints. However, PMQR is considered clinically relevant for a number of reasons; (I) it contributes to higher MICs for (fluoro)quinolones; (II) it facilitates the acquisition of total quinolone resistance; and (III) it is horizontally transferable, in contrast to other fluoroquinolone resistance mechanisms.
Before the start of this thesis, data on the occurrence and prevalence of PMQR in

Enterobacteriaceae from humans and animals in The Netherlands and Europe were limited. Therefore, studies were designed to determine the occurrence and prevalence of PMQR genes in *Salmonella* and *E. coli* from The Netherlands and other European countries and to characterize the genetic background of these genes.

As described in **chapter 2a**, the earliest identified PMQR-positive *Salmonella* isolate in The Netherlands was a *qnrS1*-positive *S*. Corvallis isolate obtained from a human patient in 2003. In this study, PMQR-suspected *Salmonella* isolates were screened for the presence of PMQR-genes. These isolates were all reduced susceptible to nalidixic acid and ciprofloxacin. *S*. Corvallis was predominantly identified next to seven other *Salmonella* serovars (Anatum, Bredeney, Kentucky, Montevideo, Saintpaul, Stanley and Typhimurium) harbouring different PMQR genes (*qnrS1*, *qnrB2* and *qnrB19*). Except for one *S*. Bredeny isolate obtained from a broiler product, all isolates were of human origin. The estimated overall prevalence of PMQR in the total strain collection was low (0.2%). More recently, a continuous low prevalence of PMQR-suspected isolates was observed in The Netherlands with a tendency to increase from 0.56% in 2007 to 1.68% in 2013. Furthermore, the proportion of PMQR-suspected isolates of animal origin (living animals and meat) showed a slight tendency to increase over the years from 0.05% in 2007 to 0.47% in 2013 (**chapter 5**).

Chapter 2b covers a molecular study of the PMQR-positive *Salmonella* from The Netherlands revealed IncN plasmids carrying *qnrS1* (S. Kentucky and *S*. Saintpaul), *qnrB2* (*S*. Bredeney) and *qnrB19* (*S*. Typhimurium PT507). Furthermore *qnrS1* was identified on ColE (*S*. Corvallis and *S*. Anatum), IncR (in *S*. Montevideo) and incHI2 plasmids (*S*. Stanley). Additional sequencing was performed to analyse the genetic environment of the *qnr* genes. As a result, a variety of plasmid replicon types and resistant genes was identified indicating a complex dissemination of PMQR genes in different *Salmonella* serovars. The majority of these relatively large plasmids were self-transferable by conjugation. However, PMQR-genes can also be present on smaller non- conjugative plasmids.

As described in **chapter 2c**, the earliest identified PMQR-positive *E. coli* in animals in The Netherlands was a *qnrS1*-carrying *E. coli* obtained from a broiler in 2009 followed by two PMQR-positive *E. coli* isolates in 2010 obtained from a veal calf (*qnrS1*) and a broiler chicken (*qnrB19*), respectively. Molecular analysis revealed that these *qnr*-genes were present on relatively small plasmids. This study suggests that PMQR in commensal *E. coli* from food-

producing animals in The Netherlands emerged after 2008. The estimated prevalence of PMQR in *E. coli* from food-producing animals in The Netherlands remained low (< 1%) in the period 2010 – 2013 (**chapter 5**). The fact that PMQR genes have so far not been identified in *E. coli* from food-producing animals in The Netherlands with the lowest usage of (fluoro)quinolones (pigs and cows) points towards an association between the occurrence of PMQR and the usage of fluoroquinolones in food-producing animals.

In **chapter 3a**, a large strain European collection was screened for PMQR-suspected *Salmonella* isolates based on their phenotype. This resulted in a similar low prevalence (0.2%) of PMQR-positive *Salmonella* isolates, with *qnrS1* and variants of *qnrB* (*qnrB19* and *qnrB2*) as the predominant PMQR-determinants identified. Remarkably, the rare *qnrD1* was identified in eight different serovars from three different countries. PMQR genes were identified in different *Salmonella* serovars from eleven European countries obtained from humans, animals, food and the environment. This indicates a the wide spread of PMQR genes in Europe among *Salmonella* from different sources despite the low prevalence. Although, the database was influenced by differences in the number of isolates per country and source, it suggested that poultry (*Gallus gallus* and turkeys) is the main source for PMQR-determinants in Europe with predominant serovars Derby, London, Saintpaul and Hadar. In addition, human PMQR-positive *Salmonella* were often associated with foreign travel, particularly *qnrS1*-positive *S*. Corvallis and *S*. Typhimurium isolates. In this study, the prevalence of PMQR-positive *E. coli* isolates was also low (< 0.1%) and mainly originated from food-producing animals in Poland, but also from Finland, Denmark and The Netherlands indicating the emergence of PMQR in commensal *E. coli* from food-producing animals in different European countries. Similar to *Salmonella*, the majority of the *E. coli* isolates harboured *qnrS1* and were mainly identified in poultry (*Gallus gallus* and turkeys).

The study described in **chapter 3b** reveals the presence of *qnrD1* in *Salmonella* isolates from Italy, Spain and The Netherlands from different serovars and sources (poultry and human patients). All *qnrD1* genes were identified on small, often similar, plasmids in *Salmonella*. Analysis of the total sequence of these plasmids revealed a striking similarity in the genetic environment of *qnrD1* indicative for a common progenitor plasmid from *P. mirabilis*.

Antibiotic resistance bacteria can spread rapidly over great distances via travel of people or transport of animals, but also by the worldwide trade in food and feed. In this thesis two examples of import of multidrug resistant Enterobacteriaceae are discussed: PMQR-positive, multidrug resistant *Salmonella* enterica obtained from humans associated to the African continent (**chapter 4a**), PMQR-positive, multidrug resistant Enterobacteriaceae identified on culinary herbs from Southeast Asia (**chapter 4b**).

Despite the strong reduction of (fluoro)quinolone usage in animals in The Netherlands since 2007, resistance to (fluoro)quinolones is still high in commensal *E. coli* and *Campylobacter* spp. in food-producing animal species with a history of widespread usage of (fluoro)quinolones. This is most probably mainly due to chromosomal point mutations in the quinolone resistance determining region (QRDR) of topoisomerase genes. The prevalence of PMQR in animal bacteria, which merely results in a slight reduction of susceptibility to (fluoro)quinolones, is relatively low but shows a tendency to increase. The fact that PMQR complicates the development and spread of fluoroquinolone resistance makes it important to include this in the monitoring system in order to determine the long-term effect of reduction of usage of (fluoro)quinolones in food-producing animals on (fluoro)quinolone resistance.

Samenvatting

Fluorochinolonen zijn potente antibiotica die werkzaam zijn tegen veel soorten bacteriën. Om deze reden worden deze antibiotica wereldwijd gebruikt voor behandeling van infecties in mensen en dieren. Snel na het gebruik van fluorochinolonen werden de eerste resistente bacteriën gevonden. Ten gevolge van overmatig gebruik van fluorochinolonen nam deze resistentie in de jaren daarna snel toe in niet-ziekteverwekkende en ziekteverwekkende bacteriën bij mens en dier. Resistentie tegen fluorochinolonen wordt gezien als een risico voor de volksgezondheid, vanwege therapiefalen bij behandeling van infecties.

De Nederlandse overheid heeft recentelijk samen met de verschillende diersectoren, dierenartsen en de Autoriteit Diergeneesmiddelen (SDa) maatregelen genomen om het gebruik van antibiotica in de dierhouderij terug te dringen en het gebruik van antibiotica die van kritisch belang zijn voor de humane geneeskunde te minimaliseren. De kritische middelen zijn 3e en 4e generatie cefalosporines en fluorochinolonen. Door het invoeren van deze maatregelen is de hoeveelheid verkochte antibiotica in de dierhouderij in vijf jaar tijd (2009 – 2013) gedaald met 58%. De totale hoeveelheid (fluoro)chinolonen verkocht voor gebruik in de dierhouderij daalde van negen ton in 2007 naar drie ton in 2012. In 2013 daalde de verkoop van (fluoro)chinolonen nog verder met 50%. Ondanks deze afname in de verkoop (en gebruik) is de resistentie tegen (fluoro)chinolonen nog steeds hoog in commensale *E. coli* en in *Campylobacter* in de darmen van sommige diersoorten, met name in pluimvee.

Resistentie tegen (fluoro)chinolonen wordt voornamelijk veroorzaakt door chromosomale mutaties in een specifiek gebied (QRDR) van topoisomerase genen. Deze genen coderen voor enzymen die een belangrijke rol spelen bij de DNA replicatie. Andere resistentie mechanismen die veroorzaakt worden door chromosomale mutaties kunnen eveneens een rol spelen in (fluoro)chinolonen resistentie: (I) verminderde doorlaatbaarheid van het buitenmembraan van een bacterie of (II) het extra activeren van zogenaamde efflux pompen in een bacterie. Vanaf 1998 zijn verschillende plasmide gemedieerde chinolonen resistentie (PMQR) genen ontdekt: *qnrA*, *qnrS*, *qnrB*, *qnrC*, *qnrD*, *aac(6')-1b-cr*, *oqxAB* en *qepA*. Enterobacteriaceae met PMQR zijn verminderd gevoelig voor fluorochinolonen, maar niet klinisch resistent. Toch wordt PMQR als klinisch relevant gezien om verschillende

redenen: (I) het verlaagt de gevoeligheid voor fluorochinolonen, (II) het kan een rol spelen bij het ontstaan van klinische resistentie, (III) in tegenstelling tot de andere vormen van (fluoro)chinolonen resistentie is dit type resistentie overdraagbaar van de ene bacterie naar de andere via overdracht van plasmiden.

Voor de start van dit onderzoek was er weinig bekend over het voorkomen van plasmide gemedieerde chinolonen resistentie (PMQR) in Enterobacteriaceae bij mens en dier in Nederland en andere delen van Europa. Om deze reden zijn verschillende studies bedacht om het voorkomen en de verspreiding van PMQR in *Salmonella* en *E. coli* verder in kaart te brengen. Daarnaast is de genetische achtergrond van deze resistentiegenen onderzocht. In **hoofdstuk 2a** wordt het vroegste PMQR-positieve *Salmonella* isolaat in Nederland beschreven. Dit is een *Salmonella* Corvallis geïsoleerd uit een patiënt in 2003. In deze studie werden PMQR-verdachte *Salmonella* isolaten gescreend op de aanwezigheid van PMQR genen. Deze *Salmonella* isolaten waren allemaal verminderd gevoelig voor nalidixinezuur en ciprofloxacine wat de aanwezigheid van een PMQR gen indiceert. In de selectie PMQR-verdachte isolaten werd *S*. Corvallis het vaakst gevonden naast zeven andere serovars (Anatum, Bredeney, Kentucky, Montevideo, Saintpaul, Stanley en Typhimurium) met verschillende soorten PMQR genen (*qnrS1*, *qnrB2* en *qnrB19*). Met uitzondering van *S*. Bredeney (geïsoleerd uit een kipproduct) waren alle PMQR-positieve isolaten afkomstig van humane patiënten. De geschatte prevalentie van PMQR-positieve isolaten in de totale collectie uit was laag (0.2%). Meer recentelijk werd eveneens een lage prevalentie gemeten van PMQR-verdachte *Salmonella* isolaten met een licht stijgende trend van 0.56% in 2007 naar 1.68% in 2013. In dieren en dierlijke producten werd eveneens een lage prevalentie gezien van PMQR-verdachte isolaten met een lichte stijging van 0.05% in 2007 naar 0.47% in 2013 (zie **hoofdstuk 5**).

Hoofdstuk 2b beschrijft de resultaten van een moleculaire studie naar de genetische achtergrond van PMQR-positieve *Salmonella* isolaten uit Nederland. In deze studie werden verschillende plasmide types geïdentificeerd met verschillende PMQR-genen in verschillende *Salmonella* serovars. Er werden IncN plasmiden gevonden met *qnrS1* (Kentucky en Saintpaul), *qnrB2* (Bredeney) en *qnrB19* (Typhimurium PT507). Daarnaast werd *qnrS1* gevonden op ColE plasmiden (Corvallis en Anatum), op een IncR plasmide (Montevideo) en een incHI2 plasmide (Stanley). Uit de resultaten van de studie blijkt dat verschillende types plasmiden een rol spelen bij de verspreiding van PMQR-genen in

Salmonella. Het merendeel van de plasmiden bleken in staat zich via conjugatie zelfstandig te kunnen verspreiden. Echter, sommige PMQR genen werden aangetroffen op kleinere (niet-conjugeerbare) plasmiden.

In **hoofdstuk 2c** worden de vroegste PMQR-positieve *E. coli* in Nederlandse landbouwhuisdieren beschreven. Het betreft een *qnrS1*-positieve *E. coli* geïsoleerd uit een vleeskuiken in 2009 en twee PMQR-positieve *E. coli* isolaten uit 2010 geïsoleerd uit een vleeskalf (*qnrS1*) en een vleeskuiken (*qnrB19*). Uit moleculaire analyses bleek dat deze PMQR genen op relatief kleine plasmiden liggen. De resultaten indiceren dat PMQR genen pas na 2008 zijn verschenen in commensale *E. coli* in de darmen van landbouwhuisdieren in Nederland. In de periode 2010-2013 bleef de prevalentie van PMQR-verdachte *E. coli* isolaten laag (**hoofdstuk 5**). Het feit dat PMQR-verdachte bacteriën niet zijn aangetroffen in de darmen van diersoorten met het laagste (fluoro)chinolonen gebruik (varkens en runderen) indiceert dat er een verband bestaat tussen het voorkomen van PMQR en het gebruik van (fluoro)chinolonen in landbouwhuisdieren.

In de studie beschreven in **hoofdstuk 3a** is een grote Europese collectie PMQR-verdachte *Salmonella* isolaten gescreend op het voorkomen van PMQR genen. Dit resulteerde in een lage prevalentie (0.2%) PMQR-positieve *Salmonella* isolaten met voornamelijk *qnrS1* en varianten van *qnrB* (*qnrB2* en *qnrB19*). Opmerkelijk was de vondst van het zeldzame *qnrD1* gen in acht verschillende *Salmonella* serovars afkomstig uit drie verschillende landen. PMQR genen werden aangetroffen in verschillende *Salmonella* serovars uit elf Europese landen afkomstig van mensen, dieren, voedsel en het milieu. Dit indiceert een wijde verspreiding van PMQR genen in *Salmonella*, ondanks de lage prevalentie. Hoewel de uitkomsten van de studie beïnvloed werden door de onevenredige verdeling in aantallen isolaten per land en bron, suggereren de uitkomsten dat pluimvee (kip en kalkoen) de belangrijkste bron vormt voor PMQR in Europa met een aantal predominante *Salmonella* serovars: Derby, London, Saintpaul en Hadar. De in de studie aangetroffen humane PMQR-positieve *Salmonella* serovars (o.a. Corvallis en Typhimurium) worden vaak geassocieerd met reizen naar het buitenland. In deze Europese studie was de prevalentie van PMQR-positieve *E. coli* laag (0.1%) en werd voornamelijk gevonden in commensale *E. coli* afkomstig uit de darmen van landbouwhuisdieren in Polen, maar ook in Finland, Denemarken en Nederland. Deze gegevens indiceren de verdere opkomst en verspreiding van PMQR in commensale *E. coli* in landbouwhuisdieren in Europa. Net als bij *Salmonella*

werd *qnrS1* het vaakst aangetroffen in *E. coli* en dan vooral in pluimvee (kip en kalkoen). In **hoofdstuk 3b** wordt de aanwezigheid van *qnrD1* beschreven in *Salmonella* uit Italië, Spanje en Nederland afkomstig van verschillende bronnen (pluimvee en mens). Alle *qnrD1* genen werden gevonden op kleine, vaak sterk op elkaar lijkende, plasmiden in verschillend *Salmonella* serovars. Uit de sequentie analyse van het DNA van de plasmiden bleek dat de *qnrD1* genen ingebed lagen in een gebied wat sterk lijkt op een plasmide wat eerder gevonden is in een *Proteus mirabilis*. Dit indiceert dat er waarschijnlijk sprake is geweest van een gemeenschappelijke voorloper.

Antibiotica resistente bacteriën kunnen zich snel over grote afstanden verspreiden via reizen van mensen of transport van dieren, maar ook door de wereldwijde handel in voedsel en voer. In dit proefschrift worden twee voorbeelden van verspreiding van multiresistente Enterobacteriaceae beschreven: PMQR-positieve, multiresistente *Salmonella* gevonden in Nederlandse, patiënten geassocieerd met Afrika **(hoofdstuk 4a)** en PMQR-positieve, multiresistente Enterobacteriaceae gevonden in geïmporteerde verse culinaire kruiden uit Zuidoost Azië **(hoofdstuk 4b)**.

Ondanks de sterke reductie in het gebruik van (fluoro)chinolonen bij landbouwhuisdieren in Nederland vanaf 2007 is resistentie tegen fluoro)chinolonen nog steeds hoog in commensale *E. coli* en in *Campylobacter* in die soorten landbouwhuisdieren waarbij in het verleden de meeste (fluoro)chinolonen zijn gebruikt. Deze resistentie wordt, zeer waarschijnlijk, voornamelijk veroorzaakt door chromosomale mutaties in the QRDR van topoisomerase genen. De prevalentie van PMQR (die alleen zorgt voor verminderde gevoeligheid voor fluorochinolonen) in landbouwhuisdieren is laag, maar vertoont recentelijk een lichte stijging. Het feit dat PMQR de ontwikkeling van (fluoro)chinolonen resistentie kan faciliteren en de mogelijkheden tot verspreiding van resistentie vergroot, maakt het belangrijk om het screenen op het voorkomen van PMQR in te passen in het monitoringsprogramma, zodat het lange termijn effect van de reductie van het (fluoro) chinolonen gebruik in landbouwhuisdieren zo goed mogelijk kan worden vastgesteld.

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About the author

Kornelis Tjipke (Kees) Veldman was born on 3 September 1965, in Twijzel, The Netherlands. In 1981, he finished lower general secondary education (MAVO) in Kollum, in 1984 higher general secondary education (HAVO) in Buitenpost and in 1989 Vocational Education (MLO) in the field of veterinary microbiology in Leeuwarden.

In September 1989 Kees started working at a laboratory of the National Inspection Service for livestock and meat (RVV) in Leeuwarden. From November 1989 till October 1990, he fulfilled his military conscription in Hilversum and Ermelo as a medical soldier. Afterwards, he returned to the RVV in Leeuwarden (October – December 1990). In January 1991, Kees started working at Central Veterinary Institute (CDI) in Lelystad as a technician at the Department of Control and Standardization. From 1994 up till now Kees works as senior technician at the Department of Bacteriology and TSEs at the laboratory of Antimicrobial Resistance under supervision of Prof. dr. Dik Mevius and is responsible for the daily routine concerning the surveillance of antimicrobial resistance in bacteria from animals. During this period Kees followed several courses at the University of Applied Sciences including Immunology, Medical Microbiology and Molecular Biology.

Kees started his PhD study in 2009 after receiving permission to admit a doctoral study from the Board for the Conferral of Doctoral Degrees (at the end of 2008). In this period he combined his daily job as senior technician with his PhD study on quinolone resistance in Enterobacteriaceae. He will continue his work at Central Veterinary Institute part of Wageningen UR at the national reference laboratory on antimicrobial resistance in animals.

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