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Salmonella enterica

MECHANISMS OF FLUOROQUINOLONE AND MACROLIDE RESISTANCE

by

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ABSTRACT

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***Salmonella enterica* – Mechanisms of fluoroquinolone and macrolide resistance**
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Severe *Salmonella* infections are usually treated with fluoroquinolones, like ciprofloxacin. Along with the extensive use of fluoroquinolones both in humans and veterinary medicine, there has been a reduction in the fluoroquinolone susceptibility among the *Salmonella enterica* isolates.

Before 2002, all isolates with reduced fluoroquinolone susceptibility were highly resistant to nalidixic acid, a first generation quinolone which is no longer used in the treatment of infections. In 2003, a new quinolone resistance phenotype of *S. enterica* was detected. These non-classical quinolone resistance phenotype isolates showed reduced susceptibility to ciprofloxacin (MIC ≥0.125 mg/L), but were susceptible or only low-level resistant to nalidixic acid (MIC ≤32 mg/L). This phenomenon has a great significance in fluoroquinolone susceptibility testing, since previously all *Salmonella* strains that were susceptible to nalidixic acid, were considered to be susceptible to ciprofloxacin as well.

In this thesis, the incidence of reduced fluoroquinolone susceptibility among domestic and foreign *S. enterica* isolates collected during 2003–2007 was determined and the epidemiology and resistance mechanisms of the non-classical quinolone resistance phenotype were investigated. Also the *in vitro* activity of azithromycin was determined, with the aim to find an alternative for the fluoroquinolone treatment of salmonellosis.

The results of this study show that reduced fluoroquinolone susceptibility among foreign *S. enterica* isolates decreased significantly during the study period. The decrease was the most prevalent in Southeast Asia. The non-classical quinolone resistance phenotype was shown to be plasmid-mediated and all isolates belonging to this phenotype were *qnr*-positive. No other plasmid-mediated quinolone resistance (PMQR) determinants were found and the isolates were also negative for all fluoroquinolone resistance causing mutations in the QRDR of the chromosomal *gyrA*, *gyrB* and *parE* genes. A transformation assay revealed that the *qnr* plasmids were transferable and the novel phenotype reproducible *in vitro*.

These results indicate that although the *qnr* phenotype in *S. enterica* is concentrated so far, in Southeast Asia, the novel phenotype is readily transferable and therefore treatment problems will occur also in the other parts of the world. The *qnr* phenotype isolates might be hard to identify with conventional screening tests. Thus, laboratories should examine both ciprofloxacin and nalidixic acid susceptibility. Finally, azithromycin proved to be *in vitro* active against *S. enterica* isolates including those with reduced fluoroquinolone susceptibility, suggesting that it may be a useful alternative for the treatment of multi-resistant *Salmonella* isolates.

Keywords: *Salmonella enterica*, fluoroquinolone, azithromycin, resistance, *qnr*

TIIVISTELMÄ

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Vakavia salmonellainfektioita on pitkään hoidettu fluorokinoloniantibiooteilla, kuten siprofloksasiinilla. Fluorokinolonien runsas käyttö niin ihmisillä kuin eläimilläkin on kuitenkin johtanut fluorokinoloniresistenttien salmonellakantojen lisääntymiseen.

Vuoteen 2002 asti kaikki matalan tason fluorokinoloniresistenssiä ilmentävät salmonellakannat olivat resistenttejä nalidiksiinihapolle, joka on vanha ensimmäisen polven kinoloniantibiootti jota ei enää käytetä infektioiden hoidossa. Vuonna 2003 havaitsimme aivan uudentyyppisen resistenssifenotyypin salmonelloissa. Kaikki uuden fenotyypin kannat osoittivat matalaa fluorokinoloniresistenssiä (MIC ≥ 0.125 mg/L), mutta useat kannat olivat yllättäen aikaisempaa herkempiä nalidiksiinihapolle (MIC ≤ 32 mg/L). Ilmiöllä on suuri merkitys salmonellan antibioottiherkkyyksien määrittämisessä, sillä jos kanta on ollut nalidiksiinihapolle herkkä, sitä on pidetty herkkänä myös fluorokinoloneille.

Väitöskirjatyössä määritettiin vuosina 2003–2007 Suomessa kerättyjen kotimaisten ja ulkomaalaisten *S. enterica* -kantojen fluorokinoloniresistenssiä sekä tutkittiin uuden salmonellafenotyypin epidemiologiaa ja resistenssimekanismeja. Lisäksi tutkittiin salmonellan hoidossa mahdollisesti käyttökelpoisen makrolidiantibioottijohdannaisen, atsitromysiinin tehoa salmonelloihin ja erityisesti matalaa fluorokinoloniresistenssiä ilmentäviin kantoihin.

Tutkimuksessa havaittiin, että matalaa fluorokinoloniresistenssiä osoittavien salmonellakantojen määrä vähenee. Lasku oli voimakkainta Kaakkois-Aasiasta tuoduissa kannoissa. Uusi resistenssifenotyyppi on plasmidivälitteinen ja *qnr*-geenit olivat ainoa plasmidivälitteinen kinoloniresistenssimekanismi, joka kannoista löydettiin. Myöskään kromosomaalisten *gyrA*, *gyrB* ja *parE* -geenien QRDR-alueelta ei löydetty fluorokinoloniresistenssiä aiheuttavia mutaatioita. Transformaatiolla osoitettiin *qnr*-plasmidien olevan siirtyviä ja uusi resistenssifenotyyppi saatiin ilmennettyä myös herkässä vastaanottajakannassa.

Nämä tulokset osoittavat, että vaikka *S. enterican qnr*-fenotyyppi on toistaiseksi levinnyt pääasiassa Kaakkois-Aasiaan, se siirtyy helposti bakteerista toiseen ja tulee todennäköisesti aiheuttamaan hoito-ongelmia myös muualla maailmassa. Uudentyyppinen *qnr*-fenotyyppi voi olla vaikea havaita perinteisellä herkkyysmäärityksellä. Siksi laboratorioissa tulisi aina määrittää sekä siprofloksasiini- että nalidiksiinihappoherkkyydet. Atsitromysiinin osoitettiin olevan herkkyys-määritysten mukaan tehokas salmonelloja kohtaan mukaanlukien matala-asteista fluorokinoloniresistenssiä ilmentävät bakteerikannat.

TABLE OF CONTENTS

ABBREVIATIONS

23S	Major component of the prokaryotic subunit 50S
50S	Larger subunit of prokaryotic 70S ribosome
aac(6')-Ib-cr	Gene encoding ciprofloxacin modifying enzyme AAC(6')-Ib-cr
AAC(6')-Ib-cr	Aminoglycoside acetyltransferase enzyme
AcrAB	Multridrug transporter in the efflux system
Ala	Alanine (A)
Arg	Arginine (R)
Asn	Asparagine (N)
Asp	Aspartic acid (D)
CCCP	Carbonyl cyanide *m*-chlorophenyl-hydrazone
CLSI	Clinical Laboratory Standards Institute
DHPLC	Denaturing high-performance liquid chromatography
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee for Antimicrobial Susceptibility Testing
Glu	Glutamic acid (E)
Gly	Glysine (G)
gyrA	DNA gyrase gene which encodes the GyrA subunit
GyrA	First subunit of the DNA gyrase enzyme: Primary target for fluoroquinolones in Gram-negative bacteria
gyrB	DNA gyrase gene which encodes the GyrB subunit
GyrB	Second subunit of the DNA gyrase enzyme
L4	50S ribosomal subunit of 23S ribosomal protein
L22	50S ribosomal subunit of 23S ribosomal protein
Leu	Leusine (L)
Lys	Lysine (K)
MDR	Multidrug resistant i.e. resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole
MIC	Minimal inhibitory concentration
MIC_{50}	Minimal inhibitory concentration which is required to inhibit the growth of 50% of organisms
MIC_{90}	Minimal inhibitory concentration which is required to inhibit the growth of 90% of organisms

PAβN	Phe-Arg-β-naphtylamide
parC	Topoisomerase IV gene which encodes the ParC subunit
ParC	First subunit of the DNA topoisomerase IV enzyme: Secondary target for fluoroquinolones in Gram-negative bacteria
parE	Topoisomerase IV gene which encodes the ParE subunit
ParE	Second subunit of the DNA topoisomerase IV enzyme
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
Phe	Phenylalanine (F)
PMF	Proton motive force
PMQR	Plasmid-mediated quinolone resistance
qepA	Transferable quinolone efflux pump gene
qnr	Transferable, plasmid-mediated quinolone resistance gene, which protects DNA gyrase from quinolone inhibition
QRDR	Quinolone resistance-determining region
RFLP	Restriction fragment length polymorphism
rlpD	Gene encoding L4 subunit
rlpV	Gene encoding L22 subunit
rRNA	Ribosomal RNA, central component of the ribosome, the protein manufacturing machinery of all living cells
Ser	Serine (S)
SSCP	Single-strand conformation polymorphism
TolC	Outer membrane component of the efflux system
tRNA	Transfer RNA that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation
Trp	Tryptophan (W)
Tyr	Tyrosine (Y)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals (I-IV) and some unpublished data:

I   Hakanen AJ, Lindgren M, Huovinen P, Jalava J, Siitonen A and Kotilainen P. New quinolone resistance phenomenon in *Salmonella enterica*: Nalidixic acid-susceptible isolates with reduced fluoroquinolone susceptibility. J Clin Microbiol 2005;43:5775-5778.

II   Lindgren M, Kotilainen P, Huovinen P, Hurme S, Lukinmaa S, Webber MA, Piddock LJV, Siitonen A and Hakanen AJ. Reduced fluoroquinolone susceptibility in *Salmonella enterica* isolates from travelers, Finland. Emerg Infect Dis 2009;15:809-812.

III   Gunell M (née Lindgren), Webber MA, Kotilainen P, Lilly AJ, Caddick JM, Jalava J, Huovinen P, Siitonen A, Hakanen AJ and Piddock LJV. Mechanism of resistance in nontyphoidal *Salmonella enterica* exhibiting a nonclassical quinolone resistance phenotype. Antimicrob Agents Chemother 2009;53:3832-3836.

IV   Gunell M (née Lindgren), Kotilainen P, Jalava J, Huovinen P, Siitonen A and Hakanen AJ. *In vitro* activity of azithromycin against nontyphoidal *Salmonella enterica*. Submitted, 2009.

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1. INTRODUCTION

Salmonella is one of the most important pathogens of the *Enterobacteriaceae* family. In humans, *Salmonella* causes different kinds of disease entities varying from mild intestinal infections like diarrhea to life-threatening generalized infections like typhoid fever. The generalized infections in humans are commonly caused by *Salmonella enterica* serotype Typhi or Paratyphi. Other serotypes cause mainly intestinal infections and humans may also be healthy carriers of *Salmonella* isolates. *Salmonella* is a common inhabitant of the normal flora in many animals, but it can also infect many species and since *Salmonella* is ubiquitous in animal populations, the human illness is usually linked to food which is derived from animal origin.

The fluoroquinolones are a family of synthetic broad-spectrum antibiotics. The first quinolone, nalidixic acid, was discovered in the 1960s and it was mainly used for the treatment of urinary tract infections, caused by Gram-negative enteric bacteria. Due to the limitations of the efficacy of nalidixic acid, the more effective and broad-spectrum quinolones were developed. These "second generation" quinolones, fluoroquinolones, were discovered in the late 1970s and they came into the market in the mid 1980s. Fluoroquinolones are broad-spectrum antimicrobials and they are widely used for the treatment of invasive and systemic salmonellosis that occurs both in humans and animals. However, excessive use of the fluoroquinolones both in human and in veterinary medicine has led to the emergence of reduced fluoroquinolone susceptibility in *Salmonella* isolates (Hakanen et al. 2001). Moreover, fluoroquinolone resistance is commonly linked to resistance against the extended-spectrum cephalosporins and even ESBLs (Giraud et al. 2006).

In Finland, the antimicrobial susceptibility of *S. enterica* has been monitored since January 1995 by analyzing isolates sent to the Gastrointestinal Infections Unit, National Institute for Health and Welfare (formerly the Enteric Bacteria Laboratory, National Public Health Institute), Helsinki, Finland, which serves as a National Salmonella Reference Centre in our country. Starting from January each year, the first 100 domestic and first 100 foreign *S. enterica* isolates, collected from Finnish travelers returning from abroad, were sent to the Antimicrobial Resistance Unit, National Institute for Health and Welfare, Turku, for antimicrobial susceptibility testing. Between 1995 and 2002, all *S. enterica* isolates with reduced ciprofloxacin susceptibility (MIC ≥ 0.125 mg/L) were uniformly highly resistant to nalidixic acid (MIC ≥ 128 mg/L).

In this work, reduced fluoroquinolone susceptibility among domestic *S. enterica* isolates and isolates collected from Finnish travelers returning from abroad between 2003 and 2008 were studied. The main interest was focused on the novel quinolone resistance phenomenon in *S. enterica*, which was first discovered in 2003 among isolates from Southeast Asia. These isolates showed reduced susceptibility to ciprofloxacin but were either susceptible or only low-level resistant (MIC ≤ 32 mg/L) to nalidixic acid. Here, the epidemiology of this nonclassical quinolone resistance phenotype was determined and the molecular mechanisms behind this type of resistance investigated. The *in vitro* activity of alternative antimicrobials against the isolates with reduced ciprofloxacin susceptibility was also examined.

2. REVIEW OF THE LITERATURE

2.1. The genus *Salmonella*

The genus *Salmonella* is composed of motile bacteria that are commonly found in the intestines of humans and animals, including birds and reptiles. Since *Salmonella* infects both humans and animals, bacteria are generally transmitted to humans through the consumption of contaminated food of animal origin, mainly meat, poultry, eggs and milk (WHO 2007). *Salmonella* is a widespread illness-causing agent and the intestinal infections like salmonellosis are common in people of all age. Salmonellosis is usually caused by *Salmonella* serovar Enteritidis and symptoms typically include fever, diarrhea and abdominal cramps. The other prevalent *Salmonella* infection is typhoid fever, caused by *Salmonella* Typhi, which is highly endemic in Indian subcontinent and in other developing countries. *S.* Typhi can invade into the bloodstream and cause life-threatening infections, like bacteremia. These severe generalized infections need to be treated with antimicrobials (CDC 2009; Pegues et al. 2005).

Despite the great progress in medicine and food production, *Salmonella* still remains a very remarkable problem for public health, especially in the developing countries. Since the beginning of the 1990s, the multi-drug resistance (MDR) of *S. enterica* has emerged and during the last decade, the increase in the reduced fluoroquinolone susceptibility among *S. enterica* has become an even more important problem (Hakanen et al. 2001; Hakanen et al. 2006). The emergence of MDR *Salmonella* strains with possible co-resistance to fluoroquinolones and extended-spectrum cephalosporins is a serious threat, and it can cause severe limitations for the effective treatment of human *Salmonella* infections (WHO 2005). Although *Salmonella* infections are more common in the developing countries, *Salmonella* infections are detected also in the Western countries. In Finland, for example, annually over 3000 Finnish citizens acquire *Salmonella* infection, although infections are mainly acquired abroad (80–90% of the cases). Thus, the increasing antimicrobial resistance among foreign *Salmonella* isolates is a threat also in Finland (THL 2009).

2.1.1. Classification and taxonomy

Salmonella was named after the veterinary pathologist Daniel Salmon, although it actually was Salmon's research assistant Theobald Smith, who isolated the first strain of *Salmonella enterica* (*cholerasuis)* from the porcine intestine in 1885. Before the 19th century, typhus and typhoid fever were confused and although various clinical differentiation schemes were proposed, these syndromes were not reliably distinguished. In 1850, William Jenner wondered, whether typhus and typhoid were different diseases and he was the first to distinguish the common pathologic characteristics of typhoid fever: The enlargement of Peyer's patches and mesenteric lymph nodes. In 1869, Wilson invented the name *enteric fever* and gave the anatomic site for the infection. However, even today 'typhoid fever' is a more commonly used term than 'enteric fever'. In 1873, Budd showed that typhoid fever was transmitted via

food and water, and in 1880, Eberth recognized that the typhoid fever was caused by the *Salmonella* bacterium. In 1884, the first typhoid bacillus was isolated from the spleens of infected patients by Gaffkey, and in 1896 Pfeiffer and Kalle invented the first typhoid vaccine. In that same year, Widal and his associates demonstrated agglutinin phenomenon in organisms isolated from the sera of the typhoid patients. This agglutination test, which is based on antigenic classification, is even today a standard method for serotyping *Salmonella* (Pegues et al. 2005; Selander et al. 1996).

The *Salmonella* genera belong to the *Enterobacteriaceae* family. The primary basis for the classification and identification of these bacteria has been a serological scheme, invented by White and refined by Kauffmann and others. Based on the serological identification of O (somatic) and H (flagellar) antigens, Kauffmann concluded one serotype-one species concept, which was the reason why many serotypes were originally named by italicized Latin binomials (e.g. *Salmonella typhimurium*) (Brenner et al. 2000). In 1973, Crosa et al. demonstrated with a DNA-DNA hybridization method that all *Salmonella* serovars are in a close relationship. After having this genetic evidence, it was approved that all salmonellas belonged to one single species, *S. enterica* former *S. choleraesuis* (Crosa et al. 1973). The only exception was *Salmonella bongori*, which was proved to be a distinct species based on DNA-DNA hybridization (Selander et al. 1996).

The nomenclature used to describe the genus *Salmonella* has been problematic for many years: In 1952, Edwards and Kauffmann proposed that *S. enterica* would be a proper species name, since that name was not shared by any of the serotypes, but it was until 1986, when it was generally recommended that the type species for *Salmonella* should be changed to *S. enterica*. In 1987, Le Minor and Popoff made a formal proposal about this nomenclature issue to the Judicial Commission of the International Committee of Systematic Bacteriology, but it was denied. It was thought that not enough attention was paid on the status of *Salmonella* serotype Typhi. There was a concern that if *Salmonella* serotype Typhi would be referred to as *S. enterica* subspecies *enterica* serotype Typhi, it might be missed and overlooked by physicians (Brenner et al. 2000).

Nowadays, two species of the genus *Salmonella* are accepted. *Salmonella bongori* (formerly subspecies V) and *Salmonella enterica*, which is composed of six subspecies: subsp. *enterica* (designated subspecies I), subsp. *salamae* (II), subsp. *arizonae* (IIIa), subsp. *diarizonae* (IIIb), subsp. *houtenae* (IV) and subsp. *indica* (VI). Currently there are nearly 2500 distinct serotypes and members of these seven *Salmonella* subgroups can be serotyped into one distinct serovar. Serotype is used for the species designation and therefore serotype is usually named after the city in which it was defined (e.g. *S.* Kentucky). Because the type species name has not been officially approved, it is widely accepted to use for example the name *Salmonella* serotype Typhimurium or only *Salmonella* Typhimurium instead of *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Brenner et al. 2000; Pegues et al. 2005; Selander et al. 1996).

2.1.2. Bacteriological characteristics and genome structure of *S. enterica*

Salmonella are Gram-negative, non-spore forming, facultative anaerobic bacilli, 2 to 3 by 0.4 to 0.6 μm in size. *Salmonella* produce acid on glucose fermentation, reduce nitrates and do not produce cytochrome oxidase. Most of the salmonellas are motile and do not ferment lactose. Different serotypes of *Salmonella* can be distinguished by the differential metabolism of sugars; *S.* Typhi is the only serotype that does not produce gas on sugar fermentation tests (Pegues et al. 2005). To detect *Salmonella* from the stool sample, the clinical laboratories use low-selective media like MacConkey and deoxycholate agar and intermediate-selective media such as Salmonella-Shigella or Hektoen agar. Nowadays selective chromogenic media like CHROMagar is used for the primary isolation and preliminary identification of *Salmonella* from clinical specimens (Pegues et al. 2005). For the isolation of *S.* Typhi strains and the detection of lactose ferment *Salmonella* strains, the more selective media, bismuth sulfite agar which contains hydrogen sulfite but not lactose, is used. *Salmonella* isolates may be identified also by a combination of different biochemical tests or by the slide latex agglutination test. In the latex agglutination test, antisera for *Salmonella* O antigens can be divided into serogroups A, B, C_1, C_2, D and E. Serotyping of *Salmonella* is based on the bacterium reaction against many different antibodies, and these reactions determine which antigens are present. Based on the detection of antigens, a single species can be divided into hundreds of different serotypes. *Salmonella* spp. are serotyped according to their O (somatic), Vi (capsular) and H (flagellar) antigens, and subtyping is performed with phage typing. In addition, strains can be differentiated with phenotyping to be able to distinguish outbreak-associated strains (Bopp et al. 1999; Pegues et al. 2005).

2.1.3. *Salmonella* infections

Salmonella infects both humans and animals and human illness is usually linked to foods of animal origin (Bopp et al. 1999). *Salmonella* infections are usually obtained from food or water which is contaminated with excretions from sick or infected people or animals. *Salmonella* infections are categorized based on their different disease symptoms: gastroenteritis, enteric fever, bacteremia, vascular infections, localized infections and a chronic carrier state. Compared with the food-borne transmission, the amount of bacteria ingested is usually smaller in water-borne transmission and therefore the incubation time is longer and infection rate lower. In addition, improper hand hygiene can lead to human to human transmission (Pegues et al. 2005; Selander et al. 1996).

2.1.3.1. Human infections

Strains of *Salmonella* are categorized as typhoidal and nontyphoidal, corresponding to the disease syndrome with which they are associated.

2.1.3.1.1. Typhoidal salmonellas

Humans are the only reservoir for *Salmonella* serotype Typhi and Paratyphi, and therefore disease can be acquired only through close contact with a person who has typhoid fever, or who is a chronic, healthy carrier of *S.* Typhi (Nataro et al. 2007). Typhoid fever, caused by *Salmonella* serotype Typhi, is a serious bloodstream infection, which is endemic in many of the developing countries with poor sanitation and faecal contamination of food and water (Guerrant and Steiner 2005; Pegues et al. 2005). Drinking untreated water or consumption of raw vegetables and unwashed fruits are the main risk factors for typhoid fever. Also dairy products are a common source of *S.* Typhi infection (Sharma et al. 2009). Worldwide approximately 22 million cases of typhoid fever are detected each year, and the incidence of typhoid fever is extremely high in Asia (Crump et al. 2004). Typhoid fever has a low infectious dose ($<10^3$) and a long and highly variable incubation time, ranging from one to six weeks. Typhoid fever is commonly associated with high fever, profuse sweating, a headache and abdominal pain. In addition, both diarrhea and constipation are possible although diarrhea may be more common (Guerrant and Steiner 2005; Nataro et al. 2007). In endemic areas, the incidence of *S.* Typhi infection is highest among children over one year old, probably due to the lack of acquired immunity. Although people of all age can suffer from typhoid fever, the disease symptoms are more severe for the children under one year of age and for patients with immunodeficiency, and with these patients, the disease is often associated with a higher rate of complications (Bopp et al. 1999; Pegues et al. 2005). However, typhoid fever in young children is relatively mild and the only symptom is usually a non-specific fever (Nataro et al. 2007).

Salmonella serotypes Paratyphi A, Paratyphi B and Paratyphi C cause a similar, but a less severe syndrome than typhoid fever. Serotype Paratyphi B is a diverse serotype that is associated with both paratyphoid fever and gastroenteritis. When enteric fever is caused by *S.* Typhi, the disease is called typhoid fever, whereas enteric fever caused by *S.* Paratyphi is called paratyphoid fever. Although enteric fever is classically described as an acute illness with fever and abdominal tenderness, the symptoms can be non-specific and occur very suddenly (Nataro et al. 2007; Pegues et al. 2005).

Since typhoid fever is transmitted by water or food contaminated with *S.* Typhi, the most efficient way of preventing typhoid fever in the developing countries is to install adequate water and sanitation systems and improve living standards. In addition, vaccination has long been another efficient way to prevent typhoid fever. Immunity to *S.* Typhi requires both cell-mediated and humoral immune responses, which are achieved by vaccination. The first inactivated whole-cell typhoid vaccine was introduced in 1896. Nowadays there are two typhoid vaccines commercially available: Ty21a (an attenuated strain of *S.* Typhi administered orally) and Vi (based on purified capsular polysaccharide *S.* Typhi Vi antigen, given intramuscularly), which both are safe and effective in preventing typhoid fever (Fraser et al. 2007; Pegues et al. 2005).

2.1.3.1.2. Nontyphoidal salmonellas

Strains of nontyphoidal *Salmonella* cause a large number of food-borne outbreaks and sporadic cases of gastroenteritis in developed countries. *S. enterica* subspecies *enterica*, to which all nontyphoidal and problem-causing serovars belong is one of the most important food-borne pathogens and is transmissible from food animals to humans through contaminated meat, eggs and milk products (Akiba et al. 2007; Pegues et al. 2005). Gastrointestinal infection is the most usual *Salmonella* infection, with the common symptoms of diarrhea, fever and abdominal cramps which usually start 12 to 72 hours after infection and last normally three to seven days, or even longer. Although *Salmonella* infections cause mostly mild diseases, life-threatening infections like bacteremia may also occur, especially with elderly and immunocompromised patients (Weill et al. 2006). In developing countries, nontyphoidal *Salmonella* causes a small but significant proportion of diarrhea in travelers and in young children (Pegues et al. 2005).

The nontyphoidal salmonellosis is commonly associated with food products. Food from animal origin like meat, poultry, eggs and dairy products can easily become contaminated with *Salmonella*. In the 1980s, for example, the major source of foodborne diseases in the United States and some other countries was *S*. Enteritidis, which was commonly found in eggshells. Outbreaks of *S*. Enteritidis infection have been associated with eating uncooked or lightly cooked eggs, egg-containing food products and inadequately cooked poultry. Changes in food consumption and the rapid growth of international trade in agricultural food products have facilitated the dissemination of new *Salmonella* serotypes. These novel serotypes are commonly associated with fresh fruits and vegetables, since the surface of fruits and vegetables is easily infected with *Salmonella* due to watering system that can be contaminated with human or animal faeces. In summary, eating raw or inadequately cooked food or food which is cross-contaminated with these food products, can easily lead to gastrointestinal infection (Bopp et al. 1999; Pegues et al. 2005).

2.1.3.2. Animal infections

Salmonella spp. are zoonotic pathogens and are therefore widely dispersed in nature. *Salmonella* is most commonly found in the gastrointestinal tracts of domesticated and wild animals, including reptiles, birds and insects. Due to this zoonotic feature of salmonellas, *Salmonella* infections in humans are usually derived from food of animal origin and although *Salmonella* can sustain in animal's intestines without causing any infection to that animal, it still might infect humans. Like *Salmonella* serotype Dublin which is found in cattle and *S*. Arizonae, found in reptiles, which are adapted to an animal species but still infect humans in some cases (Pegues et al. 2005).

Salmonella is an important pathogen in food poisoning in humans, although it has a much lower prevalence in farm animals, compared with pathogenic *E. coli* and *Campylobacter* (de Jong et al. 2009). *Salmonella* is commonly found in farm animals like cattle, pigs and chickens, and as shown in several studies, the prevalence of different *Salmonella* serotypes varies between these animals. *Salmonella* Enteritidis

and *S*. Typhimurium DT104, which is a well-known zoonotic pathogen, have a high prevalence in human infections in the UK, Europe and the USA and these serovars are also common in eggs and broiler poultry flocks. Therefore *Salmonella* infection in humans is most often got by eating chicken meat which is infected by *S*. Typhimurium DT104, or *S*. Enteritidis (Pegues et al. 2005; Piddock 2002). There are several serotypes commonly found in farm animals. Fricke et al. showed that although *Salmonella* Kentucky is relatively uncommon in human infections in the USA, it is widespread in poultry meat. *S*. Kentucky was commonly found in animal samples and it was shown to be the most common serotype isolated from chickens (48.8%) and chicken meat (38.8%), but it was also present in turkey (2.6%) and cattle (3.6%) (Fricke et al. 2009). Threlfall et al. have shown that in the UK, Europe and the USA, *S*. Enteritidis, *S*. Virchow and *S*. Hadar are normally associated with poultry and poultry products, of which *S*. Enteritidis and *S*. Virchow are associated with chickens and chicken products and *S*. Hadar with turkey products. In contrast, *S*. Typhimurium has a much wider host range, being most commonly associated with cattle and pigs, but also poultry and occasionally sheep (Threlfall 2002). The study of de Jong et al. showed that *Salmonella* serotype variation within farm animals is wide in Europe: *S*. Typhimurium was the main serotype (24%) found in pigs, the other serotypes found in pigs were *S*. Derby (23%), *S*. Rissen (9%) and *S*. Brandenburg (3%), whereas serotypes found only in chickens were *S*. Enteritidis (3%) and *S*. Indiana (12%) (de Jong et al. 2009). *Salmonella* can also infect horses and cause extensive outbreaks in veterinary hospitals. The most common serovar that causes *Salmonella* infection in horses is *S*. Typhimurium, but also *S*. Anatum and *S*. Infantis infections are detected (Kallio et al. 2004).

Salmonella infections in farm and production animals in Finland are very rare due to the National *Salmonella* surveillance program, which started in 1995. This program relates to cattle, pigs, chicken and chicken meat and eggs. The aim of this program was to prevent *Salmonella* transmission from farm animals to humans, and to keep the overall *Salmonella* prevalence in production animals and food products below 1%. This goal was achieved in 2008 (EVIRA 2009a). In Finland, *S*. Infantis has been the most prevalent *Salmonella* serovar in farm animals for almost four decades. *S*. Infantis is particularly common in poultry flocks, but it has been detected also in cattle (Lindqvist and Pelkonen 2007).

Antimicrobials like fluoroquinolones have various uses in farm animals and poultry production. While fluoroquinolone usage in veterinary medicine has increased, reduced fluoroquinolone susceptibility has emerged in zoonotic pathogens like *Salmonella* (Helin-Soilevaara et al. 2004). This is of concern, since strains that develop antimicrobial resistance in animals can then infect humans and thus resistance spreads very rapidly. Antimicrobial resistant *Salmonella* strains are detected the most abundantly in pigs however, fluoroquinolone resistant *Salmonella* strains are also found in chickens (de Jong et al. 2009; Randall et al. 2005).

Farm animals are the most potential source of salmonellosis. However, *Salmonella* infection is nowadays more often associated with pet birds, pet rodents, cats and dogs. Reptiles and other cold blooded animals, i.e. exotic pets, can act as reservoirs of

Salmonella and while more people have exotic pets, the risk of having salmonellosis is increasing, since salmonellosis is more commonly associated with reptiles and other exotic pets. For example, *S*. Marina and *S*. Chameleon infections are commonly associated with exposure to iguanas, especially in infants, and exposure to snakes has been linked to *S*. Arizonae infection (Pegues et al. 2005). In the USA, approximately 1.4 million cases of *Salmonella* infection occur each year and 74,000 cases are linked to exposure to reptiles and amphibians. Some cases of *Salmonella* infection in association with reptiles or other exotic pets have been described also in Europe. In Finland for example, between 2005 and 2008 three cases of salmonellosis were reported associating with a pet snake and one family outbreak with association with a pet turtle was detected (Bertrand et al. 2008).

2.1.3.3.   Epidemiology of infections

Salmonella infections are common all over the world. However, since infections are mostly associated with food or water which is contaminated with faeces, *Salmonella* infections are more common in the developing countries where water and sewer systems are inadequate. In humans, *S*. Enteritidis and *S*. Typhimurium are the most common serovars to cause salmonellosis. Since the 1980s there has been a dramatic increase in the reported findings of *S*. Enteritidis in Europe and worldwide. The incidence of different phage types of *S*. Enteritidis varies in different geographical areas: Phage type 1 (PT1) is common in Russia and Baltic countries, PT4 is common in Western European countries and PT8 is frequently found in the USA (Lukinmaa et al. 1999). Survey from 191 WHO Member States in 1995 revealed that three *Salmonella* serotypes were dominating: *S*. Enteritidis, *S*. Typhimurium and *S*. Typhi accounted for 76.1% of all isolates reported in 1995. *S*. Enteritidis was the most frequently isolated serotype in 35 countries, *S*. Typhi in 12 countries and *S*. Typhimurium in eight countries (Herikstad et al. 2002). Whereas at the beginning of 2000, the most important *Salmonella* serotypes in the UK, Europe and the USA were *S*. Enteritidis, *S*. Typhimurium, *S*. Virchow and *S*. Hadar, and the main source of infection was contaminated food (Threlfall 2002). In the USA, foodborne infections cause roughly 76 million cases of illness each year. Salmonellosis caused by nontyphoidal *Salmonella* is detected in 1.4 million cases, of which 95% are acquired from foodborne transmission. Over 800 cases of *S*. Typhi are discovered and 80% of these are derived from food associated infections. Annually, salmonellosis causes 600 deaths which is 30% of all deaths related to foodborne infections in the USA (Mead et al. 1999).

The level of endemic salmonellosis is low in all Nordic countries. In Finland, for instance, the annual incidence of salmonellosis is 40–50 cases per 100,000 inhabitants and totally 2300–3000 cases of salmonellosis are reported to the National Salmonella Reference Centre (Bertrand et al. 2008) (Figure 1). The prevalence of salmonellosis is low in Finland, and both typhoid and paratyphoid fever are extremely rare (Figure 2). Salmonellosis in Norway, Sweden and Finland is most often acquired abroad and it is most commonly caused by *S*. Enteritidis. In Finland, domestic *Salmonella* isolates cause only 8% of all food-borne infections and annually there are only 1–8 *Salmonella*

epidemics, since 80–90% of the *Salmonella* infections are associated with travel abroad. The predominant serotypes in the Nordic countries are *S*. Enteritidis and *S*. Typhimurium. *S*. Enteritidis is the most common imported serotype, whereas *S*. Typhimurium is usually of domestic origin. *S*. Typhimurium is the most prevalent domestic serotype in Finland (Bruun et al. 2009; EVIRA 2009b; Lukinmaa et al. 1999).
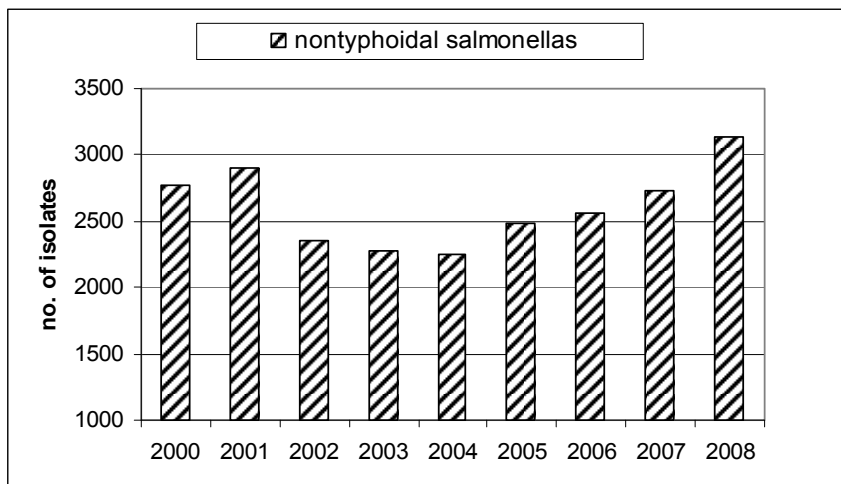
Figure 1. Prevalence of salmonellosis in Finland between 2000 and 2008. Source: Infectious Diseases Register, National Institute for Health and Welfare (http://www3.ktl.fi/).

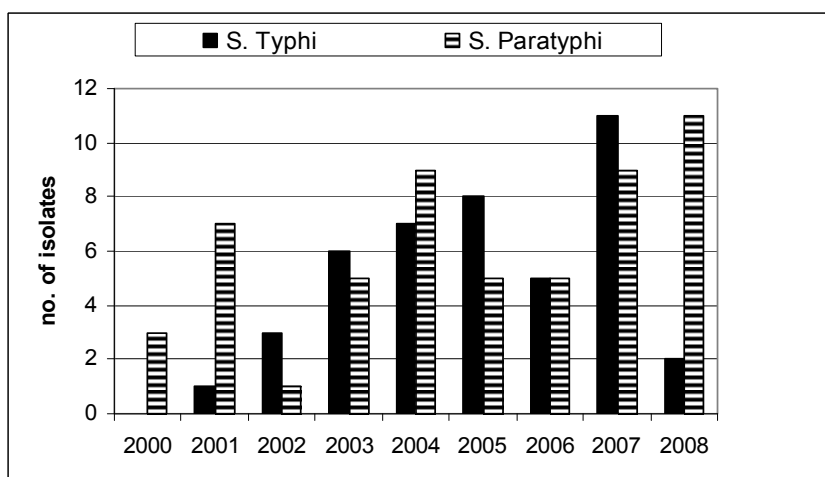**Figure 2.** Prevalence of *S*. Typhi and *S*. Paratyphi in Finland between 2000–2008. Source: Infectious Diseases Register, National Institute for Health and Welfare (http://www3.ktl.fi/)**.**

2.1.3.4. Antimicrobial treatment of *Salmonella* infections

Salmonella gastroenteritis does not usually require antibiotic treatment. However, especially in developing countries, *Salmonella* infections are often associated with a

high incidence of invasive illness which causes high mortality and in these cases, antibiotic treatment is essential (Threlfall 2002). The gastroenteritis which is commonly caused by nontyphoidal *S*. Typhimurium and *S*. Enteritidis, the predominant *Salmonella* serovars in many developed countries (Weill et al. 2006), is common among people of all ages, although the incidence is the highest among infants. Antimicrobial treatment is not required for mild *Salmonella* gastroenteritis and it is usually treated only with fluids and electrolytes. However, invasive salmonellosis, bacteremia, immunocompromised patients, infants, elderly people and patients with underlying disease should be treated with antimicrobials, mainly oral quinolones (Maraki et al. 2006; Weill et al. 2006). Although fluoroquinolones are the primary treatment for salmonellosis, fluoroquinolones are not recommended for children under 10 years of age and pregnant women due to serious side effects. Therefore, these patients should be treated with extended-spectrum cephalosporins like ceftriaxone. However, children who are having typhoid fever or MDR nontyphoidal *Salmonella* are most efficiently treated with fluoroquinolones (Cui et al. 2009; Hooper 2005; Pegues et al. 2005).

After having the gastroenteritis, the patient can remain a carrier of nontyphoidal *Salmonella* up to four to five weeks after infection. The length of the carrier state varies by *Salmonella* serotype and some studies have shown that antibiotic treatment, other than fluoroquinolones, may increase the duration of carriage (Pegues et al. 2005). Nowadays, fluoroquinolones are the primary treatment for the protracted or severe diarrhea, caused by *S. enterica*. Eradication of *Salmonella* from the long-time carriers has always been problematic, however, it has been shown that prolonged fluoroquinolone treatment can eradicate nontyphoidal *Salmonella* without development of resistant strains (Voltersvik et al. 2000). In Finland, it is agreed that two to three weeks of fluoroquinolone treatment efficiently end the carriage of *Salmonella* (over 90% of the cases) (Vaara and Huovinen 1997).

Although typhoid fever in most cases is not fatal, it is estimated to cause 600,000 deaths per year (Pegues et al. 2005), and the use of antimicrobials for the treatment of typhoid fever has reduced the case-fatality rate to 1% (Crump et al. 2004). In contrast to nontyphoidal *Salmonella* infection, typhoid fever caused by *S*. Typhi is always treated with antibiotics. The standard treatment for typhoid fever has been chloramphenicol since its introduction into the market in 1948. However, since the emergence of plasmid-mediated resistance to chloramphenicol in the 1970s, the use of amoxicillin and trimethoprim-sulfamethoxazole for the treatment of typhoid fever increased and replaced chloramphenicol (Pegues et al. 2005). The extensive use of these first-line antibiotics led, however, to the emergence of MDR *S*. Typhi isolates which were resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, in the late 1980s especially in Southeast Asia, Africa and India (Rowe et al. 1997). Thereafter, ciprofloxacin has become the primary treatment for enteric fever (Chau et al. 2007; Dutta et al. 2008; Threlfall et al. 1992). Unfortunately, nalidixic acid resistant strains of *S*. Typhi, in association with reduced fluoroquinolone susceptibility have increased in many developing countries, especially in India and Southeast Asia, which has jeopardized also the fluoroquinolone treatment of typhoid

fever (Dutta et al. 2008; Threlfall et al. 1999). Due to the increased resistance to quinolones, typhoid fever can be alternatively treated with extended-spectrum cephalosporins (Threlfall and Ward 2001).

Salmonella is commonly found in farm animals like pigs, cattle and chickens, and infections in animals are also treated with fluoroquinolones. Antibiotics are broadly used in veterinary medicine to treat infections, as prophylactics and for growth promotion. This wide use of antibiotics can cause the emergence of quinolone-resistant strains both in healthy and sick animals and therefore antimicrobial resistance in nontyphoidal *Salmonella* is very common (Threlfall 2002). There is also clear evidence that increase in the consumption of antibiotics in animals correlates with the increasing number of resistant bacterial isolates (Piddock 1996, 1998). The European Union (EU) banned the use of several antibiotics as growth promoters in 1999 as a precaution, since both consumers and scientists shared the same concern that resistant isolates which are selected from animals are easily transferred to humans and therefore jeopardize their health. However, since these banned antimicrobials had an important role as prophylactic agents, the withdrawal of these agents was accompanied with higher infection and mortality rate in animals (Casewell et al. 2003). Thus, nowadays antimicrobials like fluoroquinolones are still accepted for therapeutic use in animals and therefore the resistance problem continues.

2.2. Antimicrobial resistance

Antimicrobial resistance is a result of interactions between antimicrobial agents, micro-organisms and the environments where they are together. Antimicrobial resistance can be environmentally mediated, meaning that some physical or chemical characteristics of the environment directly alter the antimicrobial agent or change the normal response of the microbe to an antimicrobial agent. Antimicrobial resistance can also be microbially mediated, meaning that resistance is due to the genetically encoded traits of the micro-organism. Microbial-mediated resistance can be divided into intrinsic and acquired resistance. If a micro-organism is intrinsically resistant, it means that the vast majority of strains that belong to that certain bacterial group, genus or species are resistant to certain antibiotics. Since the resistance is naturally occurring, it is consistently inherited and therefore predictable. Acquired resistance means that resistance is a result from chromosomal mutations or acquisitions of foreign DNA into genome, i.e. changes in the normal genetics of bacteria. Acquired resistance is normally associated with only some strains of certain bacteria and therefore it is usually unpredictable (Quintiliani et al. 1999).

Antimicrobial resistance can evolve in four different environments. The primary place for developing resistance is human and animal microbiota, in which more than 500 bacterial species can develop resistance. The second most common places for evolving resistance are hospitals, farms and long-term care facilities, where a number of individuals are in the same place and resistant bacteria transfer easily from one individual to another. Wastewater or another type of biological residues derived from

hospitals and other similar places with high selection pressure are the third most common sites of developing resistance. Finally, wastewater ends up in the soil, where resistant bacteria can mix with envinronmental organisms and therefore antimicrobial resistance is spreading (Baquero et al. 2008).

Antimicrobial susceptibility is a very important factor in the treatment of bacterial infections. If a certain bacterium is fully resistant to some antibiotic, that antibiotic cannot be used in any of the infections which are caused by those bacteria. When bacteria are susceptible or only low-level resistant, it is important to carefully concider which antimicrobial is used, what is the proper dose, in what way the antibiotic should be given and how often. These features are investigated with pharmacokinetics and pharmacodynamics (Pk/Pd). Pharmacokinetics examines how an antimicrobial agent is distributed in organs, how it is absorbed, how it is bound to the serum proteins and in which way it is metabolized and eliminated from the body. Pharmacodynamics instead, examines the interaction between the antimicrobial in the infection site and the resulting antimicrobial effect. Antimicrobials can be divided into two different categories based on their mechanism of bacterial killing: time-dependent and concentration-dependent acitivity. The effect of time-dependent antibiotics mainly depends on the length of time that the antibiotic is in contact with the bacteria at concentrations exceeding the minimal inhibitory concentration (MIC). In concentration-dependent antibiotics, the effect of antibiotic mainly depends on the increasing concentration of antibiotic i.e., the bacterial rate of killing is seen to increase with increasing antibiotic concentration. Fluoroquinolones, for example, are concentration-dependent antibiotics and therefore short-term antibiotic course with relatively high dosage is recommended (Mueller et al. 2004; Wispelwey 2005).

Antimicrobial resistance has persisted as long as we have got antimicrobials. Already in 1945, Alexander Fleming, who discovered penicillin, warned that the misuse of penicillin may evoke resistant bacteria that can transfer to other patients who then cannot be treated with penicillin. Antimicrobial resistance is nowadays a worldwide problem: While new and more effective antibiotics are developed, bacteria are getting more and more resistant, and therefore the real efficacy of new antimicrobials has not increased (Noskin 2008). In addition, MDR among several different bacterial groups is increasing continuously.

2.2.1. Interpretation of antimicrobial susceptibility testing

For the succesfull treatment of bacterial infections, MICs of antimicrobials must be defined. The MIC value measures the susceptibility of bacteria and the efficacy of the antimicrobial agent. Especially MIC_{50} and MIC_{90} values are important for the determining the antimicrobial susceptibility and resistance limits for different bacteria. In addition to MIC values, the clinical efficacy of the tested antimicrobial agent should also be considered, since some isolates might seem susceptible against the tested antimicrobial agent in a clinical routine laboratory testing, although these antimicrobials have no efficacy *in vivo*. Therefore, antimicrobial susceptibility is not only a certain numerical value, but also a result that needs to be interpreted. To be able

to interpret results correctly, we need a standard for antimicrobial susceptibility. Most widely used susceptibility standard breakpoints are defined by an American organization, the Clinical Laboratory Standards Institute (CLSI) and Finnish susceptibility standard (FiRe) is based on this. According to the CLSI standard, different breakpoint values are determined as susceptible (S), intermediate (I) and resistant (R), and every category is between certain determined MIC values. There are, nevertheless, certain limitations concerning the CLSI standard or they are not fully recognized by regulatory agencies such as FDA (U.S. Food and Drug administration). Moreover, there is no background information available, industry is closely involved in the decisions and breakpoint stardards are chargeable. Therefore CLSI breakpoints are now being replaced by a harmonized European standard, created by the European Committee for Antimicrobial Susceptibility testing (EUCAST).

The EUCAST is a standing committee jointly organized by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), European Centre for Disease Control and Prevention (ECDC) and European national breakpoint committees. The EUCAST has joined together six European susceptibility breakpoint standards and harmonized most antimicrobial MIC breakpoints in Europe. The major task of the EUCAST is to set common European breakpoints for surveillance of antimicrobial resistance and to harmonize breakpoints for existing and new antimicrobial agents. The EUCAST has collected data on multiple MIC distributions and determined both the wild-type distributions and epidemiological cut-off values, which makes the EUCAST breakpoints more reliable. The clinical breakpoints are defined as clinically susceptible (S), clinically intermediate (I) and clinically resistant (R) and these breakpoints can be used in clinical laboratories in order to give an appropriate treatment for the patient. The epidemiological cut-off values can be used to separate the wild-type isolates from the non-wild-type isolates, which show microbiological resistance. The development of resistance both in hospitals and in the community can be measured using the epidemiological cut-off values. In the EUCAST standard, the antimicrobial breakpoints are set based on several properties: The proper dose and formulation of antimicrobial agent, the target organism, clinical indications, the MIC distribution of the target organism, resistance mechanisms, pK/pD, toxicity and the outcome versus MIC values.

Compared to the CLSI, the EUCAST breakpoints have many good qualities: The clinical breakpoints are new and well defined and they have been approved by the scientific committee. All the background information is public. Breakpoints are defined in a democratic process where industry has only a consultative role and everyone working with microbiology is entitled to contribute data. The EUCAST susceptibility breakpoints are freely available (www.eucast.org) and clinical and epidemiological breakpoints are separately defined. In addition, the EUCAST provides the Expert Rules to help the interpretation of resistance results. It is probable that in the near future, the EUCAST breakpoints will be used more frequently all over Europe, including Finland.

2.2.2. Antimicrobial resistance in *S. enterica*

Previously, salmonellosis was treated with ampicillin, chloramphenicol or trimethoprim-sulfamethoxazole. However, chloramphenicol resistance among *Salmonella* appeared already in the 1970s and since the emergence of MDR nontyphoidal *Salmonella* and *S.* Typhi isolates in the late 1980s, which were resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, treatment instructions have changed totally. Nowadays, quinolones, especially ciprofloxacin, have become the first-line treatment of *Salmonella* infections, although chloramphenicol is still used in third-world countries to treat severe diarrhea (Noskin 2008; Pegues et al. 2005; Rowe et al. 1997).

Antimicrobial resistance is readily acquired by zoonotic bacteria like *Salmonella*. Antimicrobial resistance and especially MDR became common in *S.* Typhimurium isolates already in the 1960s, but the increase was the most prevalent in the 1990s. The first MDR *S.* Typhimurium was identified in 1964, and by the end of the 1960s, this DT29 strain of *S.* Typhimurium, which was resistant to ampicillin, streptomycin, sulfonamides, tetracyclines and furazolidone had caused a significant amount of infections both in cattle and in humans (Threlfall 2002). Between the 1970s and 1980s, different types of resistant *S.* Typhimurium strains were spreading all over the world, but the real resistance problem emerged during the 1980s in the UK, Europe and North America via *S.* Typhimurium DT104 (Table 1). The phagetype DT104 was resistant to five antimicrobials: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline and by the 1990s, *S.* Typhimurium had acquired resistance also to trimethoprim-sulfamethoxazole, ciprofloxacin and extended-spectrum cephalosporins (Threlfall 2000). *Salmonella* DT104 isolates usually carry a chromosomal integron which encodes all, or a subset of these antimicrobial resistance genes (Winokur et al. 2000). The MDR *S.* Typhimurium DT104 was first identified in exotic birds in the early 1980s and by the end of the 1980s DT104 had spread to cattle and humans. Unlike other MDR *S.* Typhimurium phagetypes, DT104 was also common in poultry, particularly turkeys, and pigs and sheep, which made this phagetype a serious threat to public health (Pegues et al. 2005; Threlfall 2000, 2002; Weill et al. 2006).

Since the emergence of MDR *S.* Typhi and *S.* Typhimurium DT104, ciprofloxacin and other oral quinolones were suggested as alternatives to previous first-line antibiotics (Rowe et al. 1997). Even so, the first report on MDR *S.* Typhi with co-resistance to nalidixic acid was reported already in 1992 (Threlfall et al. 1999) and by 1999, the reduced ciprofloxacin susceptibility among *S.* Typhi had increased to 23% (Threlfall and Ward 2001). Nowadays, when reduced fluoroquinolone susceptibility has been detected also among *S. enterica* isolates (Hakanen et al. 2001; Hakanen et al. 1999b; Hakanen et al. 2006), fluoroquinolones are more often replaced by extended-spectrum cephalosporins as a first-line treatment (Threlfall 2002). To make the treatment of *Salmonella* infections even more problematic, resistance to the extended-spectrum cephalosporins has been detected in *S.* Typhimurium isolates since 1998, in combination with resistance to ciprofloxacin (Threlfall 2002; Whichard et al. 2007). In addition, these extended-spectrum cephalosporin-resistant isolates usually produce extended-spectrum β-lactamases (ESBL) (Riano et al. 2009), and with the association

of fluoroquinolone resistance genes, these *Salmonella* isolates really are a big threat to humans and a challenge for the appropriate treatment of *Salmonella* infections. The extended-spectrum cephalosporin resistance in *Salmonella* isolates is plasmid-mediated by the *bla*$_{CMY-2}$ gene which is the most prevalent AmpC-like β-lactamases and these plasmids harbour also resistance to other antimicrobial classes (Gonzalez-Sanz et al. 2009; Winokur et al. 2000; Zaidi et al. 2007). Due to this enormous resistance problem in *Salmonella*, it has been proposed to use azithromycin as a possible treatment alternative, mainly against *S*. Typhi isolates which show also reduced fluoroquinolone susceptibility (Capoor et al. 2007; Threlfall et al. 2008).

Table 1. Appearance of different *S.* Typhimurium phagetypes, their antimicrobial resistance patterns and the primary target of infection. Source: Threlfall and Ward 2001.

Year of outbreak	Serotype	Phagetype	Resistance pattern	Target
1964	*S.* Typhimurium	DT 29	AMP, STR, SUL, TET	cattle, humans
1975-1980	*S.* Typhimurium	DT 204	AMP, CHL, GEN, KAN, STR, SUL, TET, TMP	calves, humans
1975-1980	*S.* Typhimurium	DT 193	AMP, CHL, GEN, KAN, STR, SUL, TET, TMP	calves, humans
1975-1980	*S.* Typhimurium	DT 204c	AMP, CHL, KAN, STR, SUL, TET, TMP	calves, humans
1980	*S.* Typhimurium	DT 104	AMP, CHL, STR, SUL, TET	exotic birds
1990-1995	*S.* Typhimurium	DT 104	AMP, CHL, STR, SUL, TET	cattle
1990-1995	*S.* Typhimurium	DT 29, 204, 193, 204c	AMP, CHL, GEN, KAN, STR, SUL, TET, TMP	poultry, pigs, sheep
1996	*S.* Typhimurium	DT 104	AMP, CHL, STR, SUL, TET	humans, cattle
2000	*S.* Typhimurium	DT 104	AMP, CHL, STR, SUL, TET	humans
2000	*S.* Typhimurium	DT 204b	AMP, CHL, GEN, KAN, STR, SUL, TET, TMP, NAL, CIP	humans

AMP = ampicillin, STR = streptomycin, SUL = sulfonamides, TET = tetracycline, CHL = chloramphenicol, GEN = gentamicin, KAN = kanamycin, TMP = trimethoprim, NAL = nalidixic acid, CIP = ciprofloxacin

2.2.3. Fluoroquinolone resistance

2.2.3.1. Quinolone antimicrobials

Unlike some other antimicrobials which were discovered during the past century, the quinolones are a family of synthetic broad-spectrum antibiotics. The first quinolone antimicrobial agent, nalidixic acid, which has a 1,8-naphthyridine structure, was discovered in 1962 by Lesher and associates among the by-products of the synthesis of antimalarial compound chloroquine. In the 1970s, a few other (pipemidic acid, oxolinic acid and cinoxacin) first generation quinolone antibiotics were discovered, but compared with the nalidixic acid, they had only minor improvements. The real improvement of the quinolone antimicrobials happened in the 1980s, when the fluorine- and piperazinyl-substituted derivates of quinolones were developed. Especially important was the invention of ciprofloxacin, the most important and widely used fluoroquinolone, which was introduced into clinical market in 1987 (Madurga et al. 2008). These newer fluoroquinolones had a greater potency and an expanded spectrum of activity. Overall, fluoroquinolones are orally well absorbed and have good distribution in tissue and entry into phagocytic cells and the urinary concentration exceeds the MICs of many common pathogens. All these things together have assured a rapid and continuous expansion in the usage of quinolone antibiotics (Andriole 2005; Hooper 2005).

2.2.3.2. Classification of quinolones

The active structure of the fluoroquinolone class is based upon the quinolone ring system (Figure 3). All quinolone derivates in clinical use have a dual ring structure with nitrogen at position 1, a carbonyl group at position 4 and a carboxyl group attached to the carbon number 3 of the first ring. Nalidixic acid (Figure 4) has a 1,8-naphthyridine structure with 1-ethyl and 7-methyl

Figure 3. The common structure of quinolones. Figure modified from Emami et al. 2005.

substituents. The potency of quinolones was improved by the addition of a fluorine atom to the central ring system, typically at position 6, which is a typical structure for fluoroquinolones including ciprofloxacin (Figure 5). The potency against Gram-negative bacteria was further enhanced by adding a piperazinyl (ciprofloxacin, norfloxacin and enofloxacin), methyl-piperazinyl (for example ofloxacin, lomefloxacin and gatifloxacin), or dimethyl-pipezinyl (sparfloxacin) substituent in position 7. Methyl substituents on the piperazine ring can improve oral bioavailability and pyrrolidinyl substituents at position 7 (clinafloxacin) enhance activity against Gram-positive bacteria, as do dual-ring structures, derived from the pyrrolidinyl ring (moxifloxacin, sitafloxacin) (Emami et al. 2005; Hooper 2005).
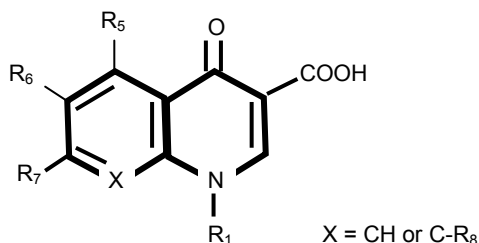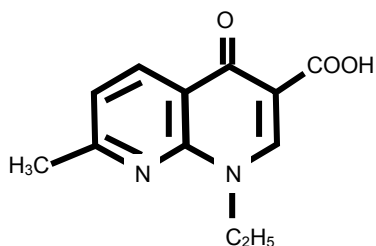
Figure 4. The structure of nalidixic acid
Figure modified from Hooper 2005.
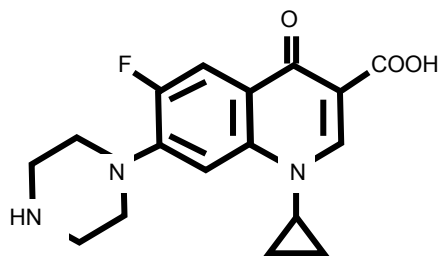
Figure 5. The structure of ciprofloxacin
Figure modified from Hooper 2005.

The quinolone antimicrobials can be divided into different generations based on their pharmacokinetic properties and antibacterial spectrum. Nalidixic acid and cinoxacin are narrow spectrum quinolones, and nowadays they are the most rarely used quinolone antimicrobials. Narrow spectrum quinolones achieve only low serum levels and therefore nalidixic acid is mainly used for the treatment of urinary tract infections. In addition to the *Enterobacteriaceae* family, quinolones also show *in vitro* activity against other Gram-negative pathogens like *Neisseria*, *Haemophilus* and *Pseudomonas aeruginosa.* Ciprofloxacin, norfloxacin and ofloxacin belong to the classical fluoroquinolones, i.e. second generation quinolones. Compared to narrow spectrum quinolones, classical fluoroquinolones have increased Gram-negative and systemic activity. Levofloxacin and moxifloxacin belong to the novel fluoroquinolones. These antimicrobials have extended activity against Gram-positive bacteria and atypical pathogens, and therefore these novel fluoroquinolones can be used for the treatment of community-acquired pneumonia, acute sinusitis and chronic bronchitis. While the narrow spectrum quinolones were mainly used for the treatment of urinary tract infections, enhanced antimicrobial activity of fluoroquinolones has extended the use of the newer fluoroquinolones beyond the traditional indications. Nowadays, several infectious diseases, including gastrointestinal infections, respiratory infections, skin infections and osteomyelitis, can be treated with quinolone antibiotics (Hooper 2005; King et al. 2000; Prescott et al. 1999; Yao and Moellering 2007).

2.2.3.3. Mechanism of action of quinolones

The fluoroquinolones rapidly inhibit the bacterial cell synthesis by targeting two essential bacterial topoisomerase II enzymes, DNA gyrase and topoisomerase IV. Both of these ezymes are large, complex enzymes, which consist of two subunits. DNA gyrase is a tetramer, composed of GyrA and GyrB subunits, which are the products of *gyrA* and *gyrB* genes, respectively. DNA gyrase is responsible for introducing negative supercoils into covalently closed double stranded DNA and removing both positive and negative supercoils. Topoisomerase IV, which is structurally related to DNA gyrase, is also composed of two subunits, products of *parC* and *parE* genes, which are homologous to *gyrA* and *gyrB*, respectively. Topoisomerase IV resolves connected

daughter DNA molecules after replication and allows their segregation into daughter cells. Thus, DNA gyrase and topoisomerase IV work together and they have an essential role in bacterial DNA replication, transcription, recombination and repairing of DNA (Jacoby 2005). The quinolones inhibit DNA gyrase and topoisomerase IV by binding to the complex of DNA gyrase or topoisomerase IV, which cause conformational changes in both enzyme-bound DNA and the enzyme itself. This results in a rapid termination of DNA synthesis. The affinities of different fluoroquinolones for these two target molecules vary, which explains why different antibiotics have diverse potencies against different bacteria. For Gram-negative bacteria, the primary target of fluoroquinolones is DNA gyrase, whereas for Gram-positive bacteria it is topoisomerase IV. The proposed reaction mechanisms of DNA gyrase and topoisomerase IV enzymes are presented in Figure 6 (Hooper 2005; Rise and Bonomo 2007; Tran and Jacoby 2002; Wiedemann and Heisig 1994).
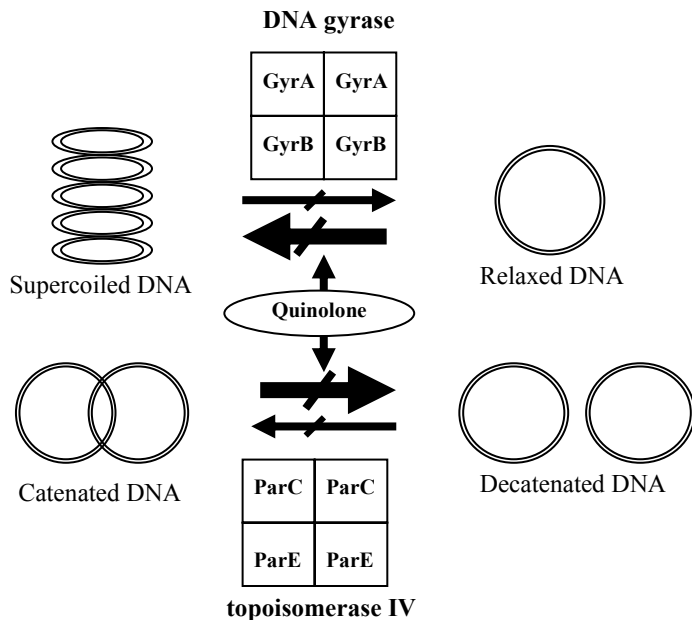
Figure 6. Reactions of DNA gyrase and topoisomerase IV.
Figure modified from Hooper 1999.

2.2.3.4.   Reduced fluoroquinolone susceptibility in *S. enterica*

Fluoroquinolones show good *in vitro* and clinical activity against *Salmonella* isolates, and therefore fluoroquinolones are commonly used for the treatment of *Salmonella* infections. Although high-level resistance to fluoroquinolones (i.e. ciprofloxacin) is relatively rare among the genus *Salmonella*, reduced fluoroquinolone susceptibility has increased and has become more prevalent among *S. enterica* isolates during the last decade (Kehrenberg et al. 2007).

The reduced fluoroquinolone susceptibility means that using currently accepted breakpoints, isolates are not fully susceptible to ciprofloxacin, but they are not resistant either, and their MIC values differ substantially from the wild population. Determining the real reduced susceptibility for fluoroquinolones is complicated due to the different MIC breakpoint values. According to the CLSI, ciprofloxacin susceptibility breakpoint for *Salmonella* and other *Enterobacteriaceae* is ≤1 mg/L. This breakpoint, however, is misguided and therefore a term 'reduced fluoroquinolone susceptibility' has become commonly used. The EUCAST has set the susceptibility value as ≤0.5 mg/L and the epidemiological cut-off of the wild-type population is 0.063 mg/L (CLSI 2007; EUCAST 2009b), i.e. these breakpoints are more precise compared to the CLSI. According to different publications, the breakpoint for reduced ciprofloxacin susceptibility of *Salmonella* isolates is determined as ≥0.125 mg/L. This breakpoint is in accordance with the EUCAST recommendations since the value differs from the wild-type population (Aarestrup et al. 2003; Hakanen et al. 2001; Molbak et al. 1999). Although *Salmonella* isolates are not usually fully resistant (MIC ≥4 mg/L) to ciprofloxacin according to CLSI breakpoints (CLSI 2007), fluoroquinolone treatment may not be effective and treatment failures may occur when patients with reduced fluoroquinolone susceptible *Salmonella* are treated with ciprofloxacin (Aarestrup et al. 2003; Molbak et al. 1999; Piddock et al. 1993; Vasallo et al. 1998).

It has been claimed that fluoroquinolone resistance in salmonellas is caused by the increased usage of fluoroquinolones in veterinary medicine, but another important reason for the increasing resistance is that fluoroquinolones are widely used also in human medicine all over the world. Unfortunately, it is widely accepted to use quinolones in pet animals and animal husbandry for prophylactic and therapeutic reasons, and this quinolone usage might select resistant bacteria which can be readily transferred from animals to humans (Fabrega et al. 2008). Isolates with reduced fluoroquinolone susceptibility are usually highly resistant to nalidixic acid (MIC ≥256 mg/L). Therefore it has been suggested that screening for reduced fluoroquinolone susceptibility could be performed with testing for susceptibility to nalidixic acid (Hakanen et al. 1999a). Since practically all *Salmonella* isolates with reduced fluoroquinolone susceptibility have been high-level resistant to nalidixic acid, some clinical laboratories do not necessarily test ciprofloxacin susceptibility, although the CLSI advises to test both ciprofloxacin and nalidixic acid against *Salmonella* isolates (CLSI 2007). It has been suggested that the most optimal method would include testing for susceptibility to nalidixic acid as a screening tool and then determine ciprofloxacin MIC for all nalidixic acid-resistant isolates (Aarestrup et al. 2003).

Reduced fluoroquinolone susceptibility has increased all over the world during the recent years (Chau et al. 2007; Stevenson et al. 2007). The increase of reduced fluoroquinolone susceptibility has been significant especially in Europe, although most *Salmonella* infections associated with reduced susceptibility to fluoroquinolones are acquired abroad, mainly in Southeast Asia (Frost et al. 1996; Hakanen et al. 2006; Kehrenberg et al. 2007; Piddock 1998). In Finland, reduced fluoroquinolone susceptibility among *S. enterica* isolates, collected from Finnish travelers returning from abroad, has increased between 1995 and 2004 from 2.0% to 39.0% (Hakanen et

al. 2001; Hakanen et al. 1999b; Hakanen et al. 2006). Reduced fluoroquinolone susceptibility in association with nalidixic acid resistance in *Salmonella* isolates is mainly caused by a single point mutation in QRDR of *gyrA*. In addition, it has been suggested that isolates with a mutation in both *gyrA* and *parC* genes are more susceptible to ciprofloxacin than those with only the *gyrA* mutation (Eaves et al. 2004). Alternatively, resistance to quinolones may arise due to decreased cell membrane permeability, the presence of efflux pump mechanisms or with plasmid-mediated quinolone resistance mechanisms (Robicsek et al. 2006a). Due to this wide variety of different quinolone resistance mechanisms, screening of QRDR mutations in the *gyrA* gene is not sufficient to detect reduced fluoroquinolone susceptibility in *Salmonella* isolates and therefore other resistance mechanisms must be tested as well.

In Finland, fluoroquinolone susceptibility in *Salmonella* has been monitored since 1995 among domestic isolates and in isolates collected from Finnish travelers returning from abroad. Between 1995 and 2002 all *Salmonella* isolates collected from Finnish travelers returning from abroad, which showed reduced fluoroquinolone susceptibility (MIC ≥0.125 mg/L), were highly resistant to nalidixic acid (MIC ≥32 mg/L) (Hakanen et al. 2001; Hakanen et al. 1999b). Similar reports were published all over the world and therefore it has been considered acceptable to use nalidixic acid for the screening of reduced fluoroquinolone susceptibility.

2.2.3.5. Mechanisms of fluoroquinolone resistance

Topoisomerase II (DNA gyrase) and IV are essential enzymes responsible for the maintenance of DNA topology within the bacterial cell and it has been shown that these enzymes are the major target of fluoroquinolones and nalidixic acid (Piddock 2002). Acquired resistance to quinolone antimicrobials is usually the result of two mechanisms: Chromosomal mutations that cause alterations in the target enzymes, DNA gyrase and topoisomerase IV, and decrease in quinolone accumulation in bacteria, typically due to overexpression of efflux pumps (e.g. AcrAB-TolC system) (Cattoir et al. 2007b; Wiedemann and Heisig 1994). Since 2006, some other mechanisms causing the fluoroquinolone resistance have been identified: Enzymatic modification or degradation of fluoroquinolones (*aac(6')-Ib-cr*), the protection of bacterial target genes from quinolone inhibition (*qnr*) and active quinolone efflux pump (*qepA*). The protection of gyrase A and topoisomerase IV is caused by plasmid-mediated quinolone resistance mechanisms, mainly due to *qnr* genes. To date, five different *qnr* alleles have been identified in many members of the *Enterobacteriaceae* family, including *Klebsiella* and *Salmonella* species (Cattoir et al. 2007b; Jacoby et al. 2008; Jacoby 2005; Jacoby et al. 2003; Robicsek et al. 2006a; Wang et al. 2003).

2.2.3.5.1. Target site modifications

Fluoroquinolone resistant isolates usually have one or more mutations in a small section of *gyrA* or *parC* genes, called quinolone resistance-determining region (QRDR). In *gyrA* gene this hotspot, QRDR, is highly conserved and is located at the 5'-terminus, close to codon Tyr122, which is temporarily covalently bound to

phosphate groups on DNA in the initial strand-breaking reaction. Mutations in the QRDR of *gyrA* induce changes in the binding site conformation which is important for quinolone-DNA gyrase interaction and reduce the quinolone affinity for the modified enzyme-DNA complex. Thus resistance is caused by reduced antibiotic affinity for the modified enzyme-DNA complex (Fluit et al. 2001; Hooper 1999). The majority of mutations causing the quinolone resistance are near the dimmer interface and DNA binding site of DNA gyrase. The mutations in this area define a binding pocket in GyrA where the drugs can simultaneously interact with DNA (Piddock 2002). The most common *gyrA* mutations in nalidixic acid-resistant strains are residues Ser83-Leu and Asp87-Gly which are commonly linked to clinical fluoroquinolone resistance. However, strains having different substitutions at codons 83 and/or 87 show different levels of decreased fluoroquinolone susceptibility (Giraud et al. 2006). There are also additional mutations within QRDR of *gyrA*, including Ala67, Asp72, Gly81 and Asp82 mutations. In addition, mutations outside the QRDR or *gyrA*, i.e. Ala131, Glu139 and Asp144 have been reported (Eaves et al. 2002). Mutations within *gyrB* are less frequently detected than in *gyrA*. However, codon Ser464 can be considered a quite common spot for quinolone resistance mutations (Giraud et al. 2006). Mutations causing decreased susceptibility to quinolones can be found also at codons Asp426 and Lys447 in *gyrB* (Piddock 2002; Yamagishi et al. 1986).

ParC mutations have been found in clinical isolates of *E. coli*, *K. pneumoniae* and *P. aeruginosa*, which show high-level fluoroquinolone resistance. In *Salmonella* however, parC and parE are the secondary target for quinolones. Mutations in *parC* and *parE* are always found together with *gyrA* mutations, which suggests that the mutations in topoisomerase IV will not occur unless the strain already has a mutated gyrase A and the sensitivity of the DNA gyrase to fluoroquinolones has already been reduced. Therefore a single mutation in *parC* does not affect the quinolone susceptibility (Bagel et al. 1999; Giraud et al. 2006; Heisig 1996). The mutations in *parC* are commonly found in the amino acids Ser80 and Glu84, causing Ser80-Phe/Tyr and Glu84-Lys/Leu mutations (Hooper 1999). Mutations in *parE* are rare in all *Enterobacteriaceae,* including salmonellas (Giraud et al. 2006; Piddock 2002).

Target site modifications within QRDR can be investigated with several methods. The initial step of these methods is PCR amplification of target nucleic acid. The presence of the desired target gene can be assured with electrophoretic mobility determinations, probe hybridization assays (including Southern blotting, enzyme-linked immunosorbent assay or liquid hybridization formats), restriction fragment length polymorphism (RFLP) analysis, mismatch amplification mutation assay (MAMA)-PCR or DNA sequencing. The specific mutations associated with antibiotic resistance in the PCR product can be detected with direct DNA sequencing methods, RFLP, single-strand conformation polymorphism (SSCP) and denaturing high-performance liquid chromatography (DHPLC) (Cockerill 1999; Eaves et al. 2002). In RFLP analysis, the amplified DNA is fragmented with restriction enzymes. Enzymes will only cleave DNA at specific sites and therefore, if the sequence of the target DNA is known, RFLP analysis can be used to confirm that the target DNA is the correct one. RFLP is also useful for identifying mutations associated with quinolone resistance. In

the SSCP method, the target DNA is first amplified with PCR, the PCR product is then denatured to a single strand and finally electrophoresed. Mutations are detected by the appearance of bands: Mutated bacterial strains have bands at different positions compared with bands seen in the wild-type strains (Cockerill 1999). In the DHPLC method, the amplified target DNA is mixed with amplified wild-type DNA. Both DNAs are denatured simultaneously in the same reaction mixture and then allowed to slowly reanneal, forming four different duplex DNA. Heteroduplexes will result in double-stranded DNA containing a mismatch bubble at the point mutation site. Under the non-denaturing conditions, all four duplexes have the same retention time during ion pair chromatography. When temperature increases, the heteroduplexes start to denature on either side of the mismatched bases and begin to emerge ahead. Thus, DHPLC is based on the DNA heteroduplex formation and separation of heteroduplex from homoduplex using an ion-pair reverse phase HPLC (Eaves et al. 2002).

2.2.3.5.2. Altered quinolone accumulation

Quinolones have to pass the bacterial cell wall to reach their intracellular targets, DNA gyrase and topoisomerase IV. Both the outer and cytoplasmic membranes are involved in the transport of small molecules like quinolones and alterations in either or both membranes are associated with decreased quinolone accumulation and increased resistance to fluoroquinolones, especially with Gram-negative bacteria. The outer cell membrane contains the pore forming proteins (porins) such as OmpC and OmpF that form channels for passive diffusion and allow solutes, including antibiotics, drift into the cell. Most of the antibiotics, like quinolones, are accumulated into cells very rapidly, usually within 1-2 minutes after the antibiotic exposure, until an equilibrium or steady state concentration is achieved (Piddock 1991). In all cells, the diffusion within the membrane involves also proton motive force (pmf). Pmf means a storage of energy as a combination of a proton and voltage gradient across a membrane. In most cases, the proton motive force is generated by an electron transport chain which is at the same time both electron and proton pump, pumping electrons in the opposite direction, creating a separation of charge. In mitochondria, free energy released from the electron transport chain is used to move protons from the mitochondrial matrix to the intermembrane space. Moving protons to the outer parts of the mitochondrion creates a higher concentration of positively charged particles, resulting in a slightly positive and slightly negative side. This charge difference results in an electrochemical gradient. Together the electrochemical gradient of protons is both a concentration and charge difference and is often called the proton motive force. When a pmf inhibitor, such as carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) or Phe-Arg-β-naphthylamide (PAβN) is added in the reaction at the equilibrium state, the fluoroquinolone accumulation is immediately increased. This phenomenon is caused by the inhibition of a pmf driven efflux pump, which is the common feature of wild-type quinolone-susceptible bacteria, which allows higher concentrations of quinolone to be accumulated within the cell (Hooper 1999; Kehrenberg et al. 2007; Piddock 2002).

Some resistant bacteria accumulate fluoroquinolones in much lower concentrations compared with wild-type strains. When CCCP or PAβN is added, the accumulated fluoroquinolone concentration increases to a similar level as seen in wild-type isolates,

which indicates that the low concentration of accumulated fluoroquinolone is due to enhanced efflux (Piddock 2002). The reduced fluoroquinolone susceptibility can be achieved via active efflux pump either by inducing the overexpression of outer membrane porin OmpF which increases the efflux, or by reducing drug export through unspecific MDR efflux pumps like AcrAB-TolC. Efflux pumps are proteins which transport antimicrobials and other toxic compounds out of the cell. The active efflux of fluoroquinolones due to AcrAB-TolC efflux system is a major resistance mechanism also in *S. enterica* isolates (Webber et al. 2008). The AcrAB-TolC system belongs to the resistance nodulation-division (RND) family of efflux pumps and it consists of AcrB, the efflux protein in the cytoplasmic membrane, AcrA, a membrane fusion protein and TolC, the outer membrane protein. In *Salmonella*, this efflux pump is regulated independently from the *mar* and *sox* regulatory systems, and mutations in the local regulators of *acrAB* (Kehrenberg et al. 2007; Piddock 2002; Preisler and Heisig 2009). In *S.* Typhimurium DT104 isolates, reduced fluoroquinolone susceptibility can involve active efflux due to overproduction of the AcrAB efflux pump (Baucheron et al. 2004). In quinolone-resistant isolates, quinolone accumulation is usually reduced as a result of the overexpression of efflux pumps, for example in *S.* Typhimurium DT104 isolates, AcrAB-TolC efflux system appears to be a primary mechanism of quinolone resistance. However, high-level resistance to fluoroquinolones in *Salmonella* is often explained by the combination of multiple target gene mutations and increased active efflux (Abouzeed et al. 2008; Baucheron et al. 2004).

The efflux pump mechanisms can be investigated with PCR. If there is a gene encoding the efflux pump, it can be amplified with specific primers and sequenced. Also, mutations in genes encoding efflux pump regulators can be detected with PCR and sequencing. Accumulation of quinolone antimicrobials can also be tested phenotypically by efflux pump inhibition. In the accumulation assay, the efflux activity of strains showing reduced susceptibility to quinolones is compared with the resistant strains, by monitoring the uptake of the fluorescent dye bis-benzimide (Hoechst 33342) or selected fluoroquinolones. In the following step, accumulation is monitored with and without CCCP or PAβN, which dissipates the proton motive force and therefore acts as an inhibitor of active efflux (Webber et al. 2008).

2.2.3.5.3. Transferable quinolone resistance

Plasmid-mediated quinolone resistance (PMQR), which can cause a horizontal transfer of fluoroquinolone resistance between strains, was discovered over a decade ago (Jacoby et al. 2003; Martinez-Martinez et al. 1998; Tran and Jacoby 2002). The first PMQR determinant, QnrA, a 218 amino acid protein, was detected from the clinical isolate of *Klebsiella pneumoniae* in the USA in 1994 (Martinez-Martinez et al. 1998). This strain carried a plasmid pMG252, which contained the quinolone resistance encoding gene *qnrA1*. Since then, several other *qnr* determinants have been found, including *qnrB*, *qnrS* and *qnrD* (Cavaco et al. 2009; Jacoby et al. 2006; Martinez-Martinez et al. 2008). All Qnr determinants belong to a pentapeptide-repeat protein family and they act by protecting DNA gyrase and probably also topoisomerase IV from the quinolone inhibition (Nordmann and Poirel 2005). The mechanism of how

Qnr protects DNA gyrase does not include changes in the intracellular quinolone accumulation or antibiotic inactivation but *qnr* genes reduce the binding of gyrase to DNA and even compete with DNA for gyrase binding (Robicsek et al. 2006a).

Unfortunately, in addition to quinolone resistance, plasmid pMG252 also encodes co-resistance to cephalosporins, chloramphenicol, kanamycin, gentamicin, streptomycin, tobramycin and trimethoprim. This genetic linkage can explain why quinolone resistance is remarkably high in some ESBL-producing isolates and plasmid-mediated quinolone resistance genes are often found in association with genes encoding ESBLs or other β-lactamases such as plasmid AmpC derivates (Hopkins et al. 2005). Thus, although *qnr* genes cause only low-level resistance to fluoroquinolones, widespread horizontal transfer of *qnr*-plasmids between different strains could compromise the use of fluoroquinolones (Hopkins et al. 2005; Nordmann and Poirel 2005). In addition, the *qnr*-containing plasmids present in *Salmonella* differ both in size and structure, which suggests that the rapid spread of *qnr* gene is based on mobile genetic elements like integrons, rather than proliferation of a single resistant plasmid (Giraud et al. 2006).

Recently, another plasmid-mediated quinolone resistance mechanism, the enzymatic inactivation of quinolones, has been reported. This *cr* variant of aminoglycoside acetyltransferase, AAC(6')-Ib is a first quinolone inactivating enzyme in bacteria and it confers reduced susceptibility to ciprofloxacin and resistance to aminoglycosides, tobramycin, amikacin and kanamycin (Robicsek et al. 2006b). The AAC(6')-Ib-cr has two amino acid changes, Trp102Arg and Asp179Tyr, which together are necessary and useful for the enzyme's ability to acetylate ciprofloxacin. The *cr* variant of *aac(6')-Ib* encodes an aminoglycoside acetyltransferase which N-acetylates the piperazinyl amine in the ciprofloxacin molecule and thus causes reduced susceptibility to ciprofloxacin. Since norfloxacin and ciprofloxacin have similar unsubstituted piperazinyl group, *aac(6')-Ib-cr* diminishes norfloxacin activity as effectively as ciprofloxacin activity. Levofloxacin and moxifloxacin activity was not reduced due to the lack of unsubstituted piperazinyl nitrogen (Park et al. 2006; Robicsek et al. 2006a; Robicsek et al. 2006b). The *aac(6')-Ib-cr* might cause problems since the selection pressure from the use of aminoglycosides can increase fluoroquinolone resistance. It has been shown that *aac(6')-Ib-cr* is commonly found in the same plasmids as *qnr* genes, and this double resistance mechanism can increase the fluoroquinolone resistance fourfold. In addition, in *Enterobacteriaceae, aac(6')-Ib-cr* and ESBL-encoding genes are also found together in the same plasmids (Jiang et al. 2008; Park et al. 2006; Pitout et al. 2009; Robicsek et al. 2006a).

The most recent transferable quinolone resistance mechanism is a plasmid-mediated efflux pump gene, *qepA*, which extrudes hydrophilic fluoroquinolones including ciprofloxacin, norfloxacin and enrofloxacin out from the bacterial cell. The *qepA* gene encodes a protein that resembles a 14-transmembrane-segment putative efflux pump, which belongs to the major facilitator superfamily of proton-dependent transporters (Poirel et al. 2008; Yamane et al. 2007). To date, this resistance mechanism has not been detected in *Salmonella* spp.

Transferable quinolone resistance is investigated with PCR, sequencing and plasmid analysis. Genes encoding fluoroquinolone resistance are amplified with specific PCR primers. The amplification of the correct gene is then assured by sequencing. Presence of plasmid-mediated quinolone resistance can be detected with plasmid analysis and transformation assay. In plasmid analysis, the plasmid DNA is extracted from the cell and digested with restriction enzymes. Resulting fragments are then separated with gel electrophoresis. Transformation assay can be used to ensure that resistance is plasmid-mediated. A plasmid which contains the gene of interest can be transferred to a susceptible recipient strain with electroporation, for example. If a similar genotype and phenotype are seen in the recipient strain after the plasmid transfer, compared with the original strain, it is probable that the resistance is plasmid-mediated. Plasmid-mediated resistance can also be detected with Southern hybridization by comparing the plasmid profile of the tested strain with the previously known plasmids.

2.2.4. Macrolide resistance

2.2.4.1. Macrolide antimicrobials

Macrolides are a heterogeneous class of antimicrobials including naturally occurring and synthetic compounds, which have been in use since the 1950s. The chemical structure of macrolides consists of a polyketide macrolactone ring which is substituted with one or more sugar residues. Macrolides differ from each other in the size (14-16 atoms) and substitutions in their lactone ring, but macrolides with 14- and 16-membered lactone rings have been proven to be clinically the most effective ones (Pfister et al. 2004).

Figure 7. The structure of erythromycin. Figure modified from Pfister et al. 2004.

Erythromycin is a naturally occurring 14-membered macrolide (Figure 7), which was first isolated in 1952 from a soil fungus called *Streptomyces erythreus*. Azithromycin is a semisynthetic 15-membered-ring erythromycin derivate, an azalide antibiotic, which has a nitrogen atom incorporated in its lactone ring (Figure 8). Nowadays, four different macrolide antibiotics are commonly used: erythromycin, azithromycin, clarithromycin and roxithromycin. Azithromycin and clarithromycin have some great advantages over erythromycin: These macrolides have expanded antimicrobial efficacy, improved pharmacokinetic parameters and they have fewer gastrointestinal side effects and drug interactions (Rise and Bonomo 2007; Steigbigel 2000; Yao and Moellering 2007). Another type of semisynthetic erythromycin derivate is telithromycin, which is a 14-membered-ring ketolide antibiotic. Telithromycin is especially effective against macrolide-resistant pneumococci (Bryskier and Butzler
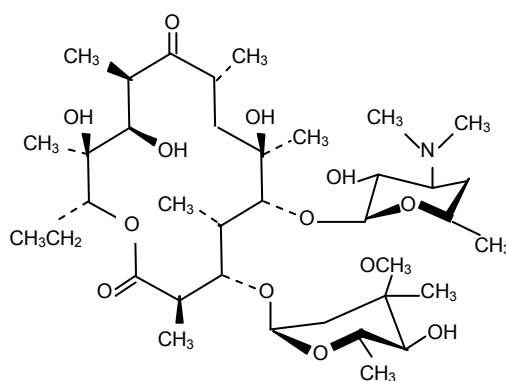
2003). Members of the *Enterobacteriaceae* family are typically resistant to erythromycin (MIC 2−256 mg/L), but since intestinal drug levels are higher than MICs, oral administration of erythromycin has been proven to decrease the intestinal *Enterobacteriaceae* colonization (Gordillo et al. 1993).
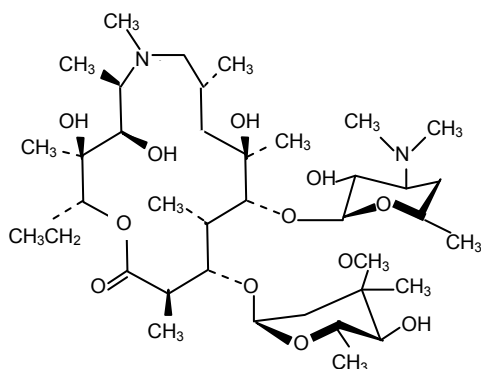

Figure 8. The structure of azithromycin. Figure modified from Pfister et al. 2004.

Macrolides are bacteriostatic agents that inhibit protein synthesis by inhibiting RNA elongation. Macrolides bind reversibly to the peptidyl-tRNA binding region of the 23S rRNA in the 50S ribosomal subunit, also called the macrolide-binding pocket, which then inhibits the translocation of a newly synthesized peptidyl-tRNA molecule from the acceptor site on the ribosome. Azithromycin binds to the same receptor on the bacterial 50S ribosomal subunit as erythromycin, and inhibits RNA-dependent protein synthesis by the same mechanism. Azithromycin is more active against Gram-negative bacteria than erythromycin and other 14-membered macrolides due to the fact that azithromycin is better penetrated to the outer membrane of Gram-negative bacteria (Bryskier and Butzler 2003; Rise and Bonomo 2007; Steigbigel 2000; Vaara 1993; Yao and Moellering 2007). Besides the antimicrobial activity, macrolide antimicrobials have immunomodulatory activities as well. Azithromycin, for example, exhibit pro-inflammatory features, like an acute stimulation of neutrophil degranulation and enzyme release, oxidative burst, which is associated with increased phagocytosis and increased serum IL-1β levels (Ribeiro et al. 2009). Azithromycin modulates the inflammation in two phases: host defence mechanisms are enhanced shortly after the initiation of antimicrobial treatment and in the following period local infection is reduced (Amsden 2005).

2.2.4.2. Erythromycin and azithromycin resistance

In most Gram-negative bacteria, the entry of erythromycin into the cell is restricted by the outer cell membrane. The outer cell membrane of Gram-negative bacteria is a relatively effective permeability barrier against all hydrophobic antibiotics, including erythromycin, which makes bacteria resistant to erythromycin (Vaara 1993). Although enteric bacteria are commonly non-susceptible to macrolide antimicrobials due to the naturally occurring chromosomal efflux pumps, for example *E. coli* isolates can also acquire *mef(A)* and *erm(B)* genes, which increase their macrolide resistance levels (Liu et al. 2009). *Enterobacteriaceae* are naturally susceptible to macrolides and therefore, neither the EUCAST nor the CLSI have determined the breakpoints for erythromycin and azithromycin.

Modification of the macrolide binding site is the primary mechanism for macrolide resistance and it is usually caused by *erm* gene-encoded rRNA methylases. Erm, methyltransferase catalyzes dimethylation of A2058 in 23S rRNA, which is critical site for the binding of macrolides, lincosamides and streptogramin B (MLS$_B$ antibiotics) and therefore the *erm* gene confers also co-resistance against MLS$_B$ antibiotics (Diner and Hayes 2009). Other resistance mechanisms which cause the macrolide resistance also in the *Enterobacteriaceae* family include macrolide-inactivating enzymes, erythromycin esterases and macrolide 2'-phosphotransferases, *mef* gene-encoded active efflux of the drug and mutations in 23S rRNA and ribosomal proteins (Gibreel and Taylor 2006; Yao and Moellering 2007). Resistance to macrolides is commonly induced by mutations in the 23S *rrn* operon, which cause a reduction in the binding of the antibiotics to the peptidyl transferase center. Nucleotides A2058C/G and A2059G (*E. coli* numbering) are the most common sites of mutations, which are associated with macrolide resistance in several bacteria. Macrolide resistance may also be caused by the alteration of the 50S ribosomal subunit proteins L4 and L22, which leads to the widening of the entrance to the peptide exit tunnel and therefore allows access to the tunnel, even in the presence of antibiotics (Rise and Bonomo 2007; Yao and Moellering 2007). In the L4 protein, amino acids in position 63–74 are the most important targets for mutations causing the macrolide resistance (Corcoran et al. 2006).

In Gram-negative bacteria, chromosomally encoded efflux pumps play an important role both in intrinsic and acquired resistance to a variety of antibiotics, including macrolides. Traditional efflux pumps excrete only one type of antibiotics, whereas multi-drug efflux systems export many different antimicrobial compounds, which can be structurally unrelated. Efflux has an important role also in the macrolide resistance of *S. enterica* isolates, since it has been shown that erythromycin resistance is mediated by AcrAB efflux pump, which belongs to the resistance nodulation division family. It has been shown as well that modifications in the cell surface hydrophobicity can cause erythromycin resistance (Braoudaki and Hilton 2005).

2.2.4.3. Alternative antimicrobials against reduced fluoroquinolone susceptible isolates

Although erythromycin and azithromycin are both macrolide antibiotics, their antimicrobial activity against *Salmonella* isolates is different. Like the majority of *Enterobacteriaceae*, *Salmonella* isolates are intrinsically highly resistant to erythromycin, probably due to an AcrAB efflux pump (Braoudaki and Hilton 2005). However, susceptibility against azithromycin varies. For example, *S*. Typhi isolates have azithromycin MICs between 4 and 16 mg/L, indicating that these *S*. Typhi isolates belong to the wild-type distribution (Butler et al. 2001).

Due to the increased fluoroquinolone resistance among *S. enterica* isolates during the recent years and the possible appearance of ESBL producing *Salmonella*, the use of fluoroquinolones and cephalosporins as a first-line treatment of *Salmonella* infections has been jeopardized. Therefore, alternative antibiotics are needed. It has been shown that azithromycin has moderate *in vitro* activity against *S*. Typhi, and high intracellular

concentration of azithromycin is achieved, which makes azithromycin effective against intracellular *S*. Typhimurium isolates (Butler and Girard 1993; Metchock 1990). The *in vitro* benefits of azithromycin are also detected in typhoid fever, with low-grade bacteremia, <10^3 bacteria per ml of blood (Butler et al. 2001). In addition, there are several reports on the successful treatment of enteric infections and uncomplicated typhoid fever in children, adolescents and adults with azithromycin. Also in typhoid fever caused by MDR *S*. Typhi, azithromycin treatment has been effective (Capoor et al. 2007; Chinh et al. 2000; Frenck et al. 2004; Frenck et al. 2000; Girgis et al. 1999; Jones et al. 1988; Threlfall et al. 2008).

There are also some reports on clinical efficacy of azithromycin compared to quinolone treament. One study showed that ciprofloxacin and azithromycin have similar effects, both clinically and bacteriologically, against typhoid fever, caused by MDR *S*. Typhi: all patients were cured within 10 days and *Salmonella* was eradicated from blood samples (Girgis et al. 1999). In another study, azithromycin and ofloxacin were compared in the treatment of MDR or nalidixic acid resistant *S*. Typhi and the results showed that a 5-day treatment with azithromycin was effective against uncomplicated enteric fever. Also the faecal carriage was eradicated and there were no relapses. It was even proved that a 5-day treatment was adequate to eradicate nontyphoidal *Salmonella,* since azithromycin has a long half-life and therefore the patients had antibiotic in their tissues for three to seven days after the end of treatment (Chinh et al. 2000; Imbert et al. 2003). DuPont et al. have suggested that although levofloxacin, or other fluoroquinolone, is recommended as a first-line treatment of salmonellosis in adult patients, nontyphoidal salmonellosis in children and in adult patients could be efficiently treated also with a 7-day course of azithromycin (DuPont 2009). In general, shorter courses of azithromycin are preferable. For example, Adachi et al. have suggested that one to three days of azithromycin is an adequate treatment for traveler's diarrhea (Adachi et al. 2003). In addition, azithromycin is also safe alternative for pregnant women, since quinolones are not recommended during pregnancy and pregnant women are at the higher risk of having traveler's diarrhea (Yates 2005). Based on these results, azithromycin treatment could be a possible alternative for patients having *Salmonella* with reduced fluoroquinolone susceptibility. However, to date, there is no conclusive evidence on the clinical efficacy of azithromycin in the treatment of salmonellosis (Capoor et al. 2009). Therefore, more investigation is needed.

3.   AIMS OF THE STUDY

The purpose of the present study was to describe and examine the novel quinolone resistance phenomenon in *Salmonella enterica*, which was first discovered in 2003 among isolates from Finnish travelers returning from Southeast Asia. These isolates showed reduced susceptibility to ciprofloxacin but were either susceptible (MIC <32 mg/L) or only low-level resistant (MIC =32 mg/L) to nalidixic acid, i.e. they exhibited a nonclassical quinolone resistance phenotype.

The specific aims of the present study were:

1)   To determine the epidemiology of the nonclassical quinolone resistance phenotype (**II**)

2)   To investigate the molecular mechanisms causing the nonclassical quinolone resistance phenotype (**I & III**)

3)   To determine the recent epidemiology of reduced fluoroquinolone susceptibility among *S. enterica* (**I & II**)

4)   To examine the *in vitro* activity of alternative antibiotics, like azithromycin to *S. enterica* isolates in general and especially to quinolone-resistant isolates (**IV**)

4. MATERIALS AND METHODS

4.1. Bacterial isolates (I-IV)

The bacterial isolates that were used in the papers **I-IV** are shown in Table 2.

Table 2. Bacterial isolates used in the study (**I-IV**)

Paper/ Study	Bacterium	No.of isolates	Isolation period	Geographical origin of isolates	Criteria for inclusion in the study
I	*S. enterica*	95	I-II/2003 I-II/2004	Thailand, Malesia, Vietnam, Indonesia	Isolates collected from Finnish travellers returning from Southeast Asia
II	*S. enterica*	499	Study I + I-II/2005, I-II/2006, I-II/2007	Mainly Southeast Asia and the Mediterranean Area	Consecutive foreign isolates from Finnish travelers
III	*S. enterica*	10	Study I + Study II	Thailand and Malaysia	Isolates showing reduced CIP susceptibility and being susceptible or only low-level resistant to NAL
IV	*S. enterica*	1237	Study I + Study II + I-II/2008 + domestic isolates collected 2003–2008	Mainly Southeast Asia, the Mediterranean Area and Finland	Consecutive foreign isolates from Finnish travelers and similarly collected domestic isolates

CIP = ciprofloxacin
NAL = nalidixic acid

4.1.1. *Salmonella* isolates (I-IV, unpublished)

In Finland, 100% of the domestic and 80-90% of foreign *S. enterica* isolates recovered from humans are sent to the Gastrointestinal Infections Unit, National Institute for Health and Welfare, Helsinki, Finland, which serves as the National Salmonella Reference Centre. Starting from January each year, the first 100 domestic and first 100 foreign *S. enterica* isolates collected from Finnish travelers returning from abroad are

sent to the Antimicrobial Resistance Unit, National Institute for Health and Welfare, Turku, Finland, for analyses of antimicrobial susceptibility. A total of 1237 epidemiologically unrelated *Salmonella* isolates collected between 2003 and 2008 were included in this study. Each isolate was accompanied with a form containing the epidemiological information. The isolates were determined to be epidemiologically unrelated if they were recovered from the distinct sources. For each *Salmonella* outbreak recognized, only one representative isolate of the epidemic was included.

The *Salmonella* isolates were divided into two different groups according to the origin of the infection. An isolate was designated to be of foreign origin (i.e. from Finnish traveler) if the patient had reported a travel abroad during one month before the specimen date. All other isolates were designated to be of domestic (i.e. Finnish) origin. The isolates were consecutively collected starting in January each year: Foreign isolates were collected between January and February, whereas domestic isolates were collected between January and July. Serotyping of all isolates and phage typing of *S.* Enteritidis and *S.* Typhimurium isolates were performed in the Salmonella Reference Centre in Helsinki.

4.2. The antimicrobial susceptibility testing (I-IV)

4.2.1. Standard agar plate dilution method (I-IV)

The MICs were determined by the standard agar plate dilution method according to the CLSI guidelines (CLSI 2006). The plates contained a series of doubling dilutions of each antimicrobial agent. In the studies **I** and **II**, susceptibility was determined to nalidixic acid and ciprofloxacin (Sigma, Steinheim, Germany). In the study **III**, susceptibility was determined to nalidixic acid, ciprofloxacin and five additional fluoroquinolones, also ampicillin, amoxicillin-clavulanic acid, cefalothin, cefuroxime, cefotaxime and ceftazidime (all from Sigma) were tested. In study **IV**, susceptibility was determined to ciprofloxacin, nalidixic acid, azithromycin, erythromycin, clindamycin (all from Sigma) and telithromycin (Sanofi Aventis, Paris, France). In addition, susceptibility to chloramphenicol and trimethoprim-sulfamethoxazole (Both from Sigma) has been tested. Antimicrobial susceptibility of *S. enterica* was tested with a final inoculum of approximately 10^3 cfu, which was transferred with a Denley Multipoint Inoculator (Denley Instruments Ltd., West Sussex, UK) onto the antimicrobial plates, 30 isolates were tested on each plate. Mueller-Hinton II agar (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) was used as a culture medium. The plates were incubated at +35˚C for 18 hours in air.

4.2.2. MIC breakpoints (I-IV)

The MIC breakpoints for the tested antimicrobial agents for *S. enterica* were chosen according to the CLSI breakpoints (CLSI 2007). Resistance breakpoints for ampicillin, amoxicillin-clavulanic acid, cephalothin, cefuroxime and ceftazidime were ≥32 mg/L,

≥64 mg/L for cefotaxime and ≥32 mg/L for nalidixic acid. The MIC breakpoint value for the reduced ciprofloxacin susceptibility was chosen to be ≥0.125 mg/L, on the basis of earlier publications (Aarestrup et al. 2003; Hakanen et al. 2001; Molbak et al. 1999). In addition, since the CLSI or EUCAST has not given any azithromycin resistance breakpoint for *S. enterica*, MIC ≥32 mg/L was chosen to be the resistance breakpoint on the basis of histogram analysis (Study **IV**).

4.2.3. Quality control strains (I-IV)

Staphylococcus aureus ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in the antimicrobial susceptibility testing of the *Salmonella* isolates. In addition, one local *S. enterica* C5 strain, highly resistant to ciprofloxacin, was used as a control.

4.3. Detection of fluoroquinolone resistance mechanisms

4.3.1. Preparation of DNA (I-IV)

Chromosomal DNA was prepared from overnight culture of each strain by dissolving a colony of the *S. enterica* isolate into 100 µl sterile water in microcentrifuge tube and boiling the suspension at 95°C for 10 minutes.

4.3.2. Detection of chromosomal mutations (I & III)

4.3.2.1. Primers

The oligonucleotide primers used in the PCR amplification and DNA sequencing are presented in Table 3. The primers were synthesized by Thermo Fisher Scientific (Ulm, Germany), except the *gyrA* primers which were synthesized by Eurogentec (Hersal, Belgium).

4.3.2.2. PCR amplification of QRDR of the *gyrA*, *gyrB*, *parC* and *parE* (**III**)

The PCR amplification of QRDR of the DNA gyrase and topoisomerase IV genes *gyrA*, *gyrB*, *parC* and *parE* was performed in a 50 µl reaction volume with previously described primers (Table 3) and protocols (Eaves et al. 2002).

4.3.2.3. Sequencing of the whole *gyrA* gene (**I, III, unpublished**)

The whole *gyrA* gene was amplified by PCR and the DNA was sequenced, using five different primer pairs (Table 3), from the six *S. enterica* isolates showing the nonclassical quinolone resistance phenotype, collected from travelers returning from Southeast Asia. The PCR amplification of the *gyrA* gene fragments was performed in a

Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, MA, USA) using 30 cycles of denaturation at 95˚C for 1 min, annealing at 54˚C for 1 min and extension at 72˚C for 2 min, followed by final extension at 72˚C for 5 min. Sequencing was performed with the ABI PRISM BigDye Terminator Cycle sequencing Ready reaction Kit (Applied Biosystems Inc, Foster City, CA, USA) and analyzed with the ABI PRISM 377 DNA Sequencer (Applied Biosystems). PCR and sequencing reactions were performed as previously described by Hakanen et al. (Hakanen et al. 1999a).

4.3.2.4.   Detection of mutations in *gyrA* with pyrosequencing (**I**)

Pyrosequencing method was used to detect point mutations at the positions Ser83 and Asp87 in the quinolone resistance-determining region (QRDR) of the *gyrA* gene in 16 *S. enterica* isolates which were collected from travelers returning from Southeast Asia. PCR was performed using primers (Table 3) and protocols, described in paper **I**. Pyrosequencing is based on sequencing-by-synthesis technology and it was performed according to the instructions of the manufacturer by using streptavidin-coated sepharose beads (Amersham Biosciences, Little Chalfont, UK), a PSQ 96 MA instrument, a vacuum prep workstation and PyroGold SQA reagents (Biotage AB, Uppsala, Sweden). 20 µl of the PCR product and 15 pmol of the sequencing primer (Table 3) were used in each sequencing reaction. In pyrosequencing, a PCR product is first attached to sepharose particles and then denatured and washed. Single-stranded DNA is then transferred to a 96-well microtiter plate and used as a template in sequencing reaction. Sequencing primer is hybridized to a single-stranded DNA and mixed with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates adenosine 5'phosphosulfate (APS) and luciferin. DNA polymerase catalyses the incorporation of the nucleotide into the DNA strand if it is complementary to the base in the template strand. While the nucleotide is incorporated, it releases inorganic pyrofosfate (PPi), the amount of which is proportional to the number of incorporated nucleotides.The liberated PPi is monitored using a coupled enzymatic reaction in which ATP sulfurylase converts PPi to ATP in the presence of APS. The produced ATP is sensed by luciferase, which then turns into visible light that can be detected with charge couples device (CCD) camera (Nyren 1987). Pyrosequencing is a rapid and accurate method to produce short DNA sequences, and it is an especially convenient method in detection of single point mutations.

Table 3. Primers used in PCR amplification and sequencing of QRDR of the *gyrA*, *gyrB*, *parC* and *parE* genes.

Primer	Sequence (5' to 3')	Use	Reference
stmgyrB1	GCGCTGTCCGAACTGTACCT	*gyrB* PCR	(Eaves et al. 2004)
stmgyrB2	TGATCAGCGTCGCCACTTCC		
stmparC1	CTATGCGATGTCAGAGCTGG	*parC* PCR	(Eaves et al. 2004)
stmparC2	TAACAGCAGCTCGGCGTATT		
stmparE1	TCTCTTCCGATGAAGTGCTG	*parE* PCR	(Eaves et al. 2004)
stmparE2	ATACGGTATAGCGGGCGGTAG		
Salm_gyrA_k_PCR_for	ATGAGCGACCTTGCGAGAGAAATTA	*gyrA* PCR	This study
Salm_gyrA_k_PCR_rev	TTACTCGTCAGCGTCATCCGCA		
Salm_gyrA_seq1f	GCGGCGGCAATGCGTTATAC	*gyrA* sequencing	(Ouabdesselam et al. 1996)
Salm_gyrA_seq2r	TTGGTCGGCATGACGTCCG		
Salm_gyrA_seq3f	ATCAGCGCGCTGCGTGAC	*gyrA* sequencing	(Ouabdesselam et al. 1996)
Salm_gyrA_seq4r	CGAAGGAAACCTGTAGCTGG		
Salm_gyrA_seq5f	AGGCGATTCTGGATCTGCGT	*gyrA* sequencing	(Ouabdesselam et al. 1996)
Salm_gyrA_seq6r	ACTTCCATCAGGCGATCGGC		
Salm_gyrA_seq7r	AACGCCAATCAGCTCGTCGC	*gyrA* sequencing	(Ouabdesselam et al. 1996)
Salm_gyrA_seq8f	CGACCGGTGTGCGCGGTA		
Salm_gyrA_seq9r	CATCTACCGGTTCAGCAACGC	*gyrA* sequencing	(Ouabdesselam et al. 1996)
Salm_gyrA_seq10f	TATCGACCGTCTGCTGGTG		
Salm_gyrA_307_for	AAGCCGGTACACCGTCGCGTACTT	*gyrA* PCR	Paper **I**
Salm_gyrA_570_rev	bio-TTTCGCCAGACGGATTTCCG		
Salm_gyrAser83seq	AATACCATCCCCACGGCGAT	pyrosequencing	Paper **I**

4.3.2.5.   Detection of QRDR mutations in target genes with DHPLC (**III**)

Mutations in the QRDR of DNA gyrase and topoisomerase IV were screened from ten selected *S. enterica* isolates, showing the nonclassical quinolone resistance phenotype. Mutations in the target genes *gyrA*, *gyrB*, *parC* and *parE* were identified with denaturing high pressure liquid chromatography (DHPLC) method with the Wave DNA fragment analysis system (Transgenomic Inc., Glasgow, UK) which employs a combination of temperature-dependent denaturation of DNA and ion pair chromatography as described by Eaves et al. (Eaves et al. 2002).

The Transgenomic WAVE® System Instruments utilize DHPLC, which is among the most sensitive and accurate technologies for detection of unknown genetic mutations and single nucleotide polymorphisms (SNPs). DHPLC identifies mutations and polymorphisms based on detection of heteroduplex formation between mismatched nucleotides in double-stranded DNA. Sequence variation creates a mixed population of heteroduplexes and homoduplexes during reannealing of wild-type and mutant DNA. When this mixed population is analyzed with HPLC under partially denaturing temperatures, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature (Transgenomic 2009).

4.3.3.   Detection of transferable resistance (II & III)

4.3.3.1.   Primers

The oligonucleotide primers used in the PCR amplification PMQR determinant and DNA sequencing are presented in Table 4. The primers were synthesized by Thermo Fisher Scientific (Ulm, Germany).

4.3.3.2.   PCR Amplification of *qnrA*, *qnrB*, *qnrS* and *qnrD* (**III, unpublished**)

PCR was used to screen for plasmid-mediated quinolone resistance (PMQR) determinants in all *S. enterica* isolates showing the nonclassical quinolone resistant phenotype. Totally 46 isolates, collected between 2003 and 2008, were tested. Amplification of *qnrA*, *qnrB* and *qnrS* alleles was performed with Multiplex PCR with a previously described method (Robicsek et al. 2006a) and specific primers (Table 4) in a Biometra T Personal instrument (Biometra GmbH i.L., Goettingen, Germany). Amplification included 32 cycles of 94˚C for 45 s, 53˚C for 45 s and 72˚C for 1 min. The positive controls were *E. coli* J53 pMG252 (positive for *qnrA*, kindly provided by Dr Neil Woodford) and pMG298 (positive for *qnrB*, strain kindly provided by Dr George Jacoby). The *qnrD* allele was separately amplified by PCR using previously described primers (Table 4) and protocols (Cavaco et al. 2009). As a positive control we used *Klebsiella pneumoniae* (U58), an isolate in our collection which has previously been confirmed carrying *qnrD*.

4.3.3.3.   Screening of *aac(6')-Ib-cr* and *qepA* genes with PCR (**III, unpublished**)

46 nonclassical quinolone-resistant isolates, collected from 2003 to 2008 were also screened for two other PMQR genes, *aac(6')-Ib-cr* and *qepA*. The *aac(6')-Ib-cr* and *qepA* genes were amplified in separate reactions with previously described primers (Table 4) and protocols (Park et al. 2006; Yamane et al. 2008), respectively. PCR amplification of *aac(6')-Ib-cr* included the following steps: Initial denaturation at 94˚C for 10 min, followed by 35 cycles of 94˚C for 30 s, 59˚C for 30 s and 72˚C for 1 min. The *qepA* was amplified as follows: Initial denaturation at 96˚C for 1 min, followed by 30 cycles of 96˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min and final extension at 72˚C for 5 min. As positive controls, *E. coli* J53 (pEK499) (positive for *aac(6')-Ib-cr*, kindly provided by Dr Neil Woodford) and *E. coli* KAM32 (pSTVqepA) (positive for *qepA*, kindly provided by Dr Kunikazu Yamane) were used.

4.3.3.4.   Sequencing of *qnr* genes (**III, unpublished**)

Of the 46 *S. enterica* isolates, which were *qnr*-positive, PCR results were confirmed by direct sequencing of both strands of amplicons using specific PCR primers (Table 4). The PCR products were purified with High Pure PCR Product Purification Kits (Roche Diagnostics Corporation, Roche Applied Science, IN, USA). Sequencing reactions were performed using an ABI BigDye™ Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) and sequencing was performed with an Applied Biosystems 3730 DNA Analyser (Hakanen et al. 1999a).

4.3.3.5.   The *qnr* plasmid analysis (**III**)

Plasmid DNA from ten selected *qnr* positive *S. enterica* isolates was extracted using the QIAprep Spin Miniprep kit (QIAGEN Finland, Helsinki, Finland). The presence of *qnr* genes was ensured using Southern hybridization as previously described by Randall et al. (Randall et al. 2004), where positive *qnr* genotypes were compared with previously identified *qnr* plasmids.

Qnr plasmids were further analyzed by restriction mapping. The plasmid DNA was restricted with *Hind*III (New England Biolabs / Finnzymes, Espoo, Finland) enzyme. Reaction mixture contained 4 µl of plasmid DNA, 4 µl aqua, 1 µl 10 x buffer 2 and 1 µl *Hind*III. Reaction was incubated at +37°C for one hour. To observe the restriction pattern, resulting fragment sizes were determined by gel electrophoresis in a 0.8% agarose gel.

4.3.3.6.   The *qnr* plasmid transformation assay (**III**)

Electroporation was used to transfer the plasmid DNA suspected to carry *qnr* alleles into *E. coli* DH5α competent cells. Electroporation was performed in a Gene-Pulser (Bio-Rad, Hercules, CA, USA) using a 0,1 cm Gene pulser/MicroPulser cuvette (Bio-

Rad) with electroporation conditions being 2.5 kV, 25 μf and 200 Ω. Transformants were selected on LB agar plates containing 0.03 mg/L ciprofloxacin.

4.3.4. Altered quinolone accumulation (III)

The efflux activity of the ten nonclassical quinolone resistant isolates was assessed by determining the accumulation of ciprofloxacin and the fluorescent substrate Hoechst 33342 (*bis*-benzimide) (Sigma). Uptake of Hoechst 33342 was determined as described previously by Webber et al. (Webber et al. 2008). Fluorescence of Hoechst 33342 substrate was measured using excitation and emission wavelengths of 355 and 365 nm, respectively, using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). Ciprofloxacin accumulation was measured as previously described by Mortimer et al. (Mortimer and Piddock 1991), and ciprofloxacin concentration was measured using an LS45 Fluorospectrofotometer (Perkin Elmer, Cambridge, UK) at excitation and emission wavelengths of 311 and 447 nm, respectively. For both substrates, accumulation was measured in the presence and absence of 100 μM CCCP (carbonylcyanide *m*-chlorophenylhydrazone), added to cell suspensions prior to addition of Hoechst 33342 substrate or ciprofloxacin. Results from the nonclassical quinolone resistant strains were compared with *S*. Typhimurium SL1344.

4.3.5. Genotyping of reduced fluoroquinolone susceptible isolates (II)

Pulsed field gel electrophoresis (PFGE) was used to analyze for clonal relationship between the 36 *Salmonella* isolates showing the nonclassical quinolone resistance phenotype. The PFGE analysis was performed as previously described by Lukinmaa et al. (Lukinmaa et al. 2006). Restriction enzyme *Xba*I (Fermentas Life Sciences, Burlington, Ontario, Canada) was used for the digestion of DNA and the resulting DNA fragments were separated in a 1.2% PFGE-agarose gel with a CHEF DRR III system (Bio-Rad). Any difference between two profiles was considered sufficient to distinguish two different PFGE profiles. Similarity values were calculated by unweighted pair-group method with arithmetic averages and the Dice coefficient by using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

4.4. Macrolide resistance mechanisms

4.4.1. Primers (IV)

For the PCR screening of 23S rRNA mutations A2058 and A2059, and 50S ribosomal proteins L4 and L22, the primers presented in Table 5 were used. All primers were produced by Thermo Fisher Scientific.

4.4.2.   Detection of macrolide resistance determinants (**IV**)

PCR was used to screen macrolide resistance causing mutations in *S. enterica* isolates, which were resistant (MIC ≥32 mg/L) to azithromycin, showed resistance (MIC ≥32 mg/L) to erythromycin and belonged to the nonclassical quinolone resistance phenotype.

4.4.2.1.   Detection of A2058 and A2059 mutations by pyrosequencing (**IV**)

Pyrosequencing was used to detect point mutations in the ribosomal target sites A2058 and A2059 (*E. coli* numbering) of the 23S rRNA which cause macrolide resistance in *Enterobacteriaceae*. Mutations were screened among all azithromycin resistant *Salmonella* isolates and isolates belonging to the nonclassical quinolone resistance phenotype with erythromycin resistance, MIC ≥32 mg/L. PCR amplification and pyrosequencing screening of A2058 and A2059 mutations were performed with previously described primers (Table 5) and protocols (Haanperä et al. 2005). A specific nucleotide dispensation order (TAAGCAAGACCATG) was designed based on wild-type and mutated A2058 and A2059 sequences.

4.4.2.2.   PCR amplification and sequencing of genes encoding ribosomal proteins L4 and L22 (**IV**)

50S ribosomal proteins L4 and L22 of *S. enterica* were amplified by PCR with specific primers. Mutations were screened among 18 azithromycin-resistant isolates and 19 isolates showing the nonclassical quinolone resistance, of which six were azithromycin-resistant and 13 azithromycin-susceptible, but all of them were erythromycin resistant. Ribosomal protein L4 and L22 gene-specific primers (Table 5) were used to amplify the complete L4 gene *rlpD* and the L22 gene *rlpV*, respectively. The PCR conditions were as follows: 94°C for 10 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 33 cycles. Any L4 or L22-positive results were confirmed by direct sequencing of both strands of amplicons using specific PCR primers. PCR products were purified with High Pure PCR Product Purification Kits and ABI BigDye™ Terminator Cycle Sequencing Kit version 3.1 was used to prepare the sequencing reactions. Samples were sequenced with an Applied Biosystems 3730 DNA Analyser.

4.5.   Data analysis (I-IV)

4.5.1.   Susceptibility data analysis

The susceptibility data were analyzed by using the WHONET 5.4 computer program. The WHONET is a free software, developed by Thomas O'Brien and John Stelling in WHO collaborating centre for the surveillance of antibiotic resistance. The WHONET

software can be downloaded free of charge from the website www.who.int/drugresistance/whonetsoftware.

4.5.2.   Sequence analysis

Nucleotide sequence data from studies III and IV was assembled, edited and analyzed using a Vector NTI Advance™ 11 (Invitrogen, Carlsbad, CA, USA) and BioEdit Sequence Aligment Editor programs (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The analyzed sequences were compared to the published nucleotide sequences with BLAST search through the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/blast/).

4.5.3.   Statistical analysis (II & III)

Exact methods used for statistical analyses are described in respective papers (**II-III**). The statistical data were analysed using the SAS system for Windows, version 9.1.3. P values of <0.05 were considered significant.

4.5.4.   Passenger statistics (II)

The data concerning the number of travels from Finland to abroad during the study months was kindly provided from the Statistics Finland (www.stat.fi) by personal communication. These data are based on the results of an ongoing interview study of the Finnish population aged 15 – 74 years old (a total of 3.8 – 3.9 million persons). In every second month, 2000–3000 persons are taken into sample and being interviewed. One travel in sample data correlates with 1200–1300 travels on the whole population level.

Table 4. Primers used in PCR amplification and sequencing of PMQR determinants

Primer	Sequence (5' to 3')	Use	Reference
qnrA_f	ATTTCTCACGCCAGGATTTG	*qnrA* PCR	(Robicsek et al. 2006a)
qnrA_r	GATCGGCAAAGGTTAGGTCA		
qnrB_f	GATCGTGAAAGCCAGAAAGG	*qnrB* PCR	(Robicsek et al. 2006a)
qnrB_r	ACGATGCCTGGTAGTTGTCC		
qnrS_f	ACGACATTCGTCAACTGCAA	*qnrS* PCR	(Robicsek et al. 2006a)
qnrS_r	TAAATTGGCACCCTGTAGGC		
qnrD_f	GACAGGAATAGCTTGGAAGG	*qnrD* PCR	(Cavaco et al. 2009)
qnrD_r	CTGCTCTCCATCCAACTTCA		
aac(6')-Ib-cr-F_A	TTGCGATGCTCTATGAGTGGCTA	*aac(6')-Ib-cr* PCR	(Park et al. 2006)
aac(6')-Ib-cr-R_A	CTCGAATGCCTGGCGTGTTT		
qepA-F	GCAGGTCCAGCAGCGGGTAG	*qepA* PCR	(Yamane et al. 2008)
qepA-R	CTTCCTGCCCGAGTATCGTG		

Table 5. Primers used for screening mutations causing the macrolide resistance

Primer	Sequence (5' to 3')	Use	Reference
23SV_univF_1926	TAAGGTAGCGAAATTCCTTGTCG	PCR screening of A2058/A2059 mutations by pyrosequencing	(Haanperä et al. 2005)
Bio_23SV_univR_2259	Bio-CGACCGCCCCAGTCAAACT		
23SV_gpos_seq	CCGCGGCAAGACGG		
Salm_L4_f	TGAAGGCGTAAGGGGATAGCA	*rlpD* PCR	paper **IV**
Salm_L4_r	TCAGCAGACGTTCTTCACGAA		
Salm_L22_f	GAAATAAGGTAGGAGGAAGAG	*rlpV* PCR	paper **IV**
Salm_L22_r	CCATTGCTAGTCTCCAGAGTC		

5. RESULTS

5.1. Fluoroquinolone resistance among *S. enterica* in Finland

5.1.1. *S. enterica* of foreign origin (I-III, unpublished)

From 2003 to 2007, altogether 499 *S. enterica* isolates collected from Finnish travelers returning from abroad were analyzed. Of these isolates, 227 (45.5%) were collected from travelers returning from Thailand or Malaysia. Between 2003 and 2007, the reduced fluoroquinolone susceptibility among foreign *Salmonella* isolates decreased significantly from 48% to 34% (p = 0.029) (Figure 9). Among isolates collected from Thailand and Malaysia, the reduced fluoroquinolone susceptibility decreased more drastically, from 65% to 32% and the decrease was significant (p = 0.002).

Of the collected isolates, 36 (7.2%) belonged to the nonclassical quinolone resistance phenotype, i.e. they showed reduced susceptibility to ciprofloxacin (MIC ≥0.125 mg/L) and were either susceptible (MIC <32 mg/L) or only low-level resistant (MIC =32 mg/L) to nalidixic acid. All the other isolates showed conventional quinolone resistance, i.e. they were highly resistant (MIC >32 mg/L) to nalidixic acid. Among all foreign isolates, the conventional quinolone resistance decreased significantly from 39% in 2003 to 22% in 2007 (p = 0.012). The decrease was even more distinct, from 47% to 12%, in isolates collected from travelers returning from Thailand and Malaysia (p = 0.0014).

The proportion of nonclassical quinolone resistance phenotype isolates was the highest in 2007, when 12 isolates (35.3%) of the reduced ciprofloxacin susceptible isolates belonged to this novel phenotype, whereas in 2005, only two isolates (7.1%) belonged to the nonclassical quinolone phenotype (Figure 9). The nonclassical quinolone resistance phenotype among foreign *S. enterica* isolates has slightly increased between 2003 and 2007 (from 9.0% to 12.0%), but no significant difference (p = 0.720) was detected between the years. Among isolates collected from travelers returning from Thailand and Malaysia, the nonclassical quinolone resistance varied, but the variation was not significant (p = 0.878). In 2008, 63% of the foreign *S. enterica* isolates were collected from travelers returning from Southeast Asia. 36 (36%) isolates showed reduced fluoroquinolone susceptibility and 10 of them, collected from travelers returning from Thailand and Philippines, belonged to the nonclassical quinolone resistance phenotype. Between 2003 and 2008, the proportion of the nonclassical quinolone resistance phenotype among all reduced fluoroquinolone susceptible isolates has increased from 18.8% to 27.8%, being highest in 2007 (35.3%).
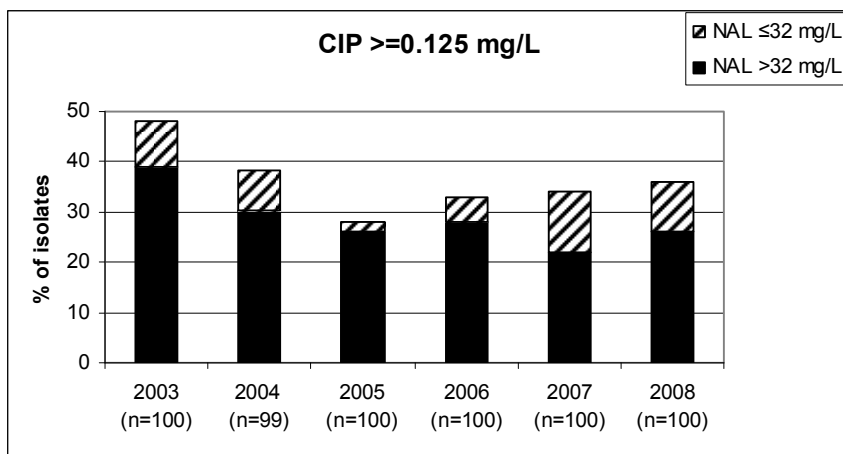
Figure 9. Trends in reduced fluoroquinolone susceptibility among foreign *S. enterica* isolates between 2003 and 2008.

5.1.2. *S. enterica* of Finnish origin (unpublished)

From 2003 to 2008, altogether 638 domestic *S. enterica* isolates were analyzed. Antimicrobial susceptibility testing revealed that 77 (12.0%) isolates showed reduced ciprofloxacin susceptibility (MIC ≥0.125 mg/L). The incidence of reduced fluoroquinolone susceptibility was highest in 2003 when 15% of the isolates had ciprofloxacin MIC ≥0.125 mg/L, and thereafter the reduced fluoroquinolone susceptibility has fluctuated between 15% and 10% (Figure 10).

Between 2003 and 2008, the majority of the reduced fluoroquinolone susceptible isolates were highly resistant to nalidixic acid (MIC ≥256 mg/L). However, from 2003 onwards the nonclassical quinolone resistant phenotype has also been detected among domestic isolates. Among domestic isolates, the nonclassical quinolone resistance phenotype was most abundant in 2003, and since then this phenotype has been detected fairly seldom. Totally seven isolates collected in different years (three isolates in 2003, one in 2005, two in 2007 and one in 2008), showed reduced fluoroquinolone susceptibility and were susceptible or only low-level resistant to nalidixic acid (MIC ≤32 mg/L) (Figure 10). Five of these isolates belonged to the *S.* Corvallis serotype, and in addition one *S.* Enteritidis and one *S.* Typhimurium serotype showed the nonclassical quinolone resistance phenotype. All the other 70 isolates with reduced fluoroquinolone susceptibility were highly resistant to nalidixic acid. The majority of the domestic isolates with high resistance to nalidixic acid belonged to *S.* Enteritidis serovar (41.4%), but also *S.* Typhimurium (17.1%) and *S.* Virchow (17.1%) serotypes were commonly detected.
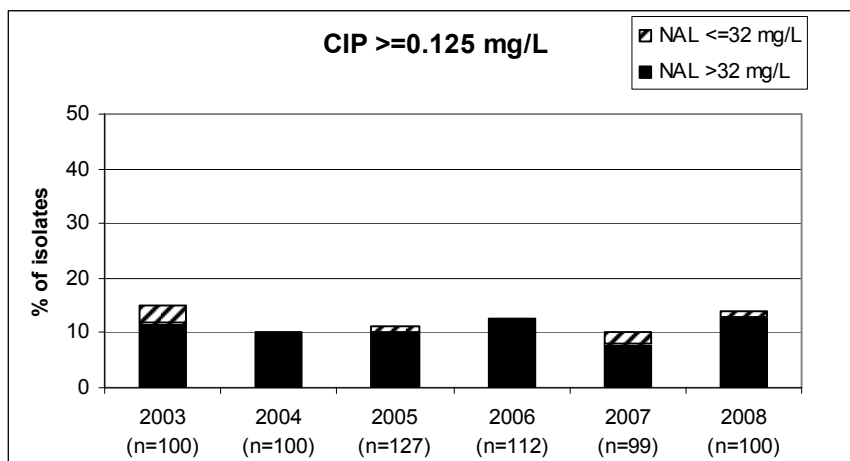
Figure 10. Trends in reduced fluoroquinolone susceptibility among domestic *S. enterica* isolates between 2003 and 2008.

5.1.3. Epidemiology of a nonclassical quinolone resistance phenotype of *S. enterica* (I-II, unpublished)

The first *S. enterica* isolates showing the nonclassical quinolone resistant phenotype were originated from Southeast Asia, specifically from Thailand and Malaysia. Between 2003 and 2008, we have totally collected 46 *S. enterica* isolates showing reduced ciprofloxacin susceptibility and being either susceptible or only low-level resistant to nalidixic acid (MIC ≤32 mg/L). 40 (87%) of these isolates have been collected from Finnish travelers returning from Thailand, five from Malaysia and one from the Philippines.

In Thailand and Malaysia, the percentage of the nonclassical quinolone resistance phenotype has been higher than among all foreign *S. enterica* isolates tested. Between 2003 and 2008, the novel quinolone-resistant phenotype has been found in 17.6%, 21.6%, 6.3%, 10.4%, 20.3% and 16.1%, respectively, of the isolates yearly tested from those travel destinations (Figure 11). The nonclassical quinolone resistance phenotype was most abundant in 2007, when 12 isolates belonged to this novel phenotype. Although the prevalence of the nonclassical quinolone resistant isolates has varied between the years, this phenotype has a major role in the reduced fluoroquinolone susceptibility in *S. enterica* isolates, especially in Southeast Asia.

Salmonella Enteritidis and *S.* Typhimurium, which are the most common serotypes isolated from Finnish travelers, were not common among the nonclassical quinolone resistance phenotype. Between 2003 and 2008, the nonclassical quinolone resistance phenotype has been found in seven different serovars, of which *S.* Corvallis (21 isolates), *S.* Stanley (7 isolates) and *S.* Typhimurium (7 isolates) were the most prevalent. In contrast, among the conventional quinolone resistant isolates *S.* Enteritidis and *S.* Virchow were the most prevalent serotypes.
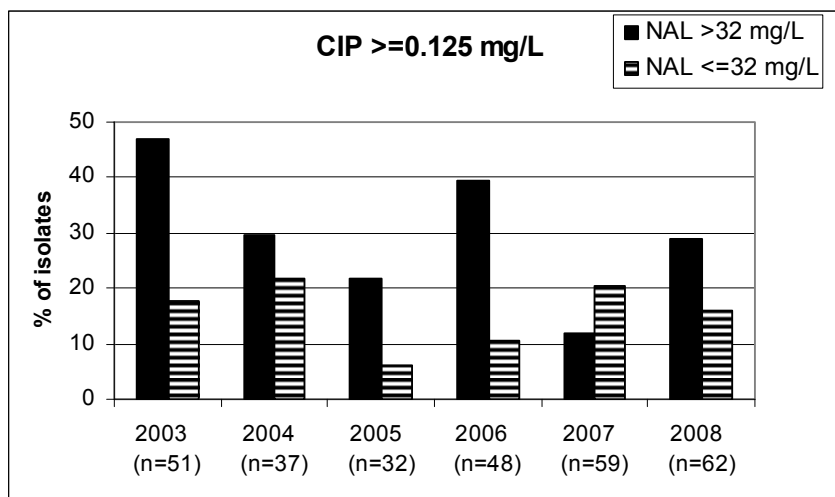
Figure 11. Prevalence of isolates showing the conventional quinolone resistance (MIC >32 mg/L) and the nonclassical quinolone resistance (MIC ≤32 mg/L) phenotype in Thailand and Malaysia.

5.1.4. PFGE analysis of the nonclassical quinolone resistant isolates (II)

The 36 isolates belonging to the nonclassical quinolone resistance phenotype were distinguished by PFGE to 16 different PFGE patterns. The *S*. Corvallis isolates (n=17) belonged to 7 different types and the *S*. Stanley (n=6) isolates to 2 different types. Two different PFGE types were observed among the *S*. Typhimurium isolates (n=5) and *S*. Montevideo isolates (n=2). One single PFGE type was detected among the *S*. Braenderup isolates (n=4) and one PFGE type each among the *S*. Mbandaka (n=1) and *S*. Virginia (n=1) isolates.

5.2. Detection of chromosomal QRDR mutations

5.2.1. Detection of *gyrA* mutations with pyrosequencing (I)

Mutations at the gene coding codons Ser83 and Asp87 of the QRDR of *gyrA*, which are the most common sites for mutation causing fluoroquinolone resistance, were not detected with pyrosequencing. Instead, all tested isolates showing the nonclassical quinolone resistance phenotype had wild-type sequence in the QRDR of *gyrA*.

5.2.2. PCR amplification and sequencing of the whole *gyrA* gene (I & III)

Sequencing of the whole *gyrA* gene from six *S. enterica* isolates revealed that two nonclassical quinolone resistant isolates possessed substitution mutation Leu396-Met in *gyrA*, but no other mutations were detected.

5.2.3. DHPLC detection of QRDR mutations (III)

None of the common mutations associated with fluoroquinolone resistance in the QRDRs of the *gyrA*, *gyrB* or *parE* genes were detected by DHPLC analysis. However, all strains with the nonclassical quinolone resistance phenotype except *S*. Typhimurium strains possessed a Thr57-Ser substitution in *parC*, including control strains.

5.3. Detection of transferable resistance

5.3.1. The *qnr* genes (II & III, unpublished)

Multiplex PCR results show that all 46 *S. enterica* isolates with a nonclassical quinolone resistance phenotype were *qnr* positive: The *S*. Mbandaka strain contained the *qnrA1* gene whereas the remainder of the strains all carried the *qnrS1* gene. The *qnrB* and *qnrD* genes were not detected.

Plasmid analysis of the ten *qnr*-positive strains showed that the *qnrA1* and *qnrS1* genes were located in plasmids ranging in size from 13 to 30 kb. Plasmid digestion with *Hind*III enzyme revealed that four of the strains had a similar restriction pattern, whereas five had unique restriction patterns and we could not obtain any restriction pattern from *S*. Stanley strain. Southern blotting analysis confirmed that the *qnr* alleles were present on the isolated plasmids.

Transformation assay revealed that *qnr* genes were transferable *in vitro*. Plasmids from seven out of ten *qnr*-positive strains could be transferred by electroporation to *E. coli* DH5α, resulting in seven *qnrS*-positive transformants. PCR amplification confirmed that the transformants harbored the same *qnrS1* gene as their donor strains. Susceptibility testing showed that the MICs of ciprofloxacin and nalidixic acid for all transformants were similar to those of the corresponding host strain.

All seven domestic *S. enterica* isolates with the nonclassical quinolone resistance phenotype were *qnrS1* positive.

5.3.2. The *aac(6')-Ib-cr* and *qepA* genes (III)

All tested isolates with the nonclassical quinolone resistance phenotype were negative for aminoglycoside acetyltransferase gene *aac(6')-Ib-cr* and quinolone efflux pump encoding gene *qepA*.

5.4. Altered drug accumulation (III)

Six out of 10 nonclassical quinolone resistant *S. enterica* isolates tested accumulated significantly less of both ciprofloxacin and Hoechst 33342 substrate than the wild-type control SL1344, indicating that increased efflux activity was present in these strains

which may contribute to the resistance phenotype observed. Of these six strains, two did not increase the level of ciprofloxacin accumulated upon the addition of CCCP, indicating that the low accumulation of quinolones in these strains is not dependent on the proton motive force.

5.5. Resistance to macrolides (IV, unpublished)

5.5.1. MICs

According to the antimicrobial susceptibility testing, there were two different populations of *S. enterica* considering azithromycin susceptibility. The majority of the isolates had azithromycin MICs of 4–8 mg/L, i.e. they represented the wild type population, whereas the azithromycin-resistant isolates had MICs of 32−≥128 mg/L. Erythromycin resistance in *S. enterica* is common, the erythromycin MIC distribution was 8–≥128 mg/L and the vast majority of isolates had erythromycin MIC ≥64 mg/L.

Between 2003 and 2008, azithromycin resistance (MIC ≥32 mg/L) was detected in 24 (1.9%) of all 1237 isolates and high-level resistance (MIC ≥128 mg/L) in 12 isolates. Nine (1.4%) out of 638 domestic isolates were azithromycin resistant and six of these showed high-level resistance. Among 599 foreign isolates, 15 (2.1%) were azithromycin resistant and six showed high-level resistance. Azithromycin resistance has been most prevalent in 2003 and 2004 among both domestic and foreign isolates. In domestic S. enterica isolates, high-level azithromycin resistance was the most common between 2003 and 2004 whereas in foreign isolates, high-level resistance was detected regularly during the whole study period.

Of the reduced fluoroquinolone susceptible isolates, four (5.2%) domestic isolates and 11 (5.1%) foreign isolates were azithromycin-resistant and three isolates from the both groups showed high-level resistance to azithromycin. Among the 53 nonclassical quinolone-resistant isolates, six (11.3%) were azithromycin resistant, one of them highly resistant. All of these resistant isolates were of foreign origin, and all were collected between 2003 and 2004.

While the azithromycin resistance is relatively rare among *S. enterica* isolates, erythromycin resistance is common: 806 (99.6%) *S. enterica* isolates had erythromycin MIC ≥32 mg/L. Erythromycin MIC distribution was as follows: MIC =8 mg/L, two isolates; MIC =16 mg/L, one isolate; MIC =32 mg/L, 42 isolates; MIC =64 mg/L, 450 isolates and MIC ≥128 mg/L, 313 isolates.

5.5.2. Macrolide resistance mechanisms

Pyrosequencing analysis revealed that there were no mutations in A2058 or A2059 of the 23S rRNA gene among azithromycin-resistant isolates, of which all but one isolate was also erythromycin-resistant, or *qnr*-phenotype isolates. The same wild type

sequence, 5'-AAAGACCCCG-3' was found both in azithromycin and erythromycin-susceptible isolates.

The sequencing of 50S ribosomal proteins L4 and L22 revealed two distinct amino acid substitutions in *rlpD* and one amino acid substitution in *rlpV* gene, respectively. G235A and C379T mutations were found in the *rlpD* gene and G25A mutation in the *rlp*V gene. A Glu79-Lys mutation in the *rlpD* was found in six isolates. Those isolates showed resistance to azithromycin, erythromycin and telithromycin, and belonged to three different serovars. An Arg127-Trp substitution in the *rlpD* gene was found in three *S.* Montevideo strains, which were erythromycin-resistant, azithromycin-susceptible and showed the nonclassical quinolone resistance phenotype. Those *S.* Montevideo strains also had Asp9-Asn mutation in *rlpV* gene, outside the coding region.

5.6. Resistance to other antimicrobials (unpublished)

Ampicillin resistance (MIC ≥128 mg/L) was very common among *S. enterica* isolates during the whole study period, between 2003 and 2008. 86 (13.5%) domestic and 84 (14.0%) foreign isolates were ampicillin resistant. Among domestic isolates, ampicillin resistance has increased from 2% to 19% between 2003 and 2008, being highest in 2005 (26%). Among foreign *S. enterica* isolates, ampicillin resistance has increased more steadily from 13% to 23%. Ampicillin resistance was the lowest in 2004, when only five foreign and none the domestic isolates were ampicillin resistant.

Resistance to chloramphenicol (MIC ≥128 mg/L) was detected in 47 (7.4%) domestic and 40 (6.7%) foreign isolates. Chloramphenicol resistance was not detected in the domestic isolates in 2003 and 2004. Unexpectedly, in 2005, chloramphenicol resistance was 20.5% and has decreased thereafter being 7.0% in 2008. Among the foreign *S. enterica* isolates, chloramphenicol resistance has decreased: Resistance rate was highest (11%) in 2003 and lowest (3%) in 2008.

Trimethoprim-sulfamethoxazole resistance was more common among foreign *S. enterica* isolates (8.0%) than in domestic isolates (1.7%). Trimethoprim-sulfamethoxazole resistance was highest among the domestic isolates in 2006 (4.5%), and in the foreign *S. enterica* isolates in 2003 (16.0%). Resistance to sulfa-trimethoprim has fluctuated both in the domestic and the foreign isolates, but resistance has been more prevalent in the foreign isolates.

The classical *Salmonella* MDR, i.e. resistance to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, was rare in domestic isolates. Only three (0.5%) domestic isolates (two isolates from 2006 and one from 2008) were MDR, whereas 13 (2.1%) foreign isolates showed MDR. The majority of the foreign MDR isolates were collected between 2006 and 2008 (2–4 isolates per year), but four MDR isolates were collected also in 2003. Six foreign MDR isolates showed reduced fluoroquinolone susceptibility and one of them belonged to the nonclassical quinolone resistance phenotype, whereas all domestic MDR isolates were ciprofloxacin susceptible.

Four *S. enterica* isolates (three foreign, collected in 2004 and 2008, and one domestic, collected in 2006), were highly resistant to ampicillin (MIC ≥128 mg/L) and amoxicillin-clavulanic acid (>64 mg/L) and showed resistance to cephalothin (>64 mg/L), ceftazidime (≥32 mg/L) and cefuroxime (=32 mg/L), i.e. they were possible β-lactamase producers. However, they did not show reduced fluoroquinolone susceptibility. In addition, three nonclassical quinolone resistance phenotype strains were ampicillin and amoxicillin-clavulanic acid resistant and two of them were highly resistant, but resistance to cephalosporins was not detected. Those two strains were TEM-1 positive, which was confirmed with sequencing.

6.   DISCUSSION

During the centuries, diseases caused by bacteria have always been a problem for humans. In the pre-antibiotic era, treatment choices for different infectious diseases have been limited due to lack of proper medicine, mortality rates have been high and an average life span has been low. When Alexander Fleming invented penicillin, it was thought to solve all problems concerning the public health, since penicillin was proved to be effective against most of the Gram-positive organisms although it had no influence on Gram-negative enteric bacteria causing diseases like typhoid and paratyphoid fever. However, as we now know, penicillin did not solve the problem at all. Already Fleming discovered that bacteria developed antibiotic resistance whenever too small doses of penicillin were used or when it was used for a too short treatment period, and this resistance problem is still continuing (Noskin 2008). Evolution in bacteria is so rapid that every time a new antimicrobial agent has come into market, we almost immediately encounter bacteria resistant to that particular agent. Thus, resistance to antimicrobials has been a problem since the discovery of the first antimicrobial agent and the invention of novel antimicrobial agents has not solved this problem yet.

Typhoid fever caused by *S. typhi* has been treated with chloramphenicol since 1948, but already in the 1970s plasmid-mediated resistance to chloramphenicol emerged, which led to changes in the therapeutic guidelines. When MDR *S. typhi* emerged in the 1990s, also ampicillin and trimethoprim-sulfamethoxazole were out of option for the treatment of typhoid fever. Thereafter, fluoroquinolones have been the drug of choice for the treatment of *Salmonella* infections. However, along with the abundant use of fluoroquinolones both in humans and in veterinary medicine, fluoroquinolone resistance has exploded during the last few decades, also among *Salmonella* isolates (Miller and Pegues 2000). It has been speculated whether fluoroquinolone usage in veterinary medicine is the ultimate reason for the increased fluoroquinolone resistance problem. Since the observance of the emerging resistance problem especially in farm animals, there have been several regulations and refusals concerning fluoroquinolone usage in animals. However, the fact that reduced fluoroquinolone susceptibility is continuously increasing indicates that fluoroquinolone-resistant clones are here to stay – which is of concern, since fluoroquinolones are still widely used antimicrobials for the treatment of different human infections.

6.1.   Reduced fluoroquinolone susceptibility in *S. enterica* isolates

6.1.1.   Classical reduced fluoroquinolone susceptibility

Fluoroquinolones are the most commonly used antimicrobial agents for the treatment of salmonellosis in adult patients (Stevenson et al. 2007). Unfortunately, the proportion of nontyphoidal strains of *Salmonella enterica* with reduced fluoroquinolone susceptibility has increased during recent years in many countries (Chau et al. 2007;

Hakanen et al. 2001; Hakanen et al. 2006; Parry et al. 2002). In Finland, fluoroquinolone susceptibility of salmonellas has been surveyed since 1995 by analyzing isolates from Finnish patients who acquired the disease either at home or abroad. Between 1995 and 2004, a significant increase (from 4% to 39%) was observed in the annual proportions of reduced fluoroquinolone susceptibility (MIC ≥0.125 mg/L) among *Salmonella* isolates of foreign origin (Hakanen et al. 2001; Hakanen et al. 2006). The increase was most prominent among the isolates collected from travelers returning from Southeast Asia, especially Thailand (Hakanen et al. 2001). Reduced fluoroquinolone susceptibility is relatively common also in Europe, especially in Spain and the Mediterranean Area (Hakanen et al. 2001; Hakanen et al. 2006).

It is shown in this study that reduced fluoroquinolone susceptibility as a whole among nontyphoidal strains of *S. enterica* from Finnish travelers showed a significant decrease during the study period, 2003–2007, after having constantly increased for several years (Hakanen et al. 2001; Hakanen et al. 2006). The significant decrease (p = 0.029) from 2003 to 2007 was driven by isolates derived from Southeast Asia, while the proportion of resistant strains among the isolates from the other travel destinations remained fairly stable. Notably, the recent decreasing trend in resistance was not linked with reduced traveling to Thailand, as the number of tourists from Finland going to Thailand has more than doubled from 2005 to 2007.

We have shown in this thesis that reduced fluoroquinolone susceptibility is fluctuating among domestic *S. enterica* isolates and no decreasing trend in the reduced susceptibility to fluoroquinolones can be observed. It is also notable that while the highest percentage of reduced fluoroquinolone susceptibility among domestic isolates has been 15%, detected in 2003, the reduced fluoroquinolone susceptibility rate is overall much lower than in foreign isolates. Although reduced fluoroquinolone susceptibility among foreign *S. enterica* isolates has decreased between 2003 and 2008 (from 48% to 36%), the percentage of reduced fluoroquinolone susceptibility has during the whole study period been evidently higher among isolates collected from Finnish travelers compared to domestic isolates.

The reasons for the earlier high incidence of quinolone resistance in Thailand and Malaysia have not been identified. Yet it is known that although quinolones are not allowed to be used as growth-promoters in any part of the world, they have been used abundantly in animal feed and veterinary medicine in Asia (Akiba et al. 2007; Fabrega et al. 2008; Hakanen et al. 2001). On this basis, it has been previously hypothesized that the significant increase in the quinolone resistance observed in Southeast Asia between 1995 and 2003 might have been due to selection pressure caused by the use of antimicrobial agents either in human medicine or in food production (Hakanen et al. 2001). By the same token, one can hypothesize that a reduction in the use of fluoroquinolones, e.g. due to improved methods of breeding of poultry, might decrease the resistance. It is also possible that the mechanisms causing the high-level fluoroquinolone resistance may have prohibitive fitness cost and therefore the emergence of high-level fluoroquinolone resistant *Salmonella* clones is limited (Giraud

et al. 2003). However, in the absence of any data on antimicrobial consumption in those countries, this is merely speculation.

6.1.2. The nonclassical quinolone resistance phenotype

Until 2002, all *Salmonella* isolates worldwide with reduced ciprofloxacin susceptibility studied were uniformly resistant to nalidixic acid (Hakanen et al. 2001; Hakanen et al. 1999a), i.e. they exhibited the classical quinolone resistance phenotype associated with point mutations in genes encoding the topoisomerase target proteins. In 2003, we identified the first *Salmonella* isolates showing reduced susceptibility to ciprofloxacin but being either susceptible (MIC <32 mg/L) or only low-level resistant (MIC = 32 mg/L) to nalidixic acid. All of the *Salmonella* isolates with this novel quinolone resistance phenotype were from travelers returning from Thailand or Malaysia. Here, the epidemiology of the *S. enterica* isolates showing this nonclassical quinolone resistance phenotype was examined.

The important finding was that after the emergence of the novel quinolone resistance phenotype among *S. enterica* in Thailand and Malaysia, these isolates have persisted in that area. During the study period, 2003–2007, there was some minor variation in the proportion of the novel resistance phenotype among the whole quinolone-resistant population, although these changes have not been significant. For example in 2005, the proportion of the nonclassical quinolone resistance phenotype was only 6%, but in that year, the proportion of all isolates with reduced ciprofloxacin susceptibility was small. This is most likely due to the collection time of foreign isolates soon after the tsunami catastrophe in December 2004. During the first months of 2005, Finnish travelers were compelled to choose a holiday destination other than Thailand, and there were over 20% fewer trips to Thailand that year.

The finding that the nonclassical quinolone resistance phenotype isolates are clustered in Thailand and Malaysia is intriguing and provokes the question of whether the emergence, persistence, and confinement of those isolates in this area might have something to do with the living conditions of the residing population. One can speculate e.g. that *Salmonella* isolates belonging to the novel resistance phenotype could be present in certain types of food commonly consumed in Southeast Asia. Moreover, any changes in the habit to use antimicrobial agents, in either human or veterinary medicine, might contribute to the frequency of this resistance pattern (Hopkins et al. 2005). The recent development even implies that the novel resistance phenotype may be associated with lower fitness costs than the classical phenotype (Giraud et al. 2003), as it seems to be replacing the classical resistance phenotype. In 2007, the proportion of the novel-phenotype isolates for the first time surpassed the proportion of the conventional phenotype isolates in Thailand and Malaysia.

Although we found isolates showing the nonclassical quinolone resistance phenotype also among domestic isolates, it is probable that these isolates are not actually of Finnish origin since the prevalence of domestic *Salmonella* outbreaks is extremely low and only certain serovars are detected in Finland. The explanation might be that these

patients have eaten in the ethnic restaurant or just ate some food supplies, which are imported from Southeast Asia. To date, the nonclassical quinolone resistance phenotype has been detected also in other Western countries, including the United Kingdom, Scotland, Denmark and the Netherlands (Cavaco et al. 2007; Hopkins et al. 2008; Murray et al. 2008; Veldman et al. 2008). However, in all of those reported cases, the novel phenotype salmonellas are originated from Southeast Asia and the source of *Salmonella* infection is mainly imported food, typically chicken meat.

The classical reduced fluoroquinolone susceptibility in association with high-level nalidixic acid resistance was the most common in *S*. Enteritidis serotype, both in domestic and foreign isolates. Also *S*. Typhimurium, *S*. Virchow and *S*. Hadar were commonly detected serotypes. However, the serotype distribution was totally different within the nonclassical quinolone resistant isolates: *S*. Corvallis was the predominant serotype and *S*. Braenderup and *S*. Stanley serotypes were also common. This indicates that the nonclassical quinolone resistance phenotype is concentrated in certain serotypes, which probably are more common in Southeast Asia. PFGE analysis revealed that although the nonclassical quinolone resistance phenotype is restricted to certain serotypes and is geographically stable, isolates showing the nonclassical quinolone resistance phenotype were non-clonal, since the phenotype was found in several PFGE clones. The nonclassical quinolone resistance phenotype might still be a worldwide problem, since although the phenotype is non-clonal, different serotypes can spread very rapidly all over the world due to the increased traveling and trading of breeding animals and food products (Archambault et al. 2006).

Reduced fluoroquinolone susceptibility is also problematic when concerning MIC-breakpoints. The CLSI has set the limits as ≤1 mg/L for susceptibility and ≥4 mg/L for resistance to ciprofloxacin (CLSI 2007), although it is commonly known that high-level resistance to ciprofloxacin among *Salmonella* is very rare. On the other hand, if the susceptibility limit is ≤1 mg/L, it is not precise enough to separate fully susceptible isolates from less fluoroquinolone susceptible isolates, since isolates having ciprofloxacin MIC ≥0.125 mg/L are considered to show reduced susceptibility. This breakpoint debate has been going on for years now, and despite suggestions (Aarestrup et al. 2003), no real action has been made to solve this problem. The EUCAST has now published its own breakpoints for *Salmonella* and ciprofloxacin which are ≤0.5 mg/L for susceptibility and >1 mg/L for resistance (EUCAST 2009a). This EUCAST proposal already is an enormous progress concerning the clinical breakpoints, but even these breakpoints might be insufficient to really detect isolates with reduced fluoroquinolone susceptibility and especially nonclassical quinolone resistant isolates. Failure to do so may lead to treatment errors.

6.2. Distribution of quinolone resistance mechanisms

It has been suggested that the Qnr proteins protect DNA gyrase and topoisomerase IV from quinolone inhibition and that isolates with a *qnr* gene may be less likely to develop topoisomerase mutations than other strains (Cesaro et al. 2008). The results

presented in this thesis are in line with these observations. All isolates showing the nonclassical quinolone resistance phenotype were *qnr*-positive. However, when mutations in the QRDR of gyrase and topoisomerase IV genes were screened, no quinolone resistance causing mutations were detected. All tested *S. enterica* strains representing seven serovars had wild-type amino acid sequences in the QRDR of *gyrA*, *gyrB* and *parE* genes. In addition, pyrosequencing screening of Ser83 or Asp87 mutations in the QRDR of *gyrA* revealed that none of the novel phenotype isolates possessed those mutations. A Thr57-Ser substitution in ParC was detected in *S.* Typhimurium strains, both in quinolone resistant and quinolone-susceptible control strains, which indicates that this mutation has no role in the novel quinolone resistance. It is more likely that there is a polymorphism in *S.* Typhimurium strains and therefore this mutation is probably serovar dependent.

Plasmid-mediated quinolone resistance has emerged very rapidly all over the world (Cavaco et al. 2007). Among the major resistance determinants the *qnr* genes have spread in *Salmonella* and in other strains of the *Enterobacteriaceae* family (Gay et al. 2006; Jacoby et al. 2003; Robicsek et al. 2006a). Plasmids carrying PMQR determinants have high heterogeneity and the spread of these plasmids is mainly due to the increased bacterial host range (Carattoli 2009). In the present study, the *qnr* genes were the only PMQR determinants found among these novel phenotype isolates. The *qnr* plasmid was successfully transferred to a recipient strain in electroporation and subsequently, the same resistance phenotype was seen both in the host and in the recipient strain. Our plasmid profile patterns indicated that horizontal transfer of plasmid-mediated quinolone resistance does occur, since the same plasmid profile was seen in different serovars. On the other hand, the plasmid size and structure of the same *qnrS1* allele could also vary widely. Collectively, these results indicated that the *qnr* plasmids are spreading, but it is not one single successful plasmid. The *qnr* genes are usually located in transferable plasmids explaining their rapid contagion. This can also explain a sudden appearance and the emergence of the novel quinolone resistance phenotype of several *Salmonella* serovars in Thailand and Malaysia.

Jiang et al. has shown that the presence of the AAC(6')-Ib-cr variant is higher among *qnr*-positive than *qnr*-negative strains and that the *qnr* genes are found together with *aac(6')-Ib-cr* gene on the same plasmids in *E. coli* and *Klebsiella* (Jiang et al. 2008). However, our results concerning *Salmonella* strains do not support this finding. We did not find any other PMQR genes, like *aac(6')-Ib-cr* or *qepA*, among these novel phenotype isolates. Although all tested strains were negative for the *qepA* gene, it is possible that enhanced efflux may also play a role in this novel resistance phenotype since six out of ten strains accumulated less ciprofloxacin and Hoechst 33342 substrate than control isolates, which indicate that in some strains efflux might have some role in the nonclassical quinolone resistance phenotype.

6.3. Clinical implications of the nonclassical quinolone resistance phenotype

The clinical significance of reduced fluoroquinolone susceptibility in the strains with the novel quinolone resistance phenotype is presently unkown. To date, no reports in the literature have focused e.g. on the outcome of the patients who have been treated with fluoroquinolones for *Salmonella* infections caused by these novel phenotype strains. However, there are several reports showing that treatment failures may occur, when fluoroquinolones are used as a first-line treatment in infections, caused by *S. enterica* isolates with classical reduced fluoroquinolone susceptibility (Brown et al. 1996; Helms et al. 2002; McCarron and Love 1997; Ouabdesselam et al. 1996; Pers et al. 1996; Piddock et al. 1993; Vasallo et al. 1998). According to our knowledge, there is no reason to anticipate any difference in the therapeutic response to fluoroquinolones between the nonclassical quinolone resistance phenotype and conventional phenotype strains, both of which exhibit reduced fluoroquinolone susceptibility. On this account, one can assume that the emergence and potential spread of the nonclassical quinolone-resistant population could potentially lead to even serious clinical consequences.

The *qnr*-positive isolates pose a major threat also because they may carry other resistant determinants. For example, *qnr* genes are commonly found in the strains that produce ESBLs (Cattoir et al. 2007a; Doublet et al. 2009; Hopkins et al. 2008; Lavilla et al. 2008; Poirel et al. 2006; Strahilevitz et al. 2007). ESBL producing strains are mainly found in *Enterobacteriaceae* other than *Salmonella*. Although we did not find any ESBL-genes among these *S. enterica* isolates, it may be just a matter of time before ESBL producing *Salmonella* isolates, which are also *qnr* positive will be found. A *Salmonella* isolate having co-resistance to extended-spectrum cephalosporins and fluoroquinolones might cause serious problems in clinical settings, since the treatment of these patients will be challenging.

The nonclassical quinolone resistance phenotype isolates may prove diagnostically very problematic in clinical laboratories, since this phenotype is difficult to recognize with conventional methods. In many microbiology laboratories, the nalidixic acid disc diffusion test is used to screen for reduced fluoroquinolone susceptibility in salmonellas, since it has been shown that high-level resistance to nalidixic acid usually correlates with reduced fluoroquinolone susceptibility (Hakanen et al. 1999a). It is to be expected that this screening approach may fail due to susceptibility or only low-level resistance to nalidixic acid in these strains. Therefore, it is important to be more precise in the quinolone susceptibility testing. Since the results from a nalidixic acid E-test or disc diffusion test may be misleading, ciprofloxacin susceptibility should be always examined in parallel. At least invasive *Salmonella* isolates from travelers returning from Thailand and Malaysia should be examined for fluoroquinolone susceptibility using an Etest or another suitable MIC method. Although the fluoroquinolone susceptibility testing may presently be the most important for these isolates, diagnostic difficulties should be kept in mind also when other *Salmonella* isolates are examined.

At the moment, the main problem with the *Salmonella* fluoroquinolone susceptibility testing is that there are no breakpoints accurate enough to detect reduced fluoroquinolone susceptible isolates or screen the novel phenotype isolates. Therefore, such MIC breakpoints including zone diameters representing resistance breakpoints for fluoroquinolones and nalidixic acid should urgently be determined. In addition, since the fluoroquinolone susceptibility among *qnr*-phenotype isolates varies and the determination of fluoroquinolone susceptibility is challenging, the nonclassical quinolone resistance phenotype may be difficult to detect based on only susceptibility testing (Lascols et al. 2007). While susceptibility testing is only a screening method, molecular methods for the detection of the nonclassical quinolone resistance phenotype are also needed. A PCR screening of PMQR determinants is, however, expensive and laborious and cannot be daily routine in clinical laboratories.

6.4. *In vitro* activity of azithromycin against *S. enterica*

It has been shown in previous studies that *Salmonella* isolates are intrinsically resistant to erythromycin but that they are naturally sensitive to another macrolide antimicrobial, azithromycin (Stock and Wiedemann 2000). Therefore it is not a surprise that azithromycin has shown a good efficacy in the treatment of patients suffering from typhoid fever (Chinh et al. 2000; Frenck et al. 2004; Girgis et al. 1999; Threlfall et al. 2008). However, its efficacy in nontyphoidal *Salmonella* infections has not been tested, or at least not been reported.

The results of the present study show that while erythromycin MICs were high, 806 (99.6%) *S. enterica* isolates had MIC $\geq$32 mg/L, azithromycin showed good *in vitro* activity against *S. enterica*. Overall, azithromycin resistance was rare both in domestic and foreign isolates (1.4% and 2.5%, respectively) and only six isolates in both groups showed high-level azithromycin resistance. Among the isolates with reduced fluoroquinolone susceptibility, only four domestic and 11 foreign isolates showed azithromycin resistance, of which three in both groups were high-level resistant. 11.3% of the nonclassical quinolone resistance phenotype isolates were azithromycin resistant and one of them showed high-level azithromycin resistance. However, all azithromycin resistant isolates which belonged to the nonclassical quinolone resistance phenotype were collected in 2003 and 2004, i.e. over five years ago and thereafter azithromycin resistance has not been detected among the novel phenotype isolates.

The mutations causing macrolide resistance were screened, and three different mutations were found. C379T and G25A mutations in L4 and L22, respectively, were detected in three *S*. Montevideo isolates showing the nonclassical quinolone resistance phenotype. Since they were erythromycin-resistant but susceptible to azithromycin, it is likely that these mutations were only serovar dependent. Although a Glu79-Lys substitution in the *rlpD* gene is located outside the target region, it could however, be associated with macrolide resistance, since this mutation was found in different serovars which all were resistant to macrolides and ketolides. The absence of macrolide resistance-associated mutations in other azithromycin- and erythromycin-

resistant isolates suggests that macrolide resistance in these *S. enterica* isolates was probably driven by efflux pump mechanisms. This hypothesis is supported by previous data indicating that macrolide resistance in *Enterobacteriaceae* is usually efflux pump mediated (Braoudaki and Hilton 2005; Caldwell et al. 2008). This remains speculation, since we have not yet tested the expression of macrolide efflux pumps.

The antimicrobial susceptibility testing revealed that ampicillin resistance was quite common among the *S. enterica* isolates tested, whereas only a few isolates showed resistance to chloramphenicol and trimethoprim-sulfamethoxazole. Thus, MDR was rare. However, in the Western countries these antimicrobials are used only seldom to treat *Salmonella* infections. Therefore the susceptibility of salmonellas to these antimicrobials has no clinical importance in the developed countries. On the contrary, since severe *Salmonella* infections are commonly treated with ciprofloxacin and extended-spectrumcephalosporins, the increasing resistance of these antimicrobials really is a problem. An increase of the reduced ciprofloxacin susceptibility may jeopardize the fluoroquinolone treatment of *Salmonella* infections. Furthermore, the emergence of the *qnr*-phenotype *Salmonella* isolates carrying ESBL genes may even complicate the use of the extended-spectrum cephalosporins. Consequently, alternative antimicrobials for the treatment of *Salmonella* infections are needed. It was shown in this study that azithromycin has good *in vitro* activity against *S. enterica* isolates. Although highly azithromycin-resistant isolates did occur, azithromycin was effective even against the reduced fluoroquinolone susceptible isolates including those showing the nonclassical quinolone resistance phenotype. Based on these results, azithromycin may be a useful alternative antimicrobial for the treatment of infections caused by antimicrobial-resistant *Salmonella* strains.

7.   SUMMARY AND CONCLUSIONS

In this thesis, it is shown that the reduced fluoroquinolone susceptibility among nontyphoidal strains of *S. enterica* among travelers from Finland decreased significantly during the study period, 2003–2007, after having constantly increased for several years. The decreasing trend was driven by isolates from Southeast Asia, while the proportion of resistant strains from the other travel destinations remained fairly stable. Neither was any decreasing trend observed among the domestic *Salmonella* isolates. Notably, the decreasing trend in resistance among the foreign isolates was not linked with reduced travel to Thailand, as the number of tourists from Finland going to Thailand more than doubled from 2005 to 2007.

Until 2002, all *Salmonella* isolates with reduced fluoroquinolone susceptibility were uniformly resistant to nalidixic acid. In 2003, we discovered a novel population of *S. enterica* strains that showed reduced susceptibility to ciprofloxacin but were either susceptible or only low-level resistant to nalidixic acid. All isolates with the nonclassical quinolone resistance phenotype were from travelers returning from Thailand or Malaysia. It is noteworthy that after the emergence of the nonclassical quinolone resistance phenotype, these isolates have persisted in that area. It is also notable that among the nonclassical quinolone resistance phenotype, *S.* Corvallis and *S.* Stanley were the most prevalent serotypes. Among the isolates from Thailand and Malaysia, the nonclassical quinolone resistance varied between 2003 and 2007; however, the difference from year to year was not significant. In 2007, the proportion of the nonclassical phenotype surpassed that of the conventional phenotype for the first time. This increase in nonclassical phenotypes may be an emerging trend that needs to be under close surveillance. At the present time, the nonclassical phenotype appears to be mainly confined to Thailand and Malaysia, but given the continuous increase in global travel, these isolates many emerge in other parts of the world.

The existence and plausible future increase of this novel resistance phenotype threatens to impede the detection of quinolone resistance of salmonellas in areas where such strains are common, since the nalidixic acid disk diffusion test may no longer be valid as a tool to screen for reduced fluoroquinolone susceptibility. Therefore, it is important to pay more attention to quinolone susceptibility testing. At least invasive *Salmonella* isolates from travelers returning from Southeast Asia should be examined for fluoroquinolone susceptibility using an Etest or another suitable MIC method. Although the fluoroquinolone susceptibility testing may be the most important for these particular isolates, diagnostic difficulties should be kept in mind also when other *Salmonella* isolates are examined.

This study demonstrates the emergence of a highly mobile *qnr* phenotype that confers the nonclassical quinolone resistance phenotype in *S. enterica* isolates. It is notable that isolates with the nonclassical resistance phenotype persist in the absence of topoisomerase mutations. Although efflux may have some role in the nonclassical phenotype, the *qnr* genes alone are adequate to produce isolates with reduced fluoroquinolone susceptibility. This is of concern since nonclassical quinolone

resistance is plasmid-mediated and therefore mobilizable. The mobile nature of the *qnr* genes may enable this novel phenotype to spread very rapidly.

Finally, azithromycin was shown to exhibit a good *in vitro* activity against *S. enterica*, also against isolates with reduced fluoroquinolone susceptibility including those showing the nonclassical quinolone resistance phenotype. Based on these data, azithromycin may prove a useful alternative for the treatment patients with salmonellosis, even when the disease is caused by fluoroquinolone- or extended-spectrum cephalosporin-resistant strains.

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