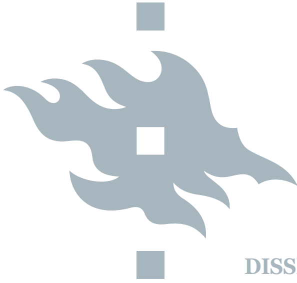

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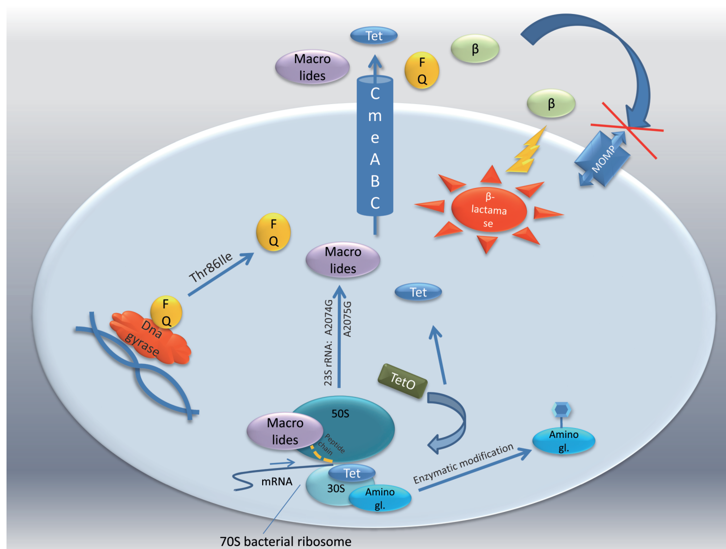

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DOCTORAL PROGRAMME IN FOOD CHAIN AND HEALTH
UNIVERSITY OF HELSINKI

Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Antimicrobial Resistance and Its Mechanisms among C*ampylobacter coli* and *Campylobacter upsaliensis* with a Special Focus on Streptomycin

SATU OLKKOLA

Academic dissertation

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”He who asks is a fool for five minutes but he
who doesn’t ask, remains a fool forever.”
A Chinese proverb

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ABSTRACT

Campylobacteriosis is the most common cause of human bacterial gastroenteritis in the developed world. *Campylobacter* spp. are known to colonize a wide range of land and sea mammals, reptiles and birds. Campylobacteriosis is also a zoonosis, with the most important reservoir considered to be chicken, but *Campylobacter* spp. are also abundantly found in other domestic animals. In particular, *Campylobacter coli* and *C. upsaliensis* are common colonizers of pigs and dogs, respectively.

The most often isolated causative agent from diseased humans is *C. jejuni*, but also *C. coli* and *C. upsaliensis* are known to cause disease, with *C. coli* estimated to cause 5-10% of the human campylobacteriosis cases. The disease is usually self-limiting but antimicrobial treatment is warranted in severe cases, with macrolides and fluoroquinolones being the first and second options, respectively. Intravenous aminoglycosides are indicated in *Campylobacter* bacteraemia.

High rates of fluoroquinolone-resistant *Campylobacter* spp. have emerged in many parts of the world. Also, in several studies, high proportions of streptomycin (STR)-resistant *C. coli* or *C. upsaliensis*, have been found. However, despite recent advances in molecular characterization of the resistance mechanisms, complete understanding of these mechanisms for certain antibiotics remains lacking. Particularly, the mechanisms of STR resistance have been only partially characterized in *C. jejuni* and *C. coli* and completely ignored in *C. upsaliensis.*

The primary aim of this thesis was to investigate the molecular mechanisms of STR resistance in porcine *C. coli* and canine *C. upsaliensis* isolates. We were able to associate high level of STR resistance (MIC>1024 mg/l) in porcine *C. coli* to mutations in the *rpsL* gene, which encodes ribosomal protein S12. In *C. upsaliensis*, a mutation in *rpsL* was also noted in all the intermediate- and high-level STR-resistant isolates. Even though STR resistance conferring mutations in *rpsL* codons 43 and 88 have been well documented in *Escherichia coli* and *Mycobacterium tuberculosis*, they were first time described in *Campylobacter* species in the present study.

In addition to *rpsL* mutations, also other mechanisms were explored to explain the different levels of STR resistance observed in both *C. coli* and *C. upsaliensis*. Using genomics and insertional mutagenesis, a novel STR resistance-conferring gene (*ant*-like gene A) was identified in the intermediately STR-resistant (MIC 128-512 mg/l) *C. coli* isolates. This *ant*-like gene A is located in a genomic region corresponding to the previously described hypervariable region 14 in *C. jejuni*, and we showed that it was naturally transformable to a STR-susceptible *C. coli* recipient. This gene is homologous, albeit at a low level, to other previously described aminoglycoside 6-adenylyltransferase encoding genes, and does not appear to originate from Gram-positive bacterial species. We hypothesize that the *ant*-like gene A could have evolved from a proto-resistance element originally having a different function in *Campylobacter* spp., as we did not find evidence of a recent jump of this gene to *Campylobacter* species.

All highly STR-resistant *C. upsaliensis* isolates had, in addition to the *rpsL* mutation, significant truncation of *rsmG*, encoding a conserved methyltransferase responsible for methylation of the STR binding site in the 500 region of 16S rRNA. Functional deletion of *rsmG* together with changes in the *rpsL* have been associated with a high-level STR-resistant phenotype in other bacterial species, such as *M. tuberculosis*, *Bacillus subtilis* and *E. coli*, but, before our studies, have not been described in *Campylobacter* species. Altogether these results provided a

significant advance in understanding the mechanisms of STR resistance in *Campylobacter* spp. and will aid in predicting the phenotypic resistance from genome data.

Fluoroquinolone resistance-associated mutations in the DNA gyrase-encoding gene *gyrA* were characterized in *C. coli* from pigs treated with danofloxacin (DANO) and in canine *C. upsaliensis*. The commonly described C257T mutation was found in both species. In *C. coli* this leads to the amino acid change T86I in DNA gyrase and high levels of ciprofloxacin (CIP) resistance, while in *C. upsaliensis* the predicted amino acid change is T86M and only slight increases in CIP MICs but high levels of nalidixic acid resistance were detected. Therefore, DANO does not seem to induce novel mutations in the QRDR region of *C. coli in vivo*. On the other hand, the same mutation appears not to be sufficient to cause a high level of fluoroquinolone resistance in *C. upsaliensis*.

Macrolide resistance-associated mutations in the 23S rRNA and in the ribosomal proteins L4 and L22 encoding genes were studied in erythromycin (ERY) –susceptible and intermediately and highly resistant *C. coli*. All highly ERY-resistant isolates were found to have the well-characterized A2122G mutation in 23S rRNA, while no resistance-associated mutations were detected in L4 and L22, indicating that the observed intermediate ERY resistance is due to other mechanisms such as the CmeABC efflux pump.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred in the text by their roman numerals:

I Olkkola S, Juntunen P, Heiska H, Hyytiäinen H, Hänninen M-L. Mutations in the *rpsL* gene are involved in streptomycin resistance in *Campylobacter coli*. Microb. Drug Res. 2010, 16(2):105–110.

II Juntunen P, Olkkola S, Hänninen M-L. Longitudinal on-farm study of the development of antimicrobial resistance in *Campylobacter coli* from pigs before and after danofloxacin and tylosin treatments. Vet Microbiol. 2011, 150(3-4):322-30. (Shared first authorship w/ Juntunen P. Data on streptomycin resistance will be published as supplemental material in study III).

III Olkkola S, Culebroe A, Juntunen P, Hänninen ML, Rossi M. Functional genomics in *Campylobacter coli* identified a novel streptomycin resistance gene located in a hypervariable genomic region. Published ahead of print in Microbiology 5th of May, 2016. doi: 10.1099/mic.0.000304.

IV Olkkola S, Kovanen S, Roine J, Hänninen ML, Hielm-Björkman A, Kivistö R. Population genetics and antimicrobial susceptibility of canine *Campylobacter* isolates collected before and after a raw feeding experiment. PLoS ONE. 2015, 10(7):e0132660.

The publications and Figure 5 have been reprinted with the kind permission of their copyright holders. In addition, some previously unpublished material is presented.

ABBREVIATIONS

16S-RMTase	16S ribosomal RNA methyltransferase
AAC	aminoglycoside acetyltransferase
ABySS	Assembly by Short Sequences
AGA	aminoglycoside group of antimicrobials
AME	aminogycoside-modifying enzyme
ANT	aminoglycoside nucleotidyltransferase
APH	aminoglycoside phosphotransferase
BLAST	Basic Local Alignement Tool
CAT	chloramphenicol acetyltransferase
CC	clonal complex
CD	coding sequence
CFU	colony-forming unit
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CNP	clusters of nucleotide polymorphism
DANO	danofloxacin
DDD	defined daily dose
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ERY	Erythromycin
ESAC-net	European Surveillance of Antimicrobial Consumption network
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIMEA	Finnish Medicines Agency
FiRe	Finnish Study Group for Antimicrobial Susceptibility Testing
GEP	Genome profiler
IM	intramuscularly
LBA	Luria Bertani agar
LOS	lipooligosaccharide
mCCDA	modified charcoal cefoperazone deoxycholate agar
MDR	multiple drug resistance
MH	Muller-Hinton
MHA	Muller-Hinton agar
MHB	Muller-Hinton Broth
MIC	minimum inhibitory concentration
MIRA	Mimicking Intelligent Read Assembly
MLST	multilocus sequence typing/type
NA	nutrient agar
NAL	nalidixic acid
NB	nutrient broth
NBA	nutrient-blood agar
NGS	next-generation sequencing

NWT	non-wild type microorganism
OD	optical density
PaβN	phenyl-arginine-β-naphthylamine
PCR	polymerase chain reaction
PCU	population correction unit
QRDR	quinolone resistance determining region
RAST	Rapid Annotation using Subsystem Technology
RFLP-PFGE	restriction fragment length polymorphisms pulsed field gel electrophoresis
SNP	single nucleotide polymorphism
SPEC	spectinomycin
ST	sequence type
STR	streptomycin
VBNC	viable but non-cultivable
wgMLST	whole genome multilocus sequence typing
WGS	whole-genome sequencing
WHO	World Health Organization
WT	wild type microorganims

1 INTRODUCTION

Campylobacteriosis is the most commonly reported bacterial zoonosis in the European Union (1). The predominant species isolated from human infections is *Campylobacter jejuni*, followed by *C. coli*, but also other *Campylobacter* spp. have been associated with human disease, including *C. upsaliensis* (2-6). While most human infections are self-limiting, antimicrobial treatment is warranted in severe cases or for immunocompromised patients, preferably using the macrolide or fluoroquinolone antimicrobials with aminoglycosides indicated for septicaemia (7).

Humans have been estimated to produce between 100 000 and 200 000 tonnes of antimicrobials annually, and increasing resistance rates to these agents have been observed in many human pathogens, including *C. jejuni* and *C. coli* (8). The World Health Organization (WHO) has warned that, unless radical measures are undertaken, we might be soon entering a post-antibiotic era (9). Resistance to antimicrobial agents has traditionally been considered to incur a fitness cost in bacteria, but nowadays there is mounting evidence that this might not be true in many cases due to, for example, compensatory mutations (10, 11).

The large-scale use of antimicrobials in agriculture has led to the emergence of resistant strains, and alarmingly high rates of resistance in *C. jejuni* and *C. coli* to certain antimicrobials, such as fluoroquinolones, have been reported in many countries, recently also against aminoglycosides in the United States and China (12-14). Also, high rates of STR-resistant *Campylobacter* have been detected in several studies (15-19). Although STR and other aminoglycosides are only infrequently used in clinical settings nowadays due to their oto- and nephrotoxic side effects, the detection of ever-increasing numbers of resistant bacteria has revived clinical interest in this group of antimicrobials (20-22).

The only reported resistance mechanisms to aminoglycosides in *Campylobacter* spp. have been the presence of genes encoding aminoglycoside-modifying enzymes (AMEs) (14, 23-25). These genes are often found as part of resistance plasmids or transposons containing also virulence or other resistance-associated genes and thought to have spread to *Campylobacter* spp. through horizontal transfer mainly from Gram-positive bacterial species (24, 26). Streptomycin resistance has often been detected in multi-resistant *Campylobacter* strains, indicating a common spread of resistance determinants. Further, we observed a statistically significant increase in the isolation frequency of STR-resistant *C. coli* from pigs treated with tylosin (27).

C. upsaliensis is commonly isolated from dog faeces and has also been reported to cause disease in humans, often associated with some underlying predisposing factor such as immunosuppression (3-6). Since dogs often are in close contact with humans, the occurrence of antimicrobial resistance of this canine-associated *Campylobacter* species is important to be assessed. In some studies, a high proportion of STR-resistant *C. upsaliensis* has been detected, but the resistance mechanisms have not been examined (5, 28). In addition, low rates of quinolone resistance have been reported, but also these mechanisms remain uncharacterized in *C. upsaliensis* (28-30).

The main aim of this thesis was to elucidate the mechanisms of high and intermediate-level STR resistance in two *Campylobacter* spp., *C. coli* and *C. upsaliensis*. Further, macrolide and quinolone resistance associated mutations were studied in porcine *C. coli* and antimicrobial susceptibility patterns and quinolone resistance associated mutations were studied in *C. upsaliensis* originating from a set of Finnish dogs.

2 REVIEW OF THE LITERATURE

2.1 Historical highlights of *Campylobacter*

In 1886, Theodor Escherich reported spiral bacteria in the faeces and colon of dead infants who had had diarrhoea ('cholera infantum') but his findings were only recognized 100 years later in 1986 by Manfred Kist and referred to be actually diarrhoea associated with *Campylobacter* (reviewed in (31)). A vibrio resembling bacterium or spirillum was also reported from aborted sheep foetuses in the early 1900s by two veterinarians, McFadyean and Stockman (31) and from foetuses of bovines with infectious abortions by Smith and Taylor, who called the bacteria *Vibrio fetus* in 1919 (31). In addition, an agent termed *V. coli* was isolated from pigs with dysentery (32).

The first well-documented human *Campylobacter* infections were described in 1938 in association with a milk-borne outbreak and involved 355 inmates of two adjacent state institutions in Illinois, USA (33). In 1947, Vinzent et al. reported having isolated *V. fetus* from the blood of three pregnant women, two of which aborted (31, 34). In 1963, the causative agent was named *Campylobacter*, meaning curved rod in Greek with the type species of the genus being *C. fetus* (35).

However, *Campylobacter* spp. as infectious agents were first isolated from diarrhoeic human faeces only in 1968 with a special filtration technique and plating of the filtrate on selective agar plates containing antimicrobials (36). In the late 1970s, Skirrow isolated *C. coli* and *C. jejuni* from the faeces of diarrhoeic individuals also using growth medium containing antimicrobials as selective agents. He found evidence that *Campylobacter* is a common cause of acute enteritis in humans and suggested that poultry might be the primary source of the infection (37). His findings started a new era in the history of zoonotic *Campylobacter* spp.

2.2 The genus *Campylobacter*

The genera *Campylobacter* and *Arcobacter* form the family *Campylobacteraeceae* belonging to the order *Campylobacterales*, which in turn is a member of the class Epsilonproteobacteria together with, for example, the genera *Wollinella* and *Helicobacter*. At present, the *Campylobacter* genus comprises 26 validated species (Table 1) (http://www.bacterio.net/campylobacter.html, accessed 10.12.2015, (38)). *Campylobacter* spp. are Gram-negative, motile, flagellated, curved or spiral rods. Most of the species require microaerobic atmosphere for growth, while some require a hydrogen-enriched atmosphere. Most *Campylobacter* spp. grow in the temperature range of 30-37 ^{o}C, but the thermophilic species (e.g. *C. jejuni, C. coli, C. upsaliensis* and *C. lari*) are able to grow at higher temperatures (up to 42°C) (39). *Campylobacter* spp. are generally very vulnerable to many environmental stresses, e.g. disinfectants, drying or UV radiation (3, 40), but they can survive certain stressful conditions, such as cold water, for long periods (3, 41, 42).

Table 1. *Campylobacter* species, the most important known hosts and reservoirs and pathogenicity to humans.[1]

Species	Most important reservoir(s) and hosts	Pathogenicity to humans
C. avium	poultry	unknown
C. canadiensis	wild birds (*Grus americana*=whooping crane)	unknown
C. coli	pigs, other domestic animals	diarrhoea, septicaemia, abortion
C. conscisus	humans	periodontal disease
C. corcagiensis	Macaque (*Macaca silenus*)	unknown
C. cuniculorum	rabbits	unknown
C. curvus	humans	gingivitis, periodontitis, periodontosis
C. fetus	cattle, sheep	septicaemia, abortion
C. gracilis	humans	periodontal disease
C. helveticus	cats, dogs	unknown
C. hominis	humans	unknown
C. hyointestinalis	pigs, cattle, deer, human	gastroenteritis
C. iguaniorum	reptiles	unknown
C. insulaenigrae	marine mammals	unknown
C. jejuni	poultry, other domestic animals, wild birds, humans	gastroenteritis, septicaemia, abortion
C. lanienae	humans, pigs, bovine	unknown
C. lari	wild birds (seagulls), other animals, water, shellfish	gastroenteritis, septicaemia
C. mucosalis	pigs	unknown
C. peloridis	humans, shellfish	unknown
C. rectus	humans	periodontal disease, abscesses, appendicitis
C. showae	humans	periodontal disease
C. sputorum	humans, cattle, sheep, pigs	abscesses, gastroenteritis
C. subantarcticus	birds	unknown
C. upsaliensis	dogs, cats, humans	gastroenteritis, septicaemia, abortion
C. ureolyticus	humans	putative gastrointestinal pathogen
C. volucris	wild birds (*Larus ridibundus* = black-headed gull)	unknown

[1]http://www.bacterio.net/campylobacter.html (38)

2.3 Human *Campylobacter* infections and their reservoirs

Several *Campylobacter* species are commensal bacteria in their natural hosts, but able to cause disease in other species such as gastroenteritis and septicaemia in humans and abortion in bovine and sheep (39). Campylobacteriosis is the most commonly reported bacterial zoonosis in the European Union (1), and the incidence of campylobacteriosis seems to have risen during the last ten years at least in countries from which consistent monitoring data are available (43). The most important human pathogen of the genus is *C. jejuni*, which has been estimated to account for up to 90% of the human infections, followed by *C. coli*, which causes approximately 5-10% (or according to some studies up to 25%) of the campylobacteriosis cases (43-45). However, discrepant reports also exist with up to two-thirds of the *Campylobacter* isolated from human diarrhoeic stools being species other than *C. jejuni* (3, 43) and several other *Campylobacter* species have been recognized as human pathogens, including *C. upsaliensis* (3-6).

Enteritis caused by *C. jejuni* or *C. coli* presents as an acute inflammatory terminal ileitis, cecitis and mesenteric adenitis (2). All age groups can be affected, but there is some evidence that the prevalence is higher in small children and young adults (43). In addition, other *Campylobacter* spp. can cause human gastroenteritis that may be milder and often go unrecognized due to the lack of appropriate cultivating techniques required by some of the species (43). Human campylobacteriosis is commonly accompanied by abdominal pain, diarrhoea and fever. The disease is usually self-limiting and no antimicrobials are needed (2). However, antimicrobial treatment is warranted in severe or prolonged infections and in immunocompromised patients, with macrolides and fluoroquinolones being the first and second treatment choices, respectively (2, 43). Intravenous aminoglycosides have been indicated as a first-line treatment in serious systemic *Campylobacter* infections and bacteraemia (7). Several post-infectious diseases have been associated with campylobacteriosis, including Guillan-Barrè syndrome (GBS) and reactive arthritis, among others, but there are also studies finding association between campylobacter infection and several other conditions, even with the increasingly common celiac disease (46).

Campylobacter spp. are widely spread in the animal kingdom, and hosts include an extensive range of land and sea mammals, reptiles and birds (39). Poultry and wild birds are among the main reservoirs for *C. jejuni* and *C. coli* (47), and eating or handling raw or uncooked chicken meat has been considered to be the most important source of human campylobacteriosis (41, 48). Pigs have a higher prevalence of *C. coli* than *C. jejuni* (27, 49, 50), and *C. upsaliensis* is the major *Campylobacter* spp. recovered from dogs (51, 52).

2.4 *Campylobacter* genome

The *Campylobacter* genome consist of a circular chromosome of approximately 1.6-1.7 Mb in size and containing about 1500-1700 coding sequences (29, 53, 54). In a study analyzing genomes of 42 *C. coli* and 43 *C. jejuni* strains, the average *C. coli* genome size was found to be larger than that of *C. jejuni* (1732 vs 1609 orthologues) and also the core genome of *C. coli* was bigger, but the proportion of core to accessory genes (ca. 83%) was similar between the species (55).

Plasmids and integrated elements are part of the accessory genome and also found in *Campylobacter* spp. (24). They are known to spread through horizontal transfer and can contain also antimicrobial resistance or virulence-associated genes. Further, also hypervariable genomic regions are part of the accessory genome, and in *C. jejuni* several of these regions (also known as regions of divergence or plasticity) have been described with variable genomic content consisting

of, for instance, genes encoding lipooligosaccharides (LOSs), motility, restriction-modification systems and metabolism (56, 57). The variation in these regions results from both gene sequence divergence and the presence or absence of specific genes (58, 59). The putative hypervariable regions in other *Campylobacter* spp. have not been extensively studied.

2.5 Antimicrobial agents

Antimicrobial agents are substances that either inhibit the growth of (microbistatic effect) or kill (microbicidal effect) susceptible micro-organisms. In the broad sense, the term also includes antiseptics and disinfectants together with antimicrobial drugs. Antimicrobial drugs can be further divided into antibacterial, antiviral and antifungal substances. While the term antibiotic was originally used to refer only to the antibacterial agents originating from living organisms, such as aminoglycoside-producing Actinomycetes, it is now used in common language also for semi-synthetic and synthetic antimicrobial drugs. The term antimicrobial or antimicrobial drug will be used in this thesis to indicate antibacterial drugs and antibiotics.

Antimicrobial drugs have several mechanisms of action against bacteria, the main ones being inhibition of synthesis of the bacterial cell wall, proteins or nucleic acids and damage to the bacterial cell membrane. They are divided into classes based on their structure and mode of action. The main classes of interest are shown in Table 2.

Table 2. Main antimicrobial groups of clinical interest, their mechanisms of action and their use in Finland.

			Consumption	
Class	Main mechanism of action	Example drugs	Human med[1]	Vet med[2]
Beta-lactams	Bactericidal, inhibition of cell wall synthesis	Penicillins, cephalosporins	10.18	9763
Tetracyclines	Bacteriostatic, protein synthesis inhibition at 50S ribosomal subunit	Tetracycline, doxycycline	4.52	2389
Sulphonamides, diaminopyrimidins	Bacteriostatic/bactericidal, folic acid synthesis inhibition	Sulfamethoxazole, trimethoprim	1.32	3129
Macrolides	Bacteriostatic, protein synthesis inhibition at 50S ribosomal subunit	Erythromycin, tylosin	1.23	456
Quinolones	Bactericidal, inhibition of DNA gyrase	Ciprofloxacin, danofloxacin	1.13	105
Aminoglycosides	Bactericidal, protein synthesis inhibition/aberrant protein synthesis at 30S ribosomal subunit	Streptomycin, gentamicin	0.02	103

[1]DDD, Defined Daily Dose/1000 inhabitants/day in 2014 (Finnish Statistics on Medicines 2014, www.kela.fi)

[2]kg of active substance in 2013 (Lääketukuista myydyt, eläimille tarkoitetut mikrobilääkkeet 2001-2013, www.fimea.fi)

2.5.1 Aminoglycosides

The aminoglycoside group of antimicrobials (AGAs) was one of the first antimicrobial classes introduced for clinical use in the 1940s. This class consists of several compounds belonging to a number of groups based on their core structure, but all share common structural components, amino-modified sugars (glycosides) connected to an aminocyclitol nucleus. The most common AGAs contain 2-deoxystreptamine as a core structure (20). They are natural or semisynthetic products of bacteria such as *Streptomyces* spp. and *Micromonospora* spp.

Aminoglycosides are positively charged, basic, highly soluble in water and relatively soluble in lipids (60) and contain several free hydroxyl groups and a minimum of two amino groups by which they bind with the 30S ribosomal structures (20). They are chemically stable (61), and oxygen and ATP are required for the transfer of aminoglycosides into the bacterial cell (60). The majority of aminoglycosides are bactericidal, inhibiting protein synthesis by affecting ribosomal translational accuracy, translocation or proofreading. They have distinct ribosomal target sites within the 30S subunit, and even those sharing the same core structure may have distinct effects on the ribosome (62). For example, spectinomycin inhibits translocation of peptidyl-tRNA between different sites of 16S rRNA, while streptomycin makes the bacterial ribosome more prone to errors by stabilizing the error-prone state (see below) (63).

Aminoglycosides have been widely used over decades and have several indications in human infections including serious *Campylobacter* bacteraemia (7) as well as other infections caused by Gram-negative bacteria (60). However, their clinical applications are limited by their nephro- and ototoxicity (64-67). After the commercial introduction of other broad-spectrum but safer antimicrobials in the 1970s, their use has declined worldwide (22) but in some countries, such as China, several aminoglycosides are still used at conventional broiler and swine farms (68). In Finland, the veterinary use of aminoglycosides has decreased steadily over the surveillance period of 2001-2013, with the sales of aminoglycosides (of active substance) decreasing from 632 kgs in 2001 to 103 kgs in 2013 (http://www.fimea.fi/elainlaakkeet/mikrobilaakkeiden_kulutus_elaimilla) and consumption in food-producing animals being 0.2 mg/population correction unit (PCU) in 2013 (69). They are also only marginally used in human medicine (Finnish Statistics on Medicines 2014, www.kela.fi). Yet, with ever-increasing resistance to the newer antimicrobial agents, aminoglycosides are again attracting clinical interest, especially in combination therapy for the treatment of serious infections caused by Gram-negative bacteria or even as last-resort antibiotics (20-22). Several aminoglycosides, including streptomycin, are found in the WHO's list of essential medicines (http://www.who.int). Gentamicin is included in the European resistance surveillance programme for *Campylobacter* (70).

2.5.1.1 Streptomycin

Streptomycin was the first aminoglycoside discovered, isolated from the soil bacterium *Streptomyces griseus* in 1943 (71, 72). It was also the first antimicrobial agent used successfully in the treatment of human tuberculosis caused by *M. tuberculosis* but also being effective against many Gram-negative bacteria that penicillin failed to affect. Its main use in humans still remains in the treatment of tuberculosis but it is also used to treat infections caused by the less common pathogens *Yersinia pestis*, *Francicella tularensis* and *Brucella* spp. (21, 60). Additionally, in some countries, it is used in plant agriculture in a spray formulation, mostly to prevent fire blight in apple and pear tree orchards (73, 74). In Finland, there is still one orally administered veterinary

streptomycin-containing drug registered for use in piglets and calves, and indicated mainly for the treatment of enteric infections (75).

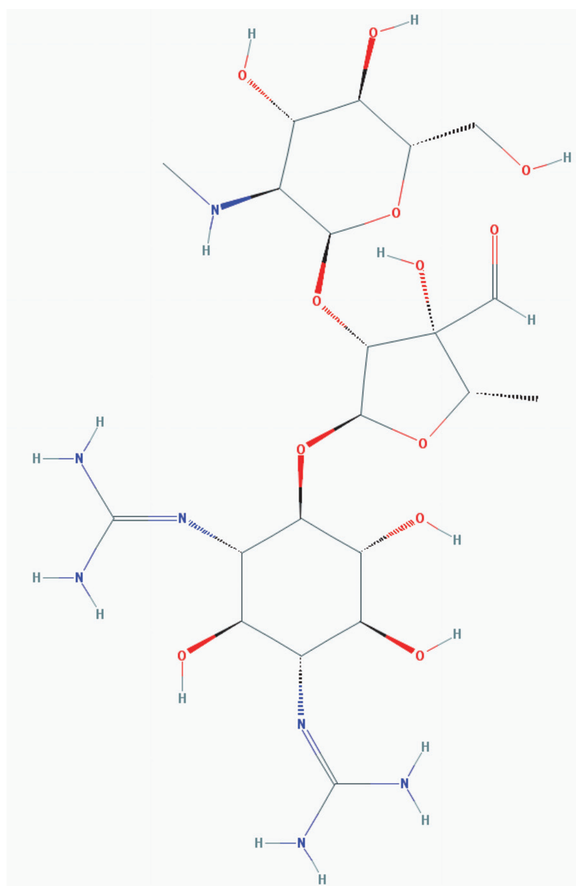

Figure 1. *Structure of streptomycin (PubChem Compound Database).*

Streptomycin is the only aminoglycoside having a guanidinylated streptamin core (20). It binds to the 16S rRNA at four different sites both through hydrogen bonds and salt bridges and is also in contact with ribosomal protein S12 encoded by the gene *rpsL* (63). It appears to stabilize the *ram* or error-prone state of the ribosomal 30S subunit helix 27 accuracy switch allowing the binding of non-cognate transfer RNAs to the ribosome and leading to aberrant protein synthesis (63). There is also evidence that it exerts its bactericidal activity partly because it becomes caged in the bacterial cell. The mechanism proposed is through insertion of mistranslated proteins in the cytoplasmic membrane, which facilitates higher influx of the agent, and thereafter bacterial proteases lyse these mistranlated proteins, trapping streptomycin and toxic protein intermediates inside the cell (76).

2.5.2 Quinolones

Quinolones are completely synthetic compounds originally derived from the antimalarial drug chloroquine. The first quinolone, nalidixic acid, was patented in 1962 (77). Quinolones are based

on a two-ring nucleus, either a naphtyridone with nitrogen at positions 1 and 8, or a quinolone with only one nitrogen at position 1 (77). They are bactericidal in action and bind primarily to DNA gyrase enzyme in Gram-negative bacteria or topoisomerase IV enzyme in Gram-positive bacteria, the DNA supercoiling and relaxing enzymes, respectively. The enzymes become trapped on DNA, thereby blocking the progression of the DNA replication fork (78-81).

While nalidixic acid has only low absorptivity from the gastrointestinal track and modest action against Gram-negative bacteria, the newer fluorinated compounds in this class, called fluoroquinolones, show marked improvements in both gastrointestinal absorption and spectrum of activity (77, 82, 83). Fluoroquinolones are also important in the treatment of enteric bacterial infections, including campylobacteriosis (84). The fluoroquinolones enrofloxacin and sarafloxacin were accepted in the United States for the treatment of respiratory colibacillosis in chickens and turkeys in the mid 1990s but have been banned since 2005 due to the rapid increase of resistance among *C. jejuni* (85).

Nowadays, there are over 10 000 compounds derived from the bicyclic parent quinolone molecules (86), with several of them registered in Finland for treatment of human (ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin) and animal (enrofloxacin, marbofloxacin, pradofloxacin, danofloxacin) infections (www.fimea.fi). Only ciprofloxacin, danofloxacin and nalidixic, which are of interest in this thesis, are discussed in more detail below.

Nalidixic acid, a quinolone, has a naphtyridone nucleus and contains an ethyl group at position 1 nitrogen (Fig. 2). Its main use is in the treatment of urinary tract infections in humans, as it concentrates in the urine (77). No quinolone antimicrobials are registered for veterinary or human use in Finland. Nalidixic acid is used in monitoring quinolone resistance and as an early indicator of emerging fluoroquinolone-resistant bacteria in the EU *Campylobacter* resistance surveillance (70).

The fluoroquinolone ciprofloxacin has a quinolone nucleus with a cyclopropyl group in position 1 nitrogen and a piperazin group in position 7 carbon (Fig. 2). It was introduced for clinical use in the 1980s and has a wide variety of indications in humans, including respiratory tract, complicated urinary tract, gastroenteric and genital infections (www.fimea.fi), and it is also included in the EU *Campylobacter* resistance monitoring (70). It is not used for animals.

Danofloxacin is a veterinary fluoroquinolone otherwise structurally similar to ciprofloxacin but containing a diazabicycloheptan in position 7 carbon (Fig. 3). It is indicated for respiratory tract infections and enteritis in bovine and swine, with one formulation currently sold in Finland (75).

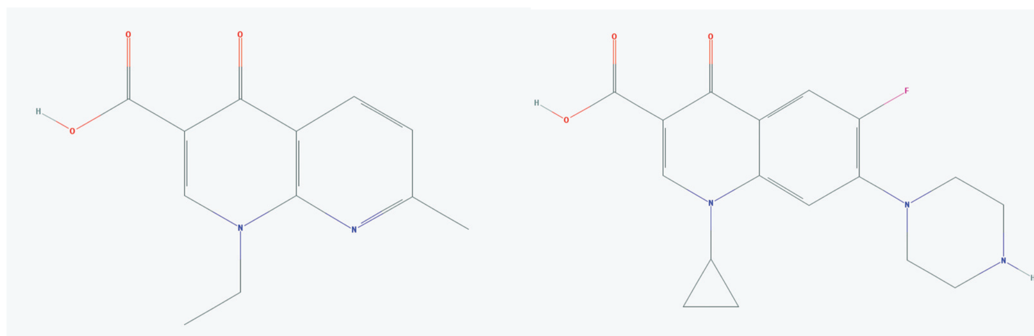

Figure 2. *Structure of nalidixic acid (left) and ciprofloxacin (right) (PubChem Compound Database).*

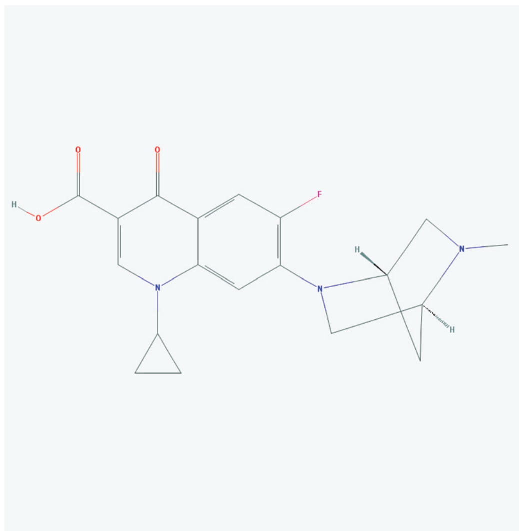

Figure 3. *Structure of danofloxacin (PubChem Compound Database).*

2.5.3 Macrolides

Macrolides are natural or semisynthetic products of soil *Streptomyces* bacteria and have been used since the 1950s (87, 88). They have a 14-, 15- or 16-membered lactone ring as a core structure which can contain several neutral or amino sugars (89). They have been shown to inhibit the elongation of bacterial peptides by blocking the ribosomal exit tunnel (90, 91). They are considered broad-spectrum and generally safe antimicrobials with only few side-effects (88). Several macrolides are accepted for use in Finland, in both humans (azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin) and animals (spiramycin, tulathromycin, tylosin) (www.fimea.fi).

Erythromycin, a 14-membered macrolide (Fig. 4), was first discovered in 1952 (92) and proved to be effective against Gram-positive pathogens, but has only modest activity against Gram-negative bacteria (88). It binds to the 23S rRNA macrolide binding pocket of the 50S subunit and this pocket is stabilized by ribosomal proteins L3, L4, L22 and L34 (88). Erythromycin causes the forming peptidyl-RNA to dissociate prematurely from the ribosome (89). Erythromycin has several clinical applications in human infectious diseases, and it is the first-choice antimicrobial for the treatment of campylobacteriosis when antimicrobial treatment is needed (2). Erythromycin resistance is monitored within the *Campylobacter* resistance monitoring programme in the EU (70).

Tylosin is a 16-membered veterinary macrolide (Fig. 4) that has a similar mechanism of action as erythromycin. It is used mainly for the treatment of respiratory, joint and enteric infections in animals. In Finland, tylosin is registered for pigs, cattle, poultry, dogs and cats and indications in pigs include the treatment of infections such as proliferative enteropathy caused by *Lawsonia intracellularis*, swine dysentery caused by *Brachyspira hyodysenteriae*, *Mycoplasma hyopneumonia* infections (tylosin is an alternative treatment) and also joint infections (75). Moreover, tylosin has been commonly used for growth-promoting purposes in pigs worldwide (93).

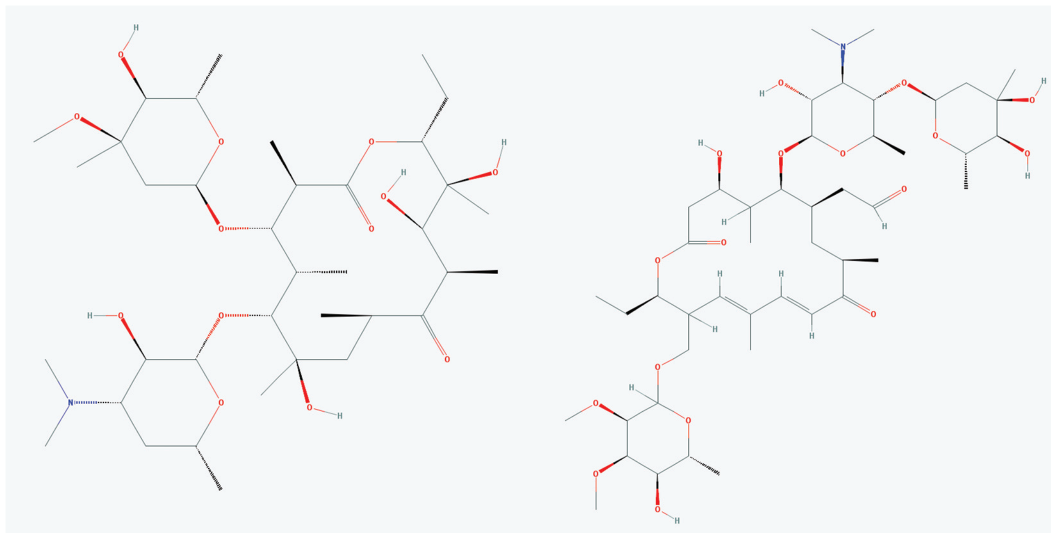

Figure 4. *Structure of erythromycin (left) and tylosin (right) (PubChem Compound Database).*

2.6 Antimicrobial consumption and surveillance

Humans have been estimated to manufacture 100 000-200 000 tonnes of antimicrobials annually (8), and antimicrobials are among the most commonly purchased drugs worldwide (94). These drugs are not used only for the treatment of human and animal infectious diseases. A large part of the produced antimicrobials is in fact consumed in agriculture for disease prophylaxis, metaphylaxis and growth promotion of food-producing animals, mainly chickens and pigs, in many parts of the world (95) and also in aquaculture and horticulture (8). In the EU member states, the use of antimicrobials for growth promotion purposes has been banned since 2006 (EU regulation no. 1831/2003). However, in several other countries such as the United States, antimicrobial use for growth promotion is allowed. In the United States alone, 15 000 tonnes of antimicrobials (of active ingredient) were sold in 2013 for use in food-producing animals (96), while, for comparison, less than 3300 tonnes were estimated to be sold for human use in 2011 (95). In the EU (a total of 26 countries), 3400 tonnes of antimicrobials (of active ingredient) were sold for humans and 7982 tonnes for use in food-producing animals in 2012 (97). Further, global consumption of antimicrobials for food-producing animals was estimated to be >60 000 tonnes in 2010 and forecasted to rise to >100 000 tonnes by 2030, mainly due to the increasing number of food-producing animals (95).

Surveillance activity of antimicrobial consumption varies between countries and regions, with well-established regular surveillance programmes in, for example, Canada, the US and most European countries. However, in many countries, surveillance is less regular (Australia, New Zealand) or non-existent (most developing countries). EU member countries report veterinary sales figures to the European Medicines Agency (EMA) overseeing the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) and human sales to the European Centre for Disease Prevention and Control (ECDC), which coordinates the European Surveillance of Antimicrobial Consumption network (ESAC-net).

In Finland, the Finnish Medicines Agency (Fimea) collects data on antimicrobial sales for animals. Total sales of veterinary antimicrobials in Finland in 2013 were 16.3 tonnes (http://www.fimea.fi/elainlaakkeet/mikrobilaakkeiden_kulutus_elaimilla) and for food-producing animals 12.5 tonnes (cf. Sweden 10 tonnes, France 681 tonnes, Italy 1318 tonnes) (69). Corrected with PCU, which takes into account the size of the food animal population in each country, the consumption for food-producing animals in Finland was 24.3 mg/PCU, which is low relative to most other EU countries (Sweden 12.6 mg/PCU, France 95 mg/PCU, Italy 302 mg/PCU) (69). The most sold classes of veterinary antimicrobials in Finland were β-lactams (G-penicillin), followed by sulfonamide-trimethoprim and tetracyclines (Table 2). However, little or no information is available on the Finnish antimicrobial consumption per animal species or indication group.

The low level of veterinary consumption in Finland can be accounted to several factors. These include efficient eradication of many common infectious animal diseases, veterinarians being allowed to hand over antimicrobials to animal owners in Finland but without profit (Lääkelaki 395/87) and all antimicrobial usage requiring a prescription. For example, in humans, the non-prescription use of antimicrobials has been estimated to range from 19% to 100% of total antimicrobial use in countries outside of the northern Europe and North America (94).

Because different units are used in surveillance of antimicrobial consumption (sales) for animals (kg of active substance) and humans (defined daily dose, DDD), comparison of the data is difficult (Table 2). However, in a recent EU report, it was estimated that approximately 47.3 tonnes of active ingredient of antimicrobials was used for humans in Finland in 2012 (as converted back from DDD) (97), while total veterinary sales were 16.0 tonnes (http://www.fimea.fi/elainlaakkeet/mikrobilaakkeiden_kulutus_elaimilla) with production animals accounting for 12.2 tonnes (97). Thus, veterinary antimicrobials accounted for 25% of total antimicrobial sales, with 19% intended for food-producing animals in Finland (97). However, these numbers are only very rough estimates of usage, as several factors cannot be accounted for, including, off-label use of antimicrobials in animals and differences between sales figures and actual consumption.

2.7 Antimicrobial resistance monitoring and susceptibility testing

Antimicrobial susceptibility testing is performed both for guiding the therapy of infectious bacterial diseases and for epidemiological monitoring of bacterial resistance rates. According to Directive 2003/99/EC, all EU member states are required to provide antimicrobial resistance monitoring data. In Finland, antimicrobial resistance monitoring from pathogenic bacteria from humans is coordinated by the National Institute for Health and Welfare and the Finnish Study Group for Antimicrobial Susceptibility Testing (FiRe). Resistance data from zoonotic and indicator bacteria from animals are collected by the Finnish Food Safety Authority. The results of monitoring are published regularly in national FINRES and FINRES-Vet reports. EU-wide surveillance data are collected by the European Food Safety Authority (EFSA) and ECDC, which publish the resistance prevalence data annually.

Two different criteria are used for dividing bacteria into susceptible and resistant populations. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines clinical resistance in the context of the likelihood of therapeutic success or failure at the achievable antimicrobial activity at the desired body site using clinical breakpoints. On the other hand, for monitoring purposes, microbial resistance is defined by the absence or presence of

acquired resistance mechanisms for the tested antimicrobial, dividing bacteria into wild-type (WT) and non-wild-type (NWT) populations using epidemiological cut-off (ECOFF) values derived from MIC (minimum inhibitory concentration) distributions of studied bacterial populations (www.eucast.org). The MIC is defined as the lowest antimicrobial concentration inhibiting all visible growth of an organism after overnight incubation (for certain organisms, including *Campylobacter* spp., the incubation period is extended due to slower growth) (98). For a given bacterial species, the clinical break-points and ECOFFs can differ from each other.

Several phenotypic and genotypic methods exist for testing antimicrobial susceptibility. Commonly used phenotypic methods are agar dilution, broth macro- and microdilution, disc diffusion and E-test (PDM Epsilometer, AB Biodisk, Solna, Sweden). In the dilution methods, generally performed by cultivating bacteria on agar plates containing serial dilutions of an antimicrobial agent or inoculating broth in test tubes or microtitre wells containing doubling concentrations of antimicrobial, the antimicrobial activity is defined by the first concentration with no visible growth of bacteria (MIC). In disc diffusion tests and E-test, the growth inhibition zone around a disc or strip containing an antimicrobial is measured.

Genetic resistance testing methods are based on detecting a known resistance associated gene or mutation by PCR and sequencing or by using genomic data acquired through next-generation sequencing (NGS) techniques. These methods provide a fast and efficient way to detect antimicrobial resistance for mechanistic and epidemiological screening.

The first standardized phenotypic susceptibility testing method for *Campylobacter* spp., agar dilution, was only available as late as in 2004 (99). At present, EUCAST has standardized disc diffusion and broth microdilution methods while the Clinical and Laboratory Standards Institute (CLSI) recommends using the agar dilution and broth microdilution methods for *C. jejuni* and *C. coli* (13). At the time of writing, the document summarizing EUCAST methodology for MIC determination of non-fastidious and fastidious organism was under preparation (www.eucast.org).

2.8 Antimicrobial resistance prevalence and mechanisms

Antimicrobial resistance is a growing problem worldwide and the World Health Organization has expressed a concern that unless drastic actions are taken we might be entering the post-antibiotic era already in the 21st century. This means that micro-organisms will become so resistant to common antimicrobials that no treatment options remain and we will face a situation in which even a minor injury/infection can lead to death (9). While the large-scale use of antimicrobials by humans only began in the 20th century, antimicrobial agents, and consequently, antimicrobial resistance-conferring genes, are ancient and very prevalent in, for example, environmental bacteria (100). The environment has been proposed to be a rich source of resistance genes readily mobilized into pathogenic bacteria upon selection and a concept of an antimicrobial resistome has been introduced and suggested to include all known antibiotic resistance genes and their precursors (100). Bacteria are known to exchange DNA through transduction, transformation and conjugation facilitating horizontal transfer of resistance-conferring genes. In addition, while harboring antimicrobial resistance-conferring mutations and genes have been traditionally thought to incur a fitness cost to an organism (101), there is now growing evidence of cost-free mutations and that several compensatory mechanisms can alleviate fitness costs, indicating that even cessation of the use of antimicrobials does not necessarily eradicate resistant strains at, for

instance, animal production facilities (10, 11). This is a concern also since there is mounting evidence that an increase in antimicrobial consumption in animals can increase the occurrence of resistant bacteria in humans (9, 97).

In general, antimicrobial resistance in bacteria can be conferred by three main mechanisms: decreasing antimicrobial entrance into the cell usually conferred by efflux mechanisms or reduced permeability of the cell membrane, drug target alteration that prevents the binding of the drug and enzymatic modification of antimicrobials (102). Resistance can also be divided into intrinsic and acquired forms. Intrinsically resistant bacterial species are naturally resistant to certain antimicrobials, for example, due to lack of a binding site for the drug or having a growth environment that does not favor the action of the antimicrobial. *Campylobacter* are considered intrinsically resistant to several antimicrobials such as polymyxin, novobiocin, cephalothin, cefoperazone and rifampin (103-105). Acquired resistance, on the other hand, can be conveyed by obtaining resistance encoding genes or mutations in bacterial genomes or increased action of certain efflux pumps. The main resistance mechanisms described in *Campylobacter* spp. are schematically presented in Fig. 5 and those relevant for this thesis, namely against aminoglycosides, quinolones and macrolides, are discussed further below.

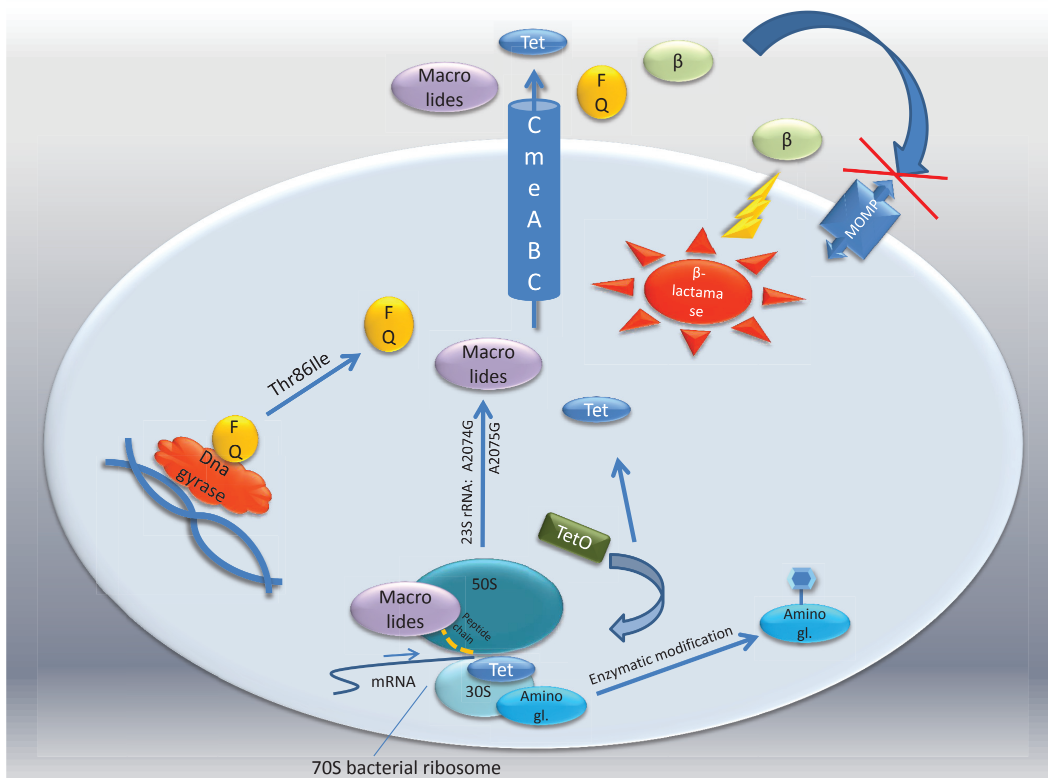

Figure 5. *Main resistance mechanisms described in Campylobacter spp. Aminogl.: aminoglycosides β: β-lactams, CmeABC: efflux pump, MOMP: major outer membrane protein, mRNA: messenger RNA, Tet: tetracycline, TetO: ribosomal protection protein, 30S, 50S: bacterial ribosomal subunits. Published in (106).*

2.8.1 Resistance to aminoglycosides

A commonly described aminoglycoside resistance mechanism in bacteria is enzymatic modification of the drugs, and a vast number of genes encoding these modification enzymes have been described in a vast number of bacterial species (107-109). The aminoglycoside-modifying enzymes (AMEs) are divided into three groups based on their mode of action, namely the phosphotransferases (APH), acetyltransferases (AAC) and nucleotidyl/adenylyltransferases (ANT), the second forming the largest and the last the smallest group (107). These enzymes function by phosphorylating, acetylating or adenylylating a specific free hydroxyl or amino group of the drug, thereby leading to inactivation (20). The AMEs form a heterologous group of enzymes, sometimes only sharing the ability to inactivate one or several aminoglycosides. Especially adenylyltransferases but also certain subgroups of phosphotransferases can share only low amino acid sequence identity with other members of the group (110-112).

There are two different nomenclature systems used for AMEs and their encoding genes, which further complicates the research of these enzymes and creates confusion. For example, in some occasions, the same name has been applied to two different enzymes (113). The system used in this thesis consists of a three-letter capitalized code specifying the enzymatic action (as above) followed by the site of modification as a number in parentheses and a Roman numeral indicating the resistance profile conveyed. In this system, the encoding genes are named with the same characters, but in lowercase and in italics (107, 113).

AME-encoding genes in bacteria are often located in transferrable genetic elements such as plasmids, integrons or transposons, commonly accompanied by other resistance or virulence genes, but they have also been found in chromosomes with no obvious indication of horizontal transfer mechanism. These genes could have evolved primarily in antibiotic-producing, mainly Gram-positive, soil bacteria and spread via horizontal transfer to other species or they could have arisen through convergent evolution in numerous bacterial species from chromosomal genes encoding enzymes originally with different functions and substrates (20, 108, 112, 114, 115). Some AMEs have been shown to have greater affinity for substrates other than aminoglycosides (116). For example, AAC(2')-Ia, a gentamicin resistance-conferring acetyltransferase encoded chromosomally in *Providencia stuartii*, has been shown to contribute to peptidoglycan O-acetylation (117). In addition to AME-encoding genes being often detected together with other resistance genes, there is at least one report of an aminoglycoside acetyltransferase that has acquired enzymatic activity capable of fluoroquinolone modification in *E. coli* (118).

Also, target binding site modification can confer aminoglycoside resistance. It can be due to mutations in the 30S ribosomal structures such as 16S rRNA (119) or enzymatic modification of the target site by 16S rRNA-methyltransferases (16S-RMTases), found in a number of pathogens, the encoding genes often also located in transferrable genetic elements such as plasmids (120, 121). However, the origins of the 16S-RMTase-encoding genes in pathogenic bacteria remain unknown (121).

For streptomycin, the most common resistance mechanism is the enzymatic drug modification mediated by ANT(6), ANT(3"), APH(6) and APH(3") classes of enzymes (107). Also, mutations at the ribosomal target sites, mainly in the *rrs* gene encoding 16S rRNA (122-124) and in *rpsL* encoding ribosomal protein S12, have been shown to result in streptomycin resistance (124-126). Mutations in 16S rRNA are clinically important merely in *M. tuberculosis*, which contains only one copy of the gene. On the contrary, mutations in the *rpsL* gene (in codons 43 and 88, *C. coli* numbering) have been associated with streptomycin resistance in

several bacterial species including *E. coli, M. tuberculosis* and *Helicobacter pylori* (122, 125, 126). Interestingly, mutations in a conserved 7-methylguanosine methyltransferase encoding gene *rsmG* (also known as *gidB*), resulting in a loss of function and consequently absence of methylation of a specific streptomycin binding site in bacterial 16S rRNA have been reported to confer a low level of streptomycin resistance in several bacterial species (127-130). In contrast, methylation of the target site by 16S-RMTases has not been associated with streptomycin resistance (121).

2.8.1.1 Aminoglycoside resistance in Campylobacter *spp.*

In recent years, increasing rates of isolation of gentamicin-resistant *Campylobacter* strains originating from humans, animals and animal products have raised concerns both in the US and in China (12, 14). In addition, in many studies high incidence of streptomycin resistance in *C. coli* originating from both humans and animals has been reported (15-19, 131) and a higher rate of streptomycin-resistant isolates has been detected in *C. coli* than in *C. jejuni* (18, 132). Especially among porcine *C. coli*, high rates (48-90%) of streptomycin-resistant strains, often also resistant to other antimicrobials, have been reported in many countries, including Canada, Japan, Denmark, Switzerland and Italy (15-17, 131, 133, 134). In Finland, in a study of antimicrobial resistance in *C. jejuni* originating from several domestic sources, only one isolate resistant to gentamicin was detected, but the streptomycin resistance rate varied from 0 to 31% between sources (135). No gentamicin-resistant *C. jejuni* strains from broilers or *C. coli* from pigs were detected in 2013 in Finland, but 2.3% of *C. coli* from broilers were resistant to gentamicin and 37.4% of *C. coli* from pigs were resistant to streptomycin ((70), www.zoonoosikeskus.fi). However, most of the STR resistant porcine *C. coli* isolates had only small increase of STR MIC (8 mg/l) and excluding these isolates from the resistant fraction, the proportion of STR resistant isolates was 10.7%.

Also, a high incidence of streptomycin resistance has been reported among *C. upsaliensis* (5, 28) and streptomycin resistance has been proposed to be intrinsic for this species (28). Another study found that *C. upsaliensis* strain isolated from a human patient was resistant (unlike most other *Campylobacter* species studied) to nalidixic acid, oxytetracycline and novobiocin, but not to streptomycin or most of the β-lactam antibiotics, including an amoxicillin-clavulanic acid combination, commonly used for various indications in small animal practice in Finland (29). However, the mechanisms of streptomycin resistance in *C. upsaliensis* have not been characterized and overall data on the prevalence of antimicrobial resistance among *Campylobacter* spp. from Finnish dogs are scarce or non-existent.

Numerous complete and partial sequences of AME-encoding genes have been detected in *C. jejuni* and *C. coli* (Table 3) (14, 24, 25, 68, 111, 136-141) often located in multidrug resistance plasmids (14, 24, 29, 141), chromosomally in genomic islands containing also other resistance or pathogenicity genes (25, 68) or as a part of integrons (140, 142). In particular, aminoglycoside 6-adenylyltransferase encoding genes, called *ant(6)*, *aadE* or *aadK*, have been frequently described as part of a resistance gene triplet *aadE-sat4-aphA3* in *Campylobacter* spp. (14, 24, 68, 141) and they have shared sequence similarity with those from Gram-positive bacteria (24, 25, 68, 143). Other aminoglycoside resistance mechanisms have not been previously described in *Campylobacter* spp.

Table 3. Aminoglycoside and streptothricin resistance genes detected in *Campylobacter* spp.

Gene[1]	Resistance conferred	Species	Location	Reference[2]
aac	kanamycin/not defined	*C. jejuni/C. coli*	plasmid/ chromosome	(24, 68)
aacA-AphD	not defined	*C. coli*	chromosome	(68)
aac(6')-Ie/ aph(2")-Ia	not defined	*C. coli*	not defined	(23)
aac(6')-Ie/ aph(2")-If$_2$	not defined	*C. jejuni*	not defined	(23)
aacA4	tobramycin, gentamicin	*C. jejuni*	not defined	(140)
aad9	spectinomycin	*C. jejuni, C. coli*	plasmid	(14, 24)
aadA	non-functional	*C. jejuni*	plasmid	(24)
aadE	streptomycin	*C. jejuni, C. coli*	plasmid	(14, 24, 68, 139, 141)
ant(6)-Ib	streptomycin	*C. fetus* subsp. *fetus*	chromosome	(25)
aph(2")-Ib	gentamicin	*C. jejuni*	not defined	(144)
aph(2")-Ic	gentamicin	*C. coli*	not defined	(144)
aph(2")-Ig	gentamicin	*C. coli*	not defined	(144)
aph(2")-Ih	gentamicin	*C. coli, C. jejuni*	not defined	(144)
aph(2")-If$_{(1-3)}$	kanamycin, gentamicin, tobramycin, dibekacin, sisomicin	*C. jejuni, C. coli*	plasmid/ not defined	(23, 24, 111)
aph(2")-Ig	gentamicin, kanamycin	*C. coli*	plasmid	(14)
aphA-3	kanamycin	*C. jejuni, C.coli*	plasmid, choromosome	(14, 24, 68, 137, 141, 145)
aphA-7	kanamycin	*C. jejuni/C. coli*	plasmid/ not defined	(68, 146)
sat4	streptothricin	*C. jejuni, C.coli*	plasmid, chromosome	(14, 24, 68, 141)

[1]Annotation used by the authors has been applied
[2]Even though several studies have described genes annotated identically (e.g. *aadE*), their nucleotide sequences might differ significantly

2.8.2 Resistance to quinolones

Common quinolone resistance mechanisms in bacteria include mutations in the quinolone resistance determining region (QRDR) of the GyrA subunit of DNA gyrase or the ParC of the topoisomerase IV. Also reduced cell permeability or increased efflux has been shown to contribute to quinolone resistance (Fig. 5) (81). Further, a plasmid-mediated quinolone resistance gene conferring a low level of resistance has been described in certain Gram-negative bacteria, but not in *Campylobacter* (147, 148).

Since *Campylobacter* spp. lack the *parC* and *parE* genes encoding the topoisomerase IV subunits, resistance to quinolones is generally mediated by mutation(s) in the *gyrA* gene and by increased efflux by an unspecific CmeABC efflux pump (53, 149-152). The most commonly described resistance-conferring mutation in *C. jejuni* and *C. coli* is the one leading to amino acid substitution T86I (Threonine86Isoleucine) in GyrA resulting in a high level of resistance. Other substitutions in QRDR of GyrA have also been associated with various levels of quinolone resistance or resistance to nalidixic acid only (150, 151, 153-156). Unlike in bacteria containing two targets for the quinolones, development of quinolone resistance in *Campylobacter* is not a stepwise process, but a single point mutation in the QRDR of *gyrA* can lead to high levels of resistance (156-158), indicating that these species are sensitive indicators on the use of fluoroquinolones. Further, it has been suggested that fluoroquinolone resistance does not incur a fitness cost in *C. jejuni*, as resistant isolates have been shown to outcompete susceptible ones in chicken (159).

After the introduction of fluoroquinolones in human and veterinary medicine in the 1980s, increasing rates of resistant *Campylobacter* isolates have been detected worldwide (160, 161). Even though the use of fluoroquinolones in poultry production in the US was banned in 2005, there are reports showing that quinolone-resistant *Campylobacter* persists in poultry for years after usage, further complicating the eradication of resistant strains (85, 162). In countries mainly located in the southern and eastern parts of Europe, high resistance rates in *C. coli* and *C. jejuni* (>90%) have been reported in humans and animals, while the Nordic countries have generally had low rates of resistance (13, 70). Further, higher rates of fluoroquinolone resistance have been detected among *C. coli* than *C. jejuni* in studies from several countries (13).

In 2013, none of the investigated Finnish *C. jejuni* isolates from broilers were resistant to ciprofloxacin, but 9.2% were resistant to nalidixic acid, and 18.3% of the *C. coli* from pigs were resistant to ciprofloxacin (70). In 2014, the national monitoring programme found 25% of the studied *C. jejuni* from broilers to be quinolone-resistant (www.zoonoosikeskus.fi).

2.8.3 Resistance to macrolides

Resistance to macrolides is mainly conferred by alterations in the ribosomal drug binding sites, either by acquisition of a methylase-encoding gene (*erm*) or through mutations in the host ribosomes, and by increased efflux (Fig. 5). The methylating genes are usually found in Gram-positive species and they methylate specific macrolide binding sites of 23S rRNA, thereby inhibiting the binding (88). The resistance conferring mutations in the host ribosomes are mainly located in the macrolide binding sites of the 23S rRNA (163). The other prevalent resistance mechanism is increased efflux by an efflux pump that is mediated through acquisition of genes encoding efflux-proteins by Gram-positive species (164, 165) or by intrinsic non-specific transport systems, mainly found in Gram-negative species (88).

The described mechanisms of macrolide resistance in *Campylobacter* include mutations in the 23S rRNA macrolide binding sites and increased efflux by the non-specific CmeABC efflux pump (29, 166-168). *Campylobacter* have three copies of the 23S rRNA gene and at least two mutated copies are needed for a resistant phenotype (166, 169). Also mutations in the ribosomal proteins L4 and L22 have been associated with a low level of macrolide resistance in *Campylobacter* (170, 171). Further, among *Campylobacter* spp., the *erm* gene was first described in *C. rectus* (172), and it was recently found to confer a high level of macrolide resistance also in several *C. coli* isolates originating from China. In *C. coli*, it was found to be a part of a transferrable multiple drug resistance genomic island probably originating from Gram-positive bacteria (173, 174). Macrolide resistance has been thought to incur a fitness cost in *Campylobacter* (175), but there are no studies on the effect on fitness in *Campylobacter* spp. after the acquisition of this new resistance determinant.

Compared with fluoroquinolones, macrolide resistance is not as common among *C. jejuni* from animals in EU countries, with the highest rate of 2.8% detected in Spain and no resistance in Finland in 2013 (70, 135). However, higher rates of macrolide-resistant *C. coli* than *C. jejuni* have been frequently reported (3, 70, 93, 163) with average ERY resistance of *C. coli* from broilers in 2013 being 13.7% compared with 0.4% in *C. jejuni* isolated from the same source in EU (70). Large differences in the prevalence of ERY-resistant *C. coli* from broilers were seen between countries, varying from 0 in several countries to 43% in Spain. Further, on average 20.7% of the porcine *C. coli* isolates were resistant to ERY in 2013 in the EU, but also varying greatly, from 2.3% in Finland to 58.3% in Spain (70).

2.9 Whole-genome sequencing and applications in resistance research

2.9.1 Sequencing technologies, assembly and annotation

Sanger sequencing technology was first introduced in 1977 (176) and relied on incorporating dideoxynucleotides in four separate reactions to DNA *in vitro*, terminating the elongation of the forming nucleotide chain. Originally, these resultant DNA fragments were visualized on an agarose gel and the order of the nucleotides in the template DNA strand was deduced by the size of the fragments in four lanes (176). Improvements were subsequently made, including radioactive labelling of dideoxynucleotides, which enabled the use of one reaction instead of four, and partly automated sequencing (177). Sanger sequencing was used to generate the first whole-genome sequence of the bacterium *Haemophilus influenza* (178) and also the first draft sequences of a human genome in 2001 (179). However, the laborious and costly nature of Sanger sequencing prevented its large-scale use for complete genomes and thus, next-generation sequencing (NGS) technologies (also known as deep sequencing, high throughput sequencing or massively parallel DNA sequencing) were developed (179).

The most important NGS techniques can be divided into two wide categories, those that rely on amplified DNA libraries of the template and those that do not require amplification but can determine the nucleotide sequence of a single DNA molecule (180). The sequencing equipment can also be divided into high-end and bench top sequencers based on the speed and number of bacterial genomes sequenced in one run (180, 181).

In amplification-based sequencing, the template DNA is first isolated in sufficient quantity, fragmented with a variety of methods in fragments of suitable length, tagged with adaptors

and amplified on a solid surface to enable large-scale parallel sequencing (180, 182). All of the amplification-based sequencing methods rely on sequencing by synthesis or sequencing by ligation principle, but vary in technical details (180, 183). For example, the Illumina Solexa technology (Illumina Inc., San Diego, CA, USA) is based on reversible termination of elongation in which terminator nucleotides with fluorescent labels emitting at different wavelengths are perfused across the sequencing cell and the resulting signals are imaged. The terminator dye is then enzymatically cleaved to allow insertion of the next nucleotide ((180), www.illumina.com). Sometimes it is challenging to discriminate the A and C signals as well as the G and T signals due to similar emission spectra of the fluorophores, and base substitution is the main error type in this technology (182, 184). The Ion Torrent (Life Technologies Corporation, Grand Island, NY, USA) technology is based on adding only one kind of nucleotide at a time on the template, and the signal (a hydrogen ion) is detected when insertion occurs (180). As all of these technologies have weaknesses, sequence quality should always be assessed considering also the sequencing technology used (182, 183).

In the newest technologies sequencing is performed on single DNA molecule templates without an amplification step (180). In 2012, a new single DNA molecule sequencing technology based on feeding the template (unamplified) DNA through protein nanopores was introduced, and a complete *E. coli* genome was assembled *de novo* (see next section) using only data generated by the nanopore technology in 2015 (180, 183, 185).

The challenge of NGS is in the handling and storing of the large amount of information generated. The sequence data, also known as reads, can vary in size from less than one hundred bases to several kilobases, depending on the technology used (183). These reads need to be assembled into longer continuous DNA sequences, called contigs, and two different approaches are used in assembly. Reads can be mapped to a known reference genome or assembly can be performed without a reference, also called *de novo* assembly (180). The *de novo* fragment assembly can be described as the problem of reconstructing a DNA string from its *k*-mers and different mathematical approaches are used in solving it (186-188). Thousands of assembly algorithms have been written and many assemblers exist, such as ABySS (Assembly By Short Sequences) (189), MIRA (Mimicking Intelligent Read Assembly), SPAdes (188) or Velvet (190). All of these are optimized for reads generated by certain sequencing technologies, performing reference-based or *de novo* assemblies or both (183).

After assembly of reads into contigs, annotation can be performed using a variety of programs, such as a free online tool, the RAST (Rapid Annotation using Subsystem Technology) server (191). Automatic annotation is usually based on putative gene detection and function prediction by comparison with known genes in databases (187). The annotation process is far from flawless and may miss or wrongly annotate several hundred coding sequences (CDS), therefore requiring human input, also called manual curation (187). Since most genomes in public databases are annotated only automatically, the putative errors need to be taken into account when utilizing these resources.

2.9.2 Whole-genome sequencing approach in antimicrobial resistance research

NGS technologies provide a means of studying bacterial genomes in more detail and with higher resolution than ever before. In relation to antimicrobial resistance, sequence data can be utilized in, for example, studies of the epidemiology of resistant bacteria, as a basis for new antimicrobial

drug development or for surveillance of bacterial resistance rates based on well-characterized resistance-conferring genes and mutations, such as those in the QRDR region of *gyrA* in the case of fluoroquinolone-resistant *Campylobacter* (181, 192). Resistance gene databases have been set up to aid the screening of WGS data and enable mining for and recognition of putative or existent resistance-conferring genes (193, 194).

Also, considerable interest has been directed at the characterization of new resistance mechanisms and genes based on whole-genome sequencing. Several new resistance-conferring mutations, genes and multidrug resistance islands have been uncovered using NGS in many bacteria, including *Campylobacter* (14, 23, 195). For instance, Chen *et al.* (2013) (14) were able to find several novel aminoglycoside resistance genes located in MDR plasmids in *C. coli* using NGS. In addition, WGS has revealed many cryptic or potential resistance genes, e.g. those coding for proteins resembling aminoglycoside-modifying enzymes, embedded in the genomes of many pathogens (100). These cryptic genes have been called resistance precursors or proto-resistance elements and have been suggested to be included in to the antibiotic resistome (100, 115).

As NGS techniques have become increasingly affordable and more user-friendly data analysis tools are being developed, it seems likely that many laborious genotyping as well as phenotypic antimicrobial resistance screening methods will be replaced by NGS techniques in the near future (196). For example, analysis of WGS data for resistance associated-genes and mutations was found to be a highly accurate method for predicting antimicrobial resistance phenotypes in *C. jejuni* and *C. coli* (144). However, these methods are unlikely to completely replace traditional phenotypic methods due to the fact that resistance conferred by, for instance, increased activity of efflux pumps or a completely new mechanism would not be detected by studying only known genetic changes. Also, in many cases, the level of resistance cannot be precisely predicted using only genomic data.

3 AIMS OF THE STUDY

The aims of this thesis were to characterize the mechanisms of high and intermediate STR resistance in porcine *C. coli* and canine *C. upsaliensis* and to find putative connections between resistance to STR and macrolides in *C. coli*. Further, the occurrence of antimicrobial resistance in *Campylobacter* spp. from a set of dogs was investigated, and mutations associated with fluoroquinolone and macrolide resistance in *C. coli* and quinolone resistance in *C. upsaliensis* were studied.

Specific objectives were as follows:

1. To characterize the mechanism of high-level STR resistance in *C. coli* and to find potential connections between macrolide and STR resistance (I)

2. To investigate the mechanisms, origins and natural transformation capability of intermediate-level STR resistance in *C. coli* (III)

3. To analyse the effect of DANO and tylosin treatments of pigs on STR resistance in *C. coli* at farm level and to investigate the mutations associated with macrolide and fluoroquinolone resistance in *C. coli* with special attention to DANO-induced changes in QRDR of *gyrA* (II, III)

4. To characterize the potential STR and quinolone resistance-associated mutations in *C. upsaliensis* from dogs and to assess the occurrence of antimicrobial resistance among canine *Campylobacter* spp. (IV)

4 MATERIALS AND METHODS

4.1 Antimicrobial treatments and sampling scheme of animals

4.1.1 Pigs

In Study I, the 17 STR- and/or ERY-resistant *C. coli* isolates originated from 15 pigs from two pig farms. At farm 1, tylosin was used for weaned piglets for the treatment of proliferative enteropathy caused by *Lawsonia intracellularis*, and occasionally penicillin, sulfadiazine-trimethoprim and aminopenicillin were used (detailed description of medications can be found in (27)). At farm 2, penicillin and sulfadiazine-trimethoprim were used if needed. No aminoglycoside treatments had been recorded from either farm at least for the past four years prior to the sampling.

In Study II, 27 piglets from farm 3 were included and part of the animals (group A, n=12 pigs) were treated intramuscularly (im) with DANO for three days at a dose of 3.3 mg/kg at 4-5 days at weaning due to diarrhoea caused by *E. coli* and tylosin in feed at a dose of 3-6 mg/kg for 10 days starting at 11 days after weaning, whereas the controls (group B=15 animals) received only tylosin without DANO treatment. Additionally, three piglets from both groups were treated with amoxicillin im before weaning. Pigs were sampled at six time points: before weaning (sampling I), after weaning (samplings II and III), after DANO medication of group A (samplings IV and V) and after tylosin treatment of both groups (sampling VI). The animals were reared in three pens, each containing animals from both groups.

4.1.2 Dogs

The 36 dogs in Study IV were Staffordshire bull terriers originating from a total of 30 households located in different parts of Southern Finland and aged between 1 and 12 years. They were sampled twice, before and at the end of a feeding period lasting 4-5 months and consisting of either raw feed or pelleted dry feed. No systemic antimicrobial treatments were recorded from the sampled dogs right before or between the samplings.

4.2 Isolation and cultivation of *Campylobacter* spp. and *E. coli*

C. coli strains (n=184) originated from five Finnish pig farms in Studies I – III. The strains were used in different studies as follows. In Study I, 17 isolates originating from farms 1 and 2 were used (primary STR-resistant isolates) to examine resistance-related mutations, and 13 isolates from five additional pig farms were used to make STR- and ERY-resistant *C. coli* variants *in vitro*. In Study II, *C. coli* isolates were collected during six samplings at farm 3 and 147 of them were tested for susceptibility to STR, ERY and CIP. Twenty-one of these isolates were characterized further by sequencing selected gene fragments and 133 were genotyped by restriction fragment length polymorphisms pulsed field gel electrophoresis (RFLP-PFGE). In Study III, 12 characterized isolates from Study II were whole genome sequenced. Additionally, 34 isolates originating from farms 1-3 (partly the same isolates used in Studies I and II) were screened by PCR for the presence of *ant*-like gene A in Study III. All *C. coli* were isolated from pig faecal samples taken from the rectum with cotton swabs (Propact, Technical Service Consultants Ltd., Heywood, UK) and cultivated on modified charcoal cefoperazone deoxycholate agar (mCCDA)

(CM739, Oxoid Ltd., Basingstoke, Hampshire, UK) with selective supplement (SR155, Oxoid Ltd.) with or without pre-enrichment in 5 ml of Bolton broth (CM983, Oxoid Ltd.). The *C. upsaliensis* and *C. jejuni* strains in Study IV were isolated from dog faeces as a three-day pooled sample and cultivated on mCCDA. Incubation was under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2 for *C. coli* and *C. jejuni* and 6% O_2, 10% CO_2, 5% H_2, 79% N_2 for *C. upsaliens*) at 37 °C. The putative *Campylobacter* isolates were confirmed as *C. coli*, *C. jejuni* or *C. upsaliensis* with species-specific PCR (27, 197-199).

The *E. coli* NEB 5-alpha cells (New England Biolabs, Frankfurt, Germany) used in Study III were grown under normal atmospheric oxygen at 37 °C on Luria-Bertani (LB) plates (Difco, Sparks, MD, USA) supplemented with ampicillin (100 mg/l, Sigma-Aldrich, Stenheim, Germany) or chloramphenicol (33 mg/l, Sigma-Aldrich), as appropriate.

All isolates were stored frozen at -70°C in either skim milk or nutrient broth (NB, Oxoid Ltd.) with 15-20% glycerol. *C. coli*, *C. jejuni* and *C. upsaliensis* were further grown on Brucella or Nutrient agars (NBA, Oxoid Ltd.) supplemented with 5% blood (Labema, Kerava, Finland).

4.3 Antimicrobial susceptibility testing

The agar dilution method (according to M31-A3 NCCLS, 2002 or M45-A CLSI, 2006) was used in all four studies (see the last paragraph for detailed description of susceptibility testing methods used in each study). Briefly, bacterial isolates were sub-cultivated twice overnight and suspended in Muller-Hinton Broth (MHB, Oxoid Ltd.) to make a suspension with turbidity corresponding to 0.5 McFarland standard. A multipoint inoculator was then used to inoculate Muller-Hinton agar plates (MHA, Oxoid Ltd.) supplemented with 5% blood with doubling concentrations of the desired antimicrobial. The plates were incubated microaerobically for 48 h at 37 °C, after which the results were read and MICs were recorded as the lowest concentration inhibiting all visible growth.

The broth microdilution method using VetMIC Camp (National Veterinary Institute, Uppsala, Sweden) microtitre plates was applied in Studies I and IV according to the procedure of the manufacturer. Briefly, after freezing, bacterial isolates were grown twice overnight, after which suspensions with turbidity corresponding to 0.5 McFarland standard were made and diluted to 1:1000 in cation-adjusted MHB (Difco, Becton-Dickinson and Company, Sparks, USA) supplemented with 5% lysed horse blood to yield suspensions containing approximately 10^5 cells. Due to the fastidious nature of some of the *C. upsaliensis* isolates, NB was used in place of cation-adjusted MHB in Study IV (30, 200). These bacterial suspensions were then inoculated in microtitre plates, which were incubated microaerobically for 48 h at 37 °C. The results were read under a magnifying lens with a lamp and MICs were recorded as the lowest concentration inhibiting all visible growth in a well.

The E-test method used in Study I was performed according to the manufacturer's instructions by preparing bacterial suspensions with turbidity corresponding to 0.5 McFarland standard and cotton swabs were used to spread suspension evenly on MHA plates. The agar was allowed to dry and then an E-test strip (Biomerieux-Suomi Oy, Helsinki, Finland) containing a concentration gradient of the desired antimicrobial was placed in the middle of the agar. The plates were incubated for 48 h at 37 °C microaerobically. MICs were recorded as the concentration at which the elliptical zone of no bacterial growth intersected with the strip.

For all susceptibility assays, *C. jejuni* reference strain ATCC 33560 was used as a control. The ECOFF values as determined by EUCAST (www.eucast.org) were applied for each species as appropriate. Since no ECOFFs have been determined for *C. upsaliensis*, those of *C. jejuni* were applied for this species.

In Study I, the preliminary antimicrobial susceptibility testing of *C. coli* was carried out with the broth microdilution method, and STR and ERY MICs were confirmed with E-tests or agar dilution. In Study II, susceptibility testing of *C. coli* isolates for STR (MP Biomedicals, Ill Cedex, France), CIP (Sigma-Aldrich) and ERY (Sigma-Aldrich) was performed with the agar dilution method. In Study III, MICs of *C. coli* for STR, CIP, ERY and spectinomycin (SPEC, Sigma-Aldrich) were determined with the agar dilution method. Additionally, STR susceptibility of selected transformant *E. coli* colonies was tested with the broth macrodilution method (CLSI M07-A9). In Study IV, the *C. upsaliensis* and *C. jejuni* isolates originating from 18 dogs were tested with the broth microdilution method, and STR MICs were confirmed with the agar dilution method.

4.4 Selection of resistant variants by exposure to STR and ERY and efflux pump inhibition assay

In Study I, 13 *C. coli* isolates with STR MICs of 2-4 mg/l were cultivated successively on MH agars with 5% blood containing increasing concentrations of STR and 20 *in vitro* generated STR-resistant colonies isolated from plates containing 8, 32 or 64 mg/l STR were selected for further MIC analysis. In addition, two of these *in vitro* acquired STR-resistant variants as well as two STR-susceptible *C. coli* isolates were used for selection of ERY-resistant variants by cultivating in increasing concentrations of ERY.

The effect of efflux pump inhibitor phenyl-arginine-β-naphthylamine (PaβN; Sigma-Aldrich) on STR MICs of 17 primary STR/ERY-resistant *C. coli* isolates in Study I was tested in two independent experiments using the agar dilution method. Briefly, bacterial isolates were grown on MHA plates containing 1-1024 mg/l STR with and without 50 mg/l PaβN and STR MICs were recorded after 48 h of microaerobic incubation at 37 °C. As a control, the isolates were also cultivated on plates containing 0.5-1024 mg/l ERY with and without 50 mg/l PaβN or only 50 mg/l PaβN.

4.5 DNA extraction, PCR, sequencing and sequence analysis of PCR products

Bacterial DNA was extracted with Pitcher's method (201) (Studies I and II) or using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) or Purelink Genomic DNA mini kit (Invitrogen, Carlsbad, CA, USA) (Studies I, III and IV). Plasmid DNA was extracted with E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA) in Study I.

In Study I, the *rpsL, rsmG* (known as *gidB* at the time), *aadE*, 23S rRNA gene and two *rrs* gene fragments (500 and 900 regions) from 17 primary STR/ERY-resistant *C. coli* isolates and the *rpsL* and *aadE* gene fragments from 33 STR-resistant variants and their parental isolates were amplified and sequenced. In addition, the rrs_{500} fragments of five *C. coli* variants (1.1, 6.1, 10.1, 10.2 and 13.1, Table 6) were amplified and sequenced. In Study II, *rplV, gyrA* and 23S rRNA gene fragments were amplified and sequenced from 19 and the *rplD* gene fragments from 12 *C.*

coli isolates. The sequences of *rplV* and *rplD* of the 12 whole-genome sequenced *C. coli* isolates in Study III were later confirmed with WGS data (see below). In Study III, the presence of *ant*-like gene A fragment was investigated from 34 *C. coli* isolates with STR MICs between 4 and >1024 mg/l using primers ccoliaadE-Fw and ccoliaadE-Rw. The *gyrA* gene fragment in Study II was amplified with primers described in (202). The other aforementioned primers used in Studies I-III are listed in Table 4.

Sequencing of amplified DNA fragments in Studies I and II was carried out at the Institute of Biotechnology, University of Helsinki, using Sanger sequencing with automated cycle sequencer with Big Dye Terminator® chemistry (PE Applied Biosystems, Foster City, CA, USA).

The sequence data were analysed using the Staden Package (http://staden.sourceforge.net/) and Bionumerics version 5.10 (Applied Maths NV, Sint-Martens-Latem, Belgium), and sequence quality was assessed with FinchTV (Geospiza Inc., Seattle, WA, USA). Consensus sequence alignment of Sanger-sequenced gene fragments was performed in ClustalW (EMBL-EBI, Cambridge, UK).

Table 4. Primers used in studies I-III.

Study	Primer	Sequence (5’-3’)	Target gene (protein)	reference genome	Reference
I	$rrsF_{500}$	cgcacgggtgagtaaggtat	*rrs* (16s rRNA 500 region)	RM2228	Study I
	$rrsR_{500}$	tcgcaatgggtattcttggt			
I	$rrsF_{900}$	ggggagcaaacaggattaga	*rrs* (16s rRNA, 900 region)	RM2228	Study I
	$rrsR_{900}$	aacaatccgaactgggacat			
I	gidBF	gggcatttactttgaatttgga	*rsmG* (RsmG)	RM2228	Study I
	gidBR	cacgcttgtgcctttaaagc			
I	rpsLF1	ccagcgcttaaaaattgtcc	*rpsL* (S12)	RM2228	Study I
	rpsLR1	tatcaagagcaccacgaacg			
I	aadEF Full	atgagatcagaaaaggaagtttat	*aadE*	pCG8245, ORF2	Study I
	aadER Full	ttatttgttatcatcttcaatgcc			
I, II	ERY23SRNA-F2	aattgatggggttagcattagc	*23S rRNA*	RM2228	(27)
	ERY23SRNA-R2	caacaatggctcatatacaactgg			
II	CcrplV F	aggccataaaggttctgtgc	*rplV* (L22)	RM2228	Study II
	CcrplV R	aaccatcttgattcccagtttc			
II	CcrplD F	aatggtgccatgggtaaaat	*rplD* (L4)	NCTC 11168	Study II
	CcrplD R	caccttgctcttgcaaattc			
II	CcrplD F5	ccaggtcgtgttcaaccag	*rplD* (L4)	NCTC 11168	Study II
	CcrplD R3	agctctttcaagcgccaat			
III	ccoliaadE-Fw	gccttgttacacttgaaggc	*ant*-like gene A (fragment)	*C. coli* 47.4 peg1084	Study III
	ccoliaadE-Rw	gtgtgtgatgctaaatggggc			
III	aadE-A-AF-KpnI	aggtaccgttgcattataaaatataagagtaacaaagcttgc	*ant*-like gene A	*C. coli* 47.4	Study III
	aadE-A-AR-PstI	atctgcagcaagcataaagattaagataaagctagaaagg			
III	aadE-A-IF-BamHI	aggatcctggttaaatcaagcctcactccactttc	Inv PCR of pUC119-ant(6)	*C. coli* 47.4 peg1084	Study III
	aadE-A-IR-SalI	accgtcgaccacactattccaaaagccccatttagc			
III	ant60-A-AF-KpnI	aggtaccgcgtggcggcggctaaattcaaagg	*ant*-like gene B	*C. jejuni* FB01514	Study III
	ant60-A-AR-PstI	atctgcaggcaaaattaaacatggtgcataagtgaagtag			

4.6 RFLP-PFGE typing

In Study II, RFLP-PFGE typing of 133 isolates originating from 12 pigs from group A and 12 pigs from group B was performed as described previously (27) using SmaI with or without additional digestion with KpnI. The banding patterns were analysed with the software Bionumerics and isolates with similarity less than 90% in the dendrogram or a minimum difference of one band were classified as different genotypes.

4.7 Whole-genome sequencing, assembly and annotation

In Studies III and IV, WGS was performed for 12 *C. coli* isolates from pigs and for 24 *C. upsaliensis* and two *C. jejuni* isolates from dogs, respectively. Further, in Study III, two *C. coli* natural transformants and a spontaneous mutant were also whole-genome-sequenced. Draft whole-genome sequences were determined using Illumina Miseq or Hiseq technology (Nextera XT library paired end kit) at the Institute for Molecular Medicine (FIMM) Technology Centre, University of Helsinki. The paired end reads were assembled into contigs using Spades 3.1.1 or 3.2.0 (188). Genomes were annotated in RAST (191) and analysed with whole-genome multilocus sequence typing (wg-MLST) using genome profiler, GEP (203).

The sequences of *gyrA, rsmG, rpsL* and *rrs* of *C. upsaliensis* in Study IV were BLAST-searched, translated in EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and aligned in MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The sequences were compared with that of *C. upsaliensis* RM3195, known to be susceptible to STR and resistant to NAL, but with no quinolone resistance-associated mutations in *gyrA* (29).

Homologues of putative STR resistance genes in the three intermediately STR-resistant whole-genome-sequenced *C. coli* in Study III and the 24 *C. upsaliensis* in Study IV were BLAST-searched using known sequences of these genes. Further, for this thesis, using WGS data, the sequences of *rplV* and *rplD* of 12 whole-genome-sequenced *C. coli* isolates were confirmed and the *rsmG* sequences of the same 12 *C. coli* were evaluated.

4.8 Screening, evolutionary analysis and enzyme modelling of *Campylobacter ant*-like gene A

In Study III, the presence of *Campylobacter ant*-like gene A was screened by PCR with the primers ccoliaadE-Fw and ccoliaadE-Rw from 34 *C. coli* isolates originating from farms 1-3 and having variable STR MICs. In addition, whole-genome sequences of ≈500 Finnish *C. jejuni* strains from several sources sequenced in other projects (204-206) were screened *in silico* for orthologues of *ant*-like gene A (peg 1084 from *C. coli* 47.4) using 40% cut-off value of the BLAST score ratio (207).

Altogether, 130 homologous sequences of *ant*-like gene A product were retrieved from NCBI nr database with blastX (http://blast.ncbi.nlm.nih.gov). These sequences were aligned using MAFFT (208), and evolutionary history was inferred in MEGA5.3 (209) using the Minimum Evolutionary Method (210). The resulting consensus tree was condensed at 95% bootstrap values and edited in iTOL web server (211).

Structural models of the ANT-like proteins A and B from *C. coli* 47.4 and *C. jejuni* 12xb_914 in Study III were built in the fully automated PHYRE2 server (212, 213), and catalytic site detection and conservation analysis were performed with Phyre2 investigator (213) using the ANT(6) from *Bacillus subtilis* as a reference structure.

4.9 Expression of *ant*-like genes in *E. coli* and construction of 47.4Δant(A)::CAT mutant

Expression of *ant*-like genes A and B in *E. coli* and construction of 47.4Δant(A)::CAT (Study III) have been schematically represented in Fig. 6. The *ant*-like genes A and B with putative promotor regions were first amplified by PCR with primers aadE-A-AF-KpnI and aadE-A-AR-PstI (*C. coli* 47.4) or ant60-A-AF-KpnI and ant60-A-AR-PstI (*C. jejuni* FB01514), respectively (Table 4), which also introduced restriction sites at each end. Each gene was then cloned into plasmid pUC119 to create pUC119antA or pUC119antB and transformed into chemically competent *E. coli* grown on LBA (Luria-Bertani agar) with 100 mg/l ampicillin. Positive transformants with plasmid containing the correct insert were confirmed by colony PCR with primers CcoliaadE-Fw and Ccoli-aadE-Rw (gene A) or by detecting correctly sized fragments after restriction-digestion with KpnI and PstI (gene B). Selected transformants were then tested for STR susceptibility by broth dilution (CLSI M07-A9). The amplified pUC119-ant(A) was isolated and used as a template for inverse PCR using primers aadE-A-IF-BamHI and aadE-A-IR-SalI (Table 4), creating linear DNA with restriction sites, and the CAT (chloramphenicol acetyltransferase) cassette with the same restriction sites was then ligated into this plasmid to obtain closed pUC119Δant(A)::CAT. This construct was amplified in *E. coli* grown on LBA with 33 mg/l chloramphenicol and introduced as a suicide plasmid into electrocompetent *C. coli* 47.4. With *C. coli* replication, the construct was inserted into the genome by homologous recombination and mutants were selected on NBA supplemented with 12.5 µg/ml chloramphenicol.

4.10 Natural transformation assays

In Study III, the natural transformability of the *Campylobacter ant*-like gene A was investigated. *C. coli* strain 18.3, susceptible to STR, was used as a recipient and DNA from *C. coli* isolate 47.4 was used as a donor in the natural transformation assays modified from previous studies (214, 215). Briefly, the recipients were incubated overnight in 2 ml of NB and the optical density (OD_{600}) was subsequently adjusted to 0.2 and 500 µl of adjusted suspension was added to test tubes containing NBA with a slanted surface. The tubes were incubated for three hours at 37 °C microaerobically and subsequently 2 µg of donor DNA diluted in a 10 µl volume was added to the recipient. The same volume of peptone water was added to the control tube to account for spontaneous mutations. Incubation was continued for three hours (one replication cycle), and bacterial suspensions were then cultivated on NBA containing 64 or 32 mg/l STR. The STR MICs and the presence of *ant*-like gene A were studied from selected transformants and spontaneous mutants by PCR using primers CcoliaadE-Fw and CcoliaadE-Rw (Table 4). Further, two natural transformants and a spontaneous mutant were whole-genome-sequenced as described above and analysed using wgMLST (GeP).

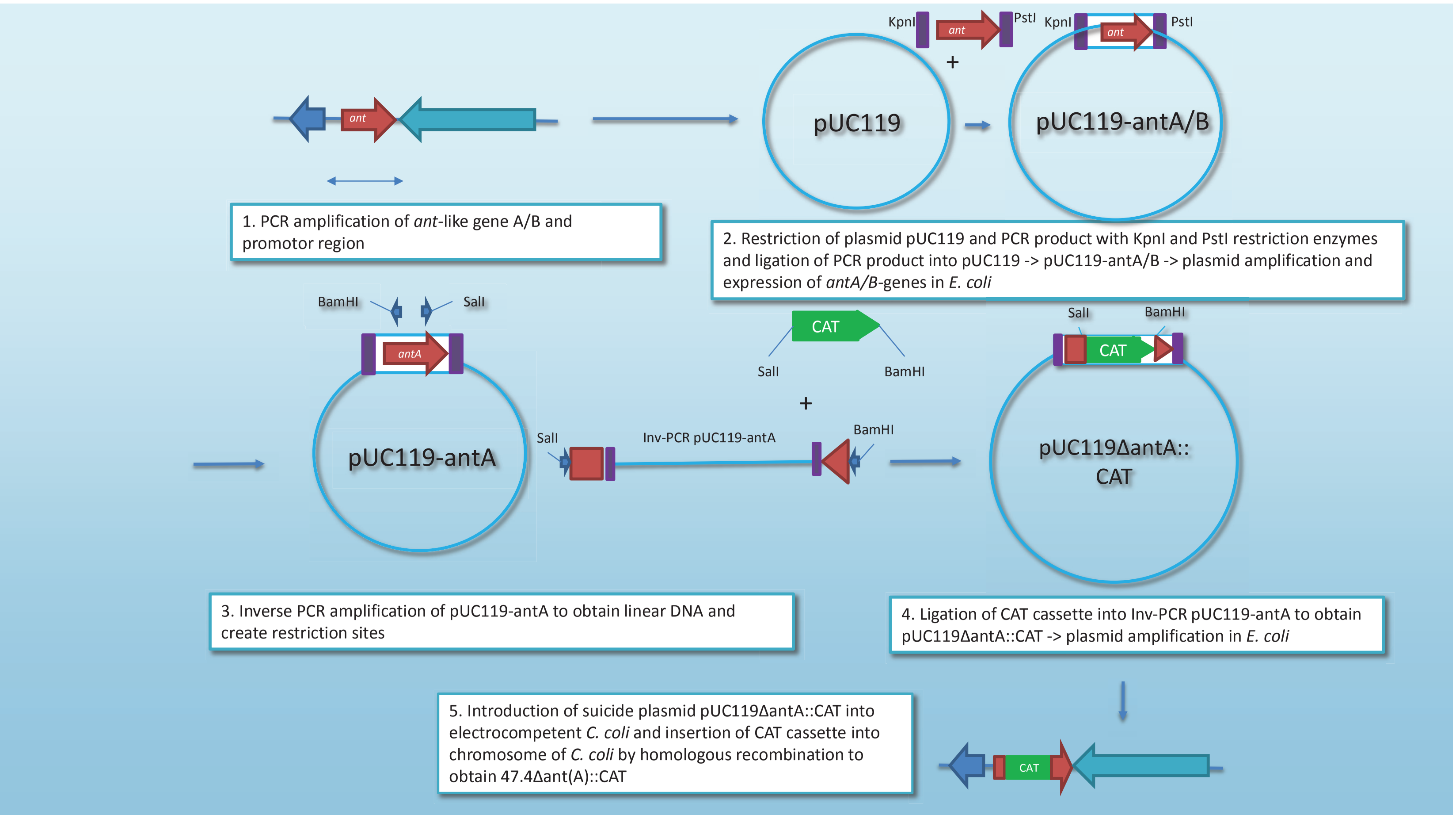

Figure 6. *Expression of* ant-*like genes A and B in* E. coli *and construction of* C. coli *47.4Δant(A)::CAT*

4.11 Statistical analyses

SPSS for Windows, Rel. 15.0.1. (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Fischer's exact test or Chi-squared test was used to test for statistically significant differences ($P<0.05$) in the prevalence of resistance among *C. coli* isolates between samplings and groups in Study II.

5 RESULTS

5.1 Characterization of antimicrobial resistance of porcine *C. coli* (I-III)

5.1.1 STR resistance-associated mutations in rpsL *(I-III)*

All analysed highly STR-resistant (MIC>1024 mg/l) *C. coli* isolates from pigs in Studies I-III (n=15) had A128G missense mutation in *rpsL*, leading to predicted K43R (Lysine43Arginine) substitution in ribosomal protein S12, but none of the studied eight STR-susceptible or of the six intermediately STR resistant (MIC 128-512 mg/l) *C. coli* had resistance associated mutations in codons 43 or 88 of *rpsL* (Table 5). No other nonsynonymous mutations were detected in the sequenced fractions of *rpsL*.

Table 5. MICs and relevant resistance conferring mutations and genes in *C. coli* from pigs.

			MIC (mg/l)			S12			23S rRNA[2]			GyrA
Study	Isolate[1]	Farm	STR	ERY	CIP	Codon 43	Codon 88	*ant*-like gene A	Base2122	L4[3]	L22[5]	codon 86
I,III	17	2	128	1	NA	K	K	+	A	NA	NA	NA
	18	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	20	2	128	1	NA	K	K	+	A	NA	NA	NA
	21	2	128	1	NA	K	K	+	A	NA	NA	NA
	22	1	1	>1024	NA	K	K	NA	G	NA	NA	NA
	36	1	1	>1024	NA	K	K	-	G	NA	NA	NA
	38	1	1	512	NA	K	K	NA	G	NA	NA	NA
	39	1	>1024	>1024	NA	R	K	-	G	NA	NA	NA
	44	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	75	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	76	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	80	1	>1024	>1024	NA	R	K	-	G	NA	NA	NA
	81	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	82	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	83	1	>1024	>1024	NA	R	K	-	G	NA	NA	NA
	84	1	>1024	>1024	NA	R	K	NA	G	NA	NA	NA
	85	1	>1024	>1024	NA	R	K	-	G	NA	NA	NA
II, III	10.4	3	4	1	≤0.125	NA	NA	NA	A	V196A	1	T
	17.3	3	4	0.5	≤0.125	NA	NA	NA	A	NA	2	T
	18.3	3	4	0.25	≤0.125	K	K	-	A	V121A, V176I, T177S, V184I, M192I, V196A	2	T
	31.1	3	4	1	≤0.125	NA	NA	NA	A	V196A	1	T
	42.1	3	4	2	0.25	K	K	-	A	V196A	1	T

Table 5. *Continued*

			MIC (mg/l)			S12			23S rRNA[2]			GyrA
Study	Isolate[1]	Farm	STR	ERY	CIP	Codon 43	Codon 88	*ant*-like gene A	Base2122	L4[3]	L22[5]	codon 86
II, III	**47.4**	3	256	16	0.5	K	K	+	A	V121A, V176I, T177S, V184I, M192I, V196A	2	T
	51.3	3	4	4	0.25	K	K	-	A	V196A	1	T
	66.4	3	>1024	32	32	R	K	-	A	P28S, V196A	1	I
	67.3	3	4	1	≤ 0.125	K	K	-	A	V196A	1	T
	72.1	3	4	8	≤ 0.125	NA	NA	NA	A	NA	2	T
	87.3	3	4	1	≤ 0.125	NA	NA	NA	A	V196A	1	T
	88.3	3	>1024	16	0.5	R	K	-	A	P28S, V196A	1	T
	94.3	3	>1024	16	32	NA	NA	NA	A	P28S, V196A	1	I
	107.3	3	4	16	0.25	K	K	-	A	V121A, V176I, T177S, V184I, M192I, V196A	2	T
	111.3	3	>1024	16	0.5	R	K	-	A	P28S, V196A	1	T
	115.3	3	>1024	32	0.5	NA	NA	NA	A	P28S, V196A	1	T
	121.1	3	>1024	32	32	R	K	-	A	P28S, V196A	1	I
	150.1	3	256	>512	128	K	K	+	G	V121A, V176I, T177S, V184I, M192I, V196A	2	I
	153.3	3	512	16	32	NA	NA	+	A	NA	2	I
	154.3	3	512	16	32	K	K	+	A	V121A, V176I, T177S, V184I, M192I, V196A	2	I
	160.3	3	4	>512	128	NA	NA	NA	G	NA	2	I

NA: not analysed
[1]Bolded if whole-genome sequenced
[2]*C. coli* RM2228 numbering, the number of mutated copies of 23S rRNA genes has not been analysed
[3] L4 predicted amino acid sequences compared with L4 of *C. jejuni* NCTC11168
[4]L22 predicted acid sequences compared with L22 of *C. jejuni* NCTC11168; 1=I65V, A74G, S109T, E111A, T114A, T120P, T121V, T122K, A124T, P125S, A126T, T129V, S131A, T132E, K134E, A135S; 2= sequence as in1 with additional A103V

The *in vitro*-generated STR resistant variants in Study I had more heterogeneous STR MICs and mutations in *rpsL*, but A128G being also the most frequently detected (n=9) followed by A263G (K88R, n=4, Table 6). Together these two transitions covered 65% of all detected mutations. In addition, transversion A262C (K88Q, Lysine88Glutamine) was detected in one highly STR-resistant mutant and transition A262G (K88E, Lysine88Glutamic acid) in two variants with STR MICs of 32 mg/l and >1024 mg/l. Altogether three *in vitro*-generated variants with STR MICs 32-128 mg/l had no change in either codon. However, variants 1.1, 6.1 and 6.2 and variants 10.2, 11.1 and 12.1 had nonsynonymous mutation A256G or G257A in *rpsL* leading to substitutions R86G (Arginine86Glysine) and R86K (Arginine86Lysine) in S12, respectively (previously unpublished). Further, in Study III, the spontaneous *rpsL* mutant *C. coli* A.2 acquired in the natural transformation experiments and having STR MIC >1024 mg/l was found to harbour both A128C and A129T transversions in *rpsL*, resulting in K43T (Lysine43Threonine) change in S12 (not shown).

Table 6. MICs and changes in codons 43 and 88 of S12 of *in vitro* STR resistant *C. coli* variants.

		MIC (mg/l)		S12[1]	
Study	Isolate[1]	STR	ERY	Codon 43	Codon 88
I	1.1	32	0.25	K	K
	1.2	>1024	0.25	K	R
	2.1	>1024	≤0.063	K	R
	2.2	>1024	≤0.063	K	R
	3.1	>1024	≤0.063	K	R
	4.1	>1024	0.25	R	K
	5.1	>1024	0.125	K	E
	6.1	64	0.25	K	K
	6.2	>1024	0.25	K	Q
	7.1	>1024	0.25	R	K
	8.1	>1024	0.5	R	K
	8.2	>1024	2	R	K
	8.3	>1024	1	R	K
	9.1	>1024	4	R	K
	9.2	>1024	4	R	K
	10.1	4	1	K	K
	10.2	128	4	K	K
	11.1	>1024	4	R	K
	12.1	>1024	0.5	R	K
	13.1	32	0.5	K	E

[1] K: Lysine, R: Arginine, E: Glutamic acid, Q: Glutamine

5.1.2 *Other STR resistance-associated mutations studied in* C. coli *(I, III)*

In Study I, no mutations in the *rpsL* codon 43 and 88 were detected in three intermediately STR-resistant isolates from pigs and in the three *in vitro*-generated *C. coli* variants, also intermediately STR-resistant, so additional genes were screened. However, no truncation in *rsmG* or previously described resistance-associated mutations in the 500 or 900 regions of *rrs* gene were detected as compared with susceptible isolates and *C. coli* RM2228, and no gene product was obtained with the primers aadEF Full and aadER Full (Table 4) from the 17 STR- and/or ERY-resistant *C. coli* isolates from pigs from farms 1 and 2 (Table 5). Also, no PCR product was obtained with the aforementioned primers from the plasmid or chromosomal DNA of any of the *in vitro* STR-resistant variants and no mutations were seen in the sequenced 500-region of the *rrs* gene in the five *in vitro*-generated intermediately STR resistant variants as compared with their susceptible parent isolates. The sequences of *rsmG* were additionally analysed from the 12 whole-genome-sequenced *C. coli* from Study III (bolded in Table 5). Also among these isolates, identical sequences were found regardless of the resistance level.

5.1.3 *Mechanism and occurrence of intermediate STR resistance (III)*

5.1.3.1 *Occurrence and location of* ant*-like genes A and B*

Twelve *C. coli* isolates with various STR resistance levels from farm 3 were whole-genome-sequenced (bolded in Table 5). Each of the three isolates exhibiting intermediate-level resistance to STR (MIC 256-512 mg/l) was found to harbour an *ant(6)*-resembling gene (referred as *ant*-like gene A) encoding a putative adenylyltransferase. The presence of this gene (peg1084 of *C. coli* 47.4) was further screened by PCR from 34 *C. coli* isolates originating from farms 1-3. All isolates with an intermediate level of STR resistance (n=10, originating from farms 2 and 3) yielded the right-sized amplicon. These included also isolates 17, 20 and 21 from farm 2 (Table 5), which had yielded a negative result with primers aadEF Full and aadER Full in Study I (Table 4).

Additionally, a 60% homologue of *ant*-like gene A (referred as *ant*-like gene B) was detected in 19 (≈4%) of the *in silico* screened genome sequences of *C. jejuni* isolates from our strain collection, but the presence of this homologue was not associated with STR resistance. Both genes, encoding ANT-like protein A in *C. coli* and protein B in *C. jejuni*, were located in a similar position in the chromosome corresponding to hypervariable region 14 described in *C. jejuni* NCTC 11168, which contains also a restriction-modification locus and genes encoding oxidoreductases, hydrolases, dehydrogenases and decarboxylases, among others (56).

The WGS data of twelve *C. coli* isolates from farm 3 (Table 5) were analysed with wgMLST. The three isolates harbouring *ant*-like A gene were two different strains as >1000 allelic differences were detected in wgMLST while the four highly STR-resistant isolates clustered together, forming a single strain.

5.1.3.2 *Evolutionary analysis of* ant*-like gene A and enzyme modelling*

A cladogram was built using the 130 closest homologues of *ant*-like gene A (peg 1084 of *C. coli* 47.4) retrieved from NCBI nr database with BlastX (see Study III, Fig. 3). The ANT-like protein A was found in a monophyletic clade, and it was detected solely in *C. coli* strains originating from several countries and hosts. The countries included the UK, Switzerland and the United States, and hosts and sources were ducks, chickens, pigs, environment and humans. A sister clade was found to include proteins similar to ANT-like protein B, and they were found in both *C. jejuni* and

C. coli. Several aminoglycoside 6-adenylyltransferase homologs described in *Campylobacter* spp. in previous studies were located in a polychotomous clade together with homologous proteins originating from Gram-positive bacterial species, including *Streptococcus* spp., *Enterococcus* spp., *Bacillus* spp. and *Clostridium* spp. Both ANT-like proteins A and B resembled the structure of aminoglycoside 6-adenylyltransferase from *B. subtilis* (accession PDB:2PBE) and lincosamide adenylyltransferase LinB from *Staphylococcus aureus* (115), albeit with a low amino acid identity. Further, the ANT-like protein A was found to share only low (<40%) amino acid identity and blast score ratios (0.11-0.24) with the previously described adenylyltransferases.

5.1.3.3 47.4ΔantA::CAT constructs and expression of ant-*like genes in* E. coli

The *ant*-like genes A and B were expressed in *E. coli* and STR MICs of *E. coli* isolates harbouring plasmid pUC119-antA (n=7) were found to increase 4- or >8-fold (16→64->128 mg/l), but no change in STR MIC was seen in *E. coli* harbouring pUC119-antB. The studied knock-out mutant colonies (n=14) of *C. coli* 47.4 with CAT cassette inserted in *ant*-like gene A (Fig. 7) showed a 64-fold decrease in STR MIC (256→4 mg/l). No changes in the MICs for SPEC, CIP or ERY were observed in studied mutants as compared with the WT *C. coli* 47.4.

5.1.3.4 Natural transformation assays

Two and 113 natural transformants were obtained from STR-susceptible *C. coli* recipient 18.3 (MIC STR 4 mg/l) in assays using 64 and 32 mg/l of STR for selection, respectively, and all tested transformants (n=9) were intermediately resistant to STR (MIC 64-512 mg/l). Altogether, one and three spontaneous mutants were generated in the same experiments, respectively, all having STR MICs >1024 mg/l. The genomes of two natural transformants and a spontaneous mutant were sequenced and analysed using wgMLST (GeP) with the genome of *C. coli* 47.4 used as a reference. The genetic exchange detected in the natural transformants as compared with the donor (47.4) and recipient (18.3) has been summarized in Tables 7 and 8. In both transformants, several genes in addition to *ant*-like gene A were found to be transferred from the donor, including also regions outside of the hypervariable region 14. A total of 24 genes in 8 regions and 18 genes in 5 regions were found to transfer from the donor to the recipient in A+.1 and A+.2, respectively.

Table 7. Genetic flow observed in the *C. coli* natural transformant A+.1 in GeP analysis.

Donor 47.4[1]	Recipient 18.3[1]	A+.1[1]	peg[2]	Annotation[3]
1	2	1	121	FIG00469778: hypothetical protein
1	2	1	122	FIG00469817: hypothetical protein
1	2	3	128	L-lactate permease
1	T	1	135	Uracil-DNA glycosylase, family 1
1	2	3	157	FIG00470444: hypothetical protein
1	2	1	304	ATP-dependent Clp protease adaptor protein ClpS
1	2	1	305	Possible periplasmic thioredoxin
1	2	1	306	tmRNA-binding protein SmpB
1	2	1	307	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148)
1	2	D	874	hypothetical protein
1	2	1	901	Gene SCO4494, often clustered with genes in menaquinone via futalosine pathway
1	2	1	904	putative periplasmic protein (vacJ homolog)
1	2	1	906	membrane protein
1	2	3	1082	sarcosine oxidase, putative
1	M	1	1083	NAD(P)H oxidoreductase YRKL (EC 1.6.99.-)
1	M	1	1084	*ant*-like gene A
1	M	1	1085	RecD-like DNA helicase YrrC
1	T	1	1086	Uncharacterized conserved protein
1	M	1	1087	Dienelactone hydrolase and related enzymes
1	M	1	1088	oxidoreductase of aldo/keto reductase family, subgroup 1
1	M	1	1089	transporter, putative
1	M	1	1090	conserved protein
1	2	1	1091	Arsenical resistance operon repressor
1	2	1	1092	Arsenate reductase (EC 1.20.4.1)

[1]Allele type; 1= donor, 2= recipient, 3= new, M= missing, T= truncated, D= doubled (203)
[2]Peg numbering according to the genome of 47.4.gbk used as a reference
[3]Annotation in RAST (191)

Table 8. Genetic flow observed in the *C. coli* natural transformant A+.2 in GeP analysis.

Donor 47.4	Recipient 18.3[1]	A+.2[1]	peg[2]	Annotation[3]
1	T	1	135	Uracil-DNA glycosylase, family 1
1	2	T	185	Ribbon-helix-helix protein, copG family domain protein
1	2	D	874	hypothetical protein
1	2	1	1079	MloA protein, putative
1	2	1	1080	Type I restriction-modification system, DNA-methyltransferase subunit M (EC 2.1.1.72)
1	2	1	1081	hypothetical protein
1	2	4	1082	sarcosine oxidase, putative
1	M	1	1083	NAD(P)H oxidoreductase YRKL (EC 1.6.99.-)
1	M	1	1084	*ant*-like gene A
1	M	1	1085	RecD-like DNA helicase YrrC
1	T	1	1086	Uncharacterized conserved protein
1	M	1	1087	Dienelactone hydrolase and related enzymes
1	M	1	1088	oxidoreductase of aldo/keto reductase family, subgroup 1
1	M	1	1089	transporter, putative
1	M	1	1090	conserved protein
1	2	1	1091	Arsenical resistance operon repressor
1	2	1	1092	Arsenate reductase (EC 1.20.4.1)
1	2	1	1571	Methionyl-tRNA synthetase (EC 6.1.1.10)

[1]Allele type; 1= donor, 2= recipient, 4= new, M= missing, T= truncated, D= doubled (203)
[2]Peg numbering according to the genome of 47.4.gbk used as a reference
[3]Annotation in RAST (191)

5.1.4 Mutations associated with ERY and CIP resistance (I, II)

All of the studied *C. coli* isolates highly resistant to ERY (MIC≥512 mg/l, n=16) had A2122G mutation in the 23S rRNA encoding gene, but this mutation was not detected in the low-level resistant isolates (ERY MIC 16-32 mg/l, n=11) (Table 5). Further, in Study II, no ERY resistance-associated mutations in the genes *rplV* (coding for ribosomal protein L22) and *rplD* (encoding ribosomal protein L4) were found, including analysed isolates with ERY MIC values of 16–32 mg/l (n=9) (Table 5).

All CIP resistant *C. coli* isolates (MIC 32-128 mg/l, n=7) from pigs from farm 3, where DANO was used, had a commonly described C257T mutation in *gyrA*, resulting in T86I substitution in DNA gyrase (Table 5).

5.1.5 Associations between STR and ERY resistance (I)

As a statistically significant increase was seen in the number of STR resistant isolates after tylosin treatment of pigs in a previous farm study (27) the putative associations were examined in Study

I. The STR MIC of an initially STR-susceptible *C. coli* isolate did not increase with exposure to increasing concentrations of ERY even when the generated variants were resistant to ERY (MIC>256 mg/l) and no increase in ERY MICs were observed in the *in vitro*-generated STR-resistant variants. No differences in the STR MICs of the primary STR- and/or ERY-resistant *C. coli* isolates (n=17) were seen with or without the efflux pump inhibitor PaβN, while the ERY MICs decreased on average fourfold.

5.1.6 *Dynamics of STR resistance in* C. coli *after DANO and tylosin treatments (II, III)*

The occurrence of STR resistance was monitored along with resistance to CIP and ERY of 147 *C. coli* isolates in Study II (published as supplemental material in Study III) as pigs were treated consecutively with DANO (group A, n=12 animals) and tylosin (groups A+B, in group B, n=15 animals). The results are summarized in Table 9. Differing from EUCAST ECOFFs, isolates with STR MIC of 8 mg/l were classified as susceptible since a clear division was observed with *C. coli* isolates having STR MICs of either 4-8 mg/l or ≥256 mg/l in this and in our previous study (27).

While no STR resistance was detected among *C. coli* isolated before weaning (sampling I, n=22), a common resistance pattern already after weaning (samplings II and III) was STR resistance (12/48, 25%). The occurrence of STR-resistant isolates increased after DANO treatment (samplings IV and V) in the group A (n=16/22, 73% $P<0.01$), and the proportion of these isolates was higher in group A than in group B (n=9/28, 32%, $P<0.01$). After tylosin treatment (sampling VI) the proportion of STR-resistant isolates decreased in group A ($P<0.05$) while no statistically significant change was seen in group B or between the groups (Table 9).

Table 9. Distribution of STR MICs after DANO and tylosin treatments of pigs in Study II.

Sampling	Pig group[a]	No. of isolates with MIC (mg/l) of:											No. (%) of resistant isolates
		1	2	4	8	16	32	64	128	256	512	≥1024	
I	A			8	1								0 (0.0)
	B			9	4								0 (0.0)
II	A			6	2							1	1 (11.1)
	B			6	4					1	1	1	3 (23.1)
III[b]	A		1	4	2							4	4 (36.4)
	B			9	2					1		3	4 (26.7)
IV	A			1	1					1		8	9 (81.8)
	B			7	3							5	5 (33.3)
V[c]	A				4							7	7 (63.6)
	B			6	3							4	4 (30.8)
VI	A			5	3							4	4 (33.3)
	B			5	4					1	3	2	6 (40.0)

Vertical line indicates the breakpoint used in this study

[a]A, DANO-treated pigs; B, control pigs

[b]a three-day DANO treatment for group A after sampling III

[c]a ten-day tylosin treatment for group A and B after sampling V

In study II, RFLP-PFGE typing of 133 isolates originating from 12 pigs from group A and 12 pigs from group B was performed. Most of the STR-resistant isolates were also resistant to at least one other antimicrobial studied (CIP, ERY), and 29/41 (70%) of the genotyped STR-resistant isolates had the same PFGE type 1 which was always highly STR-resistant (MIC>512 mg/l), but isolates having this genotype had resistance patterns STR, STR-CIP or STR-CIP-ERY. While high-level STR resistance occurred also in another PFGE type, type 11 (n=5, exhibiting resistance patterns STR or STR-ERY), the intermediately STR-resistant isolates (MIC 256-512 mg/l, n=6) had three different genotypes, (PFGE: 2, STR-CIP; PFGE: 21, STR/STR-CIP; PFGE: 19, STR-CIP-ERY). High and intermediate level STR-resistant isolates were never found to have same PFGE-types.

5.2 Resistance in canine *C. upsaliensis* and *C. jejuni* strains (IV)

5.2.1 Occurrence of resistance

Antimicrobial resistance against STR, ERY, CIP, NAL, tetracycline and gentamicin of two *C. jejuni* and 24 *C. upsaliensis* isolates originating from 18 Finnish Staffordshire bull terriers was evaluated. Both *C. jejuni* isolates were susceptible to all the tested antimicrobials while, among *C. upsaliensis*, a high rate of STR resistance was observed with 19/24 isolates (79.2%) having MICs of > 4 mg/l (Table 10) and one isolate (4.2%) having increased MIC for NAL and CIP. Resistance against other antimicrobials was not detected in *C. upsaliensis*. Isolates that originated from the same dog in two consecutive samplings and had the same STs (DR15S and DR15S_2; DR18S and DR18S_2; DR44S and DR44S_2; DR55S and DR55S_2, Table 10) always had also the same STR MICs.

5.2.2 Mutations in STR and quinolone resistant C. upsaliensis

The amino acid changes corresponding to missense mutations in *rpsL* codons 43 and 88 and the observed deletions, insertions and truncation caused by frameshift mutations in *C. upsaliensis rsmG* are shown in Table 10. In all low- (MIC 16 mg/l, n=12) and high-level (MIC≥1024 mg/l, n=7) STR-resistant *C. upsaliensi*s isolates, a missense mutation A263G in *rpsL* corresponding to amino acid change K88R was detected. In addition, in all highly STR-resistant isolates, various nucleotide deletions and insertions were seen in the sequence of *rsmG*, leading to frameshift and termination immediately downstream of the mutation site at amino acids 13, 49, 137, 139, 144 or 155 of the total of 191 amino acids of *C. upsaliensis rsmG*. All of these mutants also harboured A263G change in *rpsL* and had high STR MICs (≥1024 mg/l). Also some truncation of *rsmG* was observed in a part of the STR-susceptible and low-level resistant isolates, but only the last 3-5 amino acids were lost in these cases. In contrast, none of the STR-susceptible strains (MIC 0.5-2 mg/l, n=5) had STR resistance-associated mutations in *rpsL* or a significant truncation of *rsmG* (Table 10). Moreover, several other nucleotide substitutions in *rsmG* as compared to the sequence of *C. upsaliensis* RM3195, leading to various amino acid substitutions in the predicted sequence of RsmG, were observed in all isolates regardless of the STR MIC-level. Further, no resistance-associated mutations in *rrs* were found and no other AME-encoding genes associated with STR resistance were detected in the *C. upsaliensis* annotated genomes to explain the observed resistance levels.

One *C. upsaliensis* isolate (DR22S, Table 10) with slightly elevated MIC for CIP and resistant to NAL had C257T transition on *gyrA* leading to T86M (Threonine86Methionine) change. Further, another isolate (DR5S, previously unpublished) not included in Study IV due to it originating from a dog sampled only once had similar levels of quinolone resistance and harboured the same mutation in *gyrA* and is therefore included in Table 10, but not in the occurrence analysis.

Table 10. STR and quinolone MICs and changes in S12, RsmG and GyrA in *C. upsaliensis.*

	MIC (mg/l)				S12			GyrA
Isolate[1]	ST	STR	CIP	NAL	codon 43	codon 88	RsmG[2]	codon 86
DR5S[3]	138	>1024	0.5	>64	K	R	C415T → Q139STOP	M
DR9S	158	1024	≤0.12	2	K	R	insertion of nucleotide A at pos. 39 → L13STOP	T
DR9S_2	159	16	≤0.12	4	K	R	deletion of nucleotides 558-572→deletion of 5 aa (187-191)	T
DR15S	160	16	≤0.12	4	K	R	No indels	T
DR15S_2	160	16	≤0.12	4	K	R	No indels	T
DR18S	160	16	≤0.12	4	K	R	No indels	T
DR18S_2	160	16	≤0.12	4	K	R	No indels	T
DR22S	165	>1024	1	>64	K	R	deletion of nucleotide T395→frameshift → M132R, A134Q, D136I, L137STOP	M
DR28S	166	0.5	0.12	8	K	K	deletion of nucleotides 558-572→deletion of 5 aa (187-191)	T
DR36S_2	167	>1024	≤0.12	4	K	R	deletion of nucleotide T426 -> frameshift→L144STOP	T
DR39S	169	0.5	≤0.12	8	K	K	deletion of nucleotides 561-569→deletion of 3 aa(188-190), I191L, STOP: taa→tga	T
DR40S_2	170	1024	0.25	8	K	R	deletion of nucleotide G139→frameshift→E47K, I48S, L49STOP	T
DR42S	171	16	≤0.12	<1	K	R	No indels	T
DR44S	172	16	0.25	8	K	R	No indels	T
DR44S_2	172	16	0.25	4	K	R	No indels	T
DR45S_2	174	16	≤0.12	4	K	R	No indels	T
DR47S	166	0.5	≤0.12	4	K	K	deletion of nucleotides 558-572 → deletion of 5 aa (187-191)	T
DR48S	176	16	≤0.12	4	K	R	deletion of nucleotides 561-569→deletion of 3 aa (188-190)	T
DR53S_2	177	0.5	≤0.12	4	K	K	No indels	T
DR55S	178	16	≤0.12	4	K	R	deletion of nucleotides 561-569→deletion of 3 aa(188-190), I191L, STOP: taa →tga	T
DR55S_2	178	16	≤0.12	4	K	R	deletion of nucleotides 561-569→deletion of 3 aa(188-190), I191L, STOP: taa→tga	T

Table 10. *continued*

		MIC (mg/l)			S12			GyrA
Isolate[1]	ST	STR	CIP	NAL	codon 43	codon 88	RsmG[2]	codon 86
DR56S	167	>1024	≤0.12	4	K	R	deletion of nucleotide T426→frameshift→L144STOP	T
DR59S	181	2	0.25	8	K	K	No indels	T
DR59S_2	182	>1024	≤0.12	4	K	R	insertion of nucleotide C in pos 460→L154Y, K155STOP	T
DR60S_2	182	>1024	≤0.12	4	K	R	insertion of nucleotide C in pos 460→L154Y, K155STOP	T

[1]DRXS indicates the number of the animal, no suffix indicates that the isolate was obtained in the first sampling whereas suffix _2 indicates second sampling.
[2] Sequences were compared with *C. upsaliensis* RM3195 (susceptible to streptomycin).
[3]DR5S is a previously unpublished isolate not included in study IV or in the occurrence analysis due to it originating from a dog sampled only once during the feeding study. It is included in the table because of its quinolone resistance phenotype.

6 DISCUSSION

6.1 STR resistance in *C. coli* and *C. upsaliensis*

6.1.1 C. coli

We were the first to associate mutations in codons 43 and 88 (*C. coli* numbering) of the *rpsL* gene with STR resistance in *C. coli*, a finding concordant with previous findings in other bacterial species, such as *E. coli, M. tuberculosis* and *H. pylori* (122, 125, 126). This shared resistance mechanism between species is due to the unique way that streptomycin interacts with the bacterial 30S ribosomal structures and, unlike other aminoglycosides, makes contact also with the ribosomal protein S12 (63).

In our studies, mutation in *rpsL* leading to predicted substitution K43R in *C. coli* ribosomal protein S12 were associated with a high level of streptomycin resistance (MIC>1024 mg/l) and this was the only *rpsL* mutation detected in isolates from pigs. Mutations leading to other changes than K43R were observed only in the *in vitro*-generated *C. coli* variants and occurred mainly in codon 88, leading to more heterogeneous streptomycin MICs, as also described in *E. coli* and *M. tuberculosis* (122, 216, 217). The K88E substitution was detected in two variants with streptomycin MICs of 1024 and 32 mg/l. Similar observations have been made in *M. tuberculosis* in which K88R mutants were found to have STR MICs of 16, 256 and 1024 mg/l (217). This difference in MICs indicates that also other mechanisms could be involved in the higher level resistance phenotype, which could be, for example, mutations in *rsmG*. RsmG is a conserved methyltransferase that catalyses the methylation of G527 in 16S rRNA, a nucleotide that is in direct contact with STR (63, 127). Loss of function of RsmG has been shown to lead to a low level of STR resistance and to high rates of emergence of highly streptomycin-resistant mutants in several bacterial species including *E. coli, Bacillus subtilis* and *M. tuberculosis*, but having no effect on viability or growth rate (127, 128, 130). Observed resistance could be the result of reduced affinity of streptomycin to the unmethylated ribosome or increased translational accuracy (127, 128). Even though no truncation of *rsmG* was detected among the analysed isolates with various STR MICs from farms 1-3, the STR-resistant isolates harboured either *ant*-like gene A or mutation in *rpsL*, explaining their resistance level. The possibility that resistance-conferring mutations in this gene would not have been present in the *in vitro*-generated variants cannot be excluded and warrants further study. We also observed three *in vitro*-generated *C. coli* variants with streptomycin MICs of 32-128 mg/l which had no resistance-associated mutations in *rpsL* codons 43 or 88. However, they had nonsynonymous mutations not seen in their parent isolates leading to amino acid substitutions in codon 86, which could contribute to the intermediate streptomycin resistant phenotype.

Streptomycin resistance-conferring mutations in *rpsL* have been characterized as restrictive (increased translational accuracy through increased ribosomal binding affinity due to ribosomal conformation changes), such as K42/43T, and non-restrictive, the only known example being K42/43R (218-223). The restrictive type manifests as a hyper accurate phenotype, and consequently, a slower protein translation rate, leading to even STR dependence, whereas the non-restrictive phenotype does not differ significantly from the wild-type ribosomes in protein translation rate (221, 223, 224). We did not observe *rpsL* mutations classified as restrictive in isolates from pigs. Indeed, all mutations found in *C. coli* isolates highly resistant to streptomycin *in vivo* (e.g. from pigs) were of the non-restrictive type, which is not surprising. A small fitness

cost of the K42R (corresponding to K43R in *C. coli*) substitution *in vitro* has been reported in *E. coli*, but it was not seen when these isolates were inoculated into piglets (225). Further, in a mouse model even a minor fitness advantage was observed in *Salmonella* Typhimurium having K42R substitution in S12 as compared with the wild-type strain (226). However, also, *Salmonella* Typhimurium strains with mutation in *rpsL* leading to K42N substitution in S12 and restrictive phenotype, were found to develop compensatory mutations in a number of genes after several passages *in vitro*, and these mutations partially restored fitness (227). Similar results have also been observed in *E. coli* (219). Curiously, *S.* Typhimurium with K42N amino acid substitution in S12 was shown to grow faster than the wild type with poor carbon source conditions *in vitro* (228). Although there is one streptomycin-containing drug currently registered for use in piglets and calves in Finland, veterinary streptomycin consumption is at a low level, with annual sales of only slightly exceeding 100 kg (of active substance) (Table 2). The persistent presence of streptomycin-resistant *C. coli* strains at pig farms found in our studies could be a consequence of the *rpsL* mutation leading to K43R in S12 incurring no, low or an easily compensated fitness cost also in *C. coli*.

Interestingly, the K87/88E mutation has been associated with increased protein or antibiotic production rate in the late growth phase in *Streptomyces* spp. and *E. coli* which is suggested to be due to increased stability of the bacterial ribosome (218, 229, 230). Whether K88E substitution affects the late growth phase protein synthesis rates in *Campylobacter* spp. remains to be elucidated.

As several intermediately streptomycin-resistant *C. coli* isolates with no obvious resistance-conferring mutations or genes were detected in Studies I and II, we used the NGS approach to further examine the potential genetic mechanism(s). A novel streptomycin resistance-conferring gene, encoding a protein homologous to (albeit at the low level of <40%) the ANT(6) class of aminoglycoside-modifying enzymes, was found and further screening revealed its presence in all of the studied intermediately resistant *C. coli* isolates. A second phylogenetically close homologue (60% amino acid identity) was detected in *C. jejuni* and *C. coli* isolates, but we did not find evidence of its association with streptomycin resistance. Both *ant*-like genes were located in similar positions in the *C. coli* and *C. jejuni* genomes, corresponding to the previously described hypervariable region 14 in *C. jejuni*, which is characterized by a restriction-modification locus (56). These hypervariable genomic regions contain genes that are considered to be part of the *Campylobacter* accessory genome, but the corresponding genomic regions in *C. coli* remain largely uncharacterized. It has been proposed that these areas would not differ significantly in GC content from the rest of the genome in *C. jejuni* (57) and could be the result of horizontal gene transfer inside the species by homologous recombination (56), but more studies are needed to shed light on the dynamics of gene transfer in these regions.

AMEs have been found to be very heterogeneous, forming several subfamilies and groups, and many of them share only the ability to inactivate one or several aminoglycosides. In particular, adenylyltransferases have been found to share only little amino acid sequence identity with the other enzymes or with each other (109, 110, 112). Lincosamide and aminoglycoside nucleotidyltransferases have been previously proposed to be related to ancient nucleotide polymerases and further hypothesized that they have arisen from these so-called proto-resistant elements through divergent evolution (115, 231). Phylogenetic analysis revealed the presence of *ant*-like gene A only in a clade consisting of homologous proteins in *C. coli* derived from several sources and locations, including chicken, human and environmental sources and the countries United States, Switzerland and the UK. Among the screened *C. coli* isolates from our isolate

collection, this gene was found in isolates from 2/3 studied farms and included also the three intermediately streptomycin-resistant isolates from farm 2 that did not contain a previously described aminoglycoside adenylyltransferase gene found in a MDR plasmid in *C. jejuni* (24). Based on the phylogenetic analysis and the lack of insertion elements or other resistance genes in the region and the GC content being close to that of *Campylobacter* genome, we did not find evidence of a recent jump of this gene to *Campylobacter* spp. from another species. Therefore, it can be hypothesized that *ant*-like gene A evolved in *Campylobacter* spp. from a proto-resistance element originally having a different substrate/function.

A multidrug-resistant phenotype has been linked to most of the previously detected aminoglycoside resistance genes in *Campylobacter* spp. due to their location in MDR plasmids or genomic islands (24, 25, 68), but this does not seem to be the case with *ant*-like gene A, as no other known resistance genes were detected in the close proximity. We were also able to transfer *ant*-like gene A to another *C. coli* isolate by natural transformation, indicating that this might occur also *in vivo* in field conditions.

In conclusion, we were able to associate two mechanisms with high and intermediate streptomycin resistance in *C. coli* isolated from pigs in field conditions. Curiously, the *ant*-like gene A and streptomycin resistance-associated mutations in *rpsL* were not present concurrently in *C. coli* isolates. Further, our genotyping data from farm 3 shows, that high and intermediate level streptomycin resistance occurred consistently in different PFGE-types. This might indicate that an intermediate level of streptomycin resistance (e.g. harbouring aminoglycoside modifying enzyme encoding gene) is not required for the development of high level of streptomycin resistance (*rpsL* mutants) *in vivo*.

6.1.2 C. upsaliensis

Interestingly, in streptomycin-resistant canine *C. upsaliensis,* we did not observe any mutations in *rpsL* leading to the non-restrictive change K43R, which was the only phenotype found in the STR-resistant *C. coli* from pigs. Instead, mutations leading to amino acid change K88R in S12 were observed in all of the studied STR-resistant isolates (accounting for 79% of all *C. upsaliensis* isolates included in Study IV), and this mutation was found in isolates having both low (16 mg/l) and high (>1024 mg/l) streptomycin MICs. This mutation has been classified as semi-restrictive in *E. coli*, resulting in only small decreases in protein synthesis rate (221, 222). In addition to the *rpsL* mutation, all highly streptomycin-resistant isolates harboured frameshift mutations in *rsmG*, leading to a major truncation and probable loss of function of the gene product. Consistent with our observations in *C. upsaliensis*, *rpsL rsmG* double mutants have been associated with a high level of streptomycin resistance in several other bacterial species including *M. tuberculosis, Bacillus subtilis* and *E. coli* (127, 128, 130).

We detected also isolates susceptible to streptomycin (MIC<4 mg/l) and not harbouring any resistance-associated mutations in *rpsL* or *rsmG* implying that streptomycin resistance is not intrinsic in this species as suggested previously (28). This is also supported by the findings of Fouts et al. (2005) (29) that *C. upsaliensis* strain RM3195 was not resistant to streptomycin. However, it can be presumed that the streptomycin resistance phenotype among canine *C. upsaliensis* isolates is quite persistent not only based on the prevalence data but also on the same phenotype (and resistance-conferring mutation) being observed in isolates acquired from the same dogs 4-5 months apart in each of the four cases when the dog had retained *C. upsaliensis* with the same ST.

6.2 Quinolone resistance mutations in *C. coli* and *C. upsaliensis* and ERY resistance mutations in *C. coli*

While T86I is the most commonly described amino acid substitution in GyrA in *C. jejuni* and *C. coli*, various other substitutions have also been described (Thr86Lys, Asp90Asn, Asp90Ala, Asp90His, Asp90Tyr, Ala87Pro, Ala70Thr, Thr86Ala, Thr86Val), many of them associated with a low level of fluoroquinolone resistance or resistance to nalidixic acid only (149-151, 153-156). All ciprofloxacin-resistant *C. coli* isolates from farm 3, where danofloxacin was used, were found to harbour C257T mutation in *gyrA*, leading to amino acid substitution T86I, but no other mutations in the QRDR region were detected, indicating that danofloxacin does not induce novel mutations in the *gyrA* in *C. coli in vivo*. The same mutation was also found in two *C. upsaliensis* isolates with a slight increase in ciprofloxacin MICs but resistant to nalidixic acid. This is explained by the fact that C257T mutation leads to the substitution T86M in *C. upsaliensis* due to differing nucleotide sequences of *gyrA* between *C. upsaliensis* and *C. coli*. T86M is a novel substitution which has not been described previously in *Campylobacter* species. Interestingly, Fouts et al. (2005) found that *C. upsaliensis* strain RM3195 was resistant to nalidixic acid (but not to ciprofloxacin), but, unlike in our isolates, they were unable to detect any known quinolone resistance-associated mutations in the QRDR of *gyrA*, implying that also mechanisms other than those described in *C. jejuni* and *C. coli* play a part in quinolone resistance in this species.

All *C. coli* isolates highly resistant to erythromycin were found to have an A2122G mutation in the 23S rRNA encoding gene, which has been previously associated with erythromycin resistance in *C. coli* (29). However, we did not find evidence that mutations in *rplD* or *rplV* would explain the decreased susceptibility to erythromycin (MIC 16-32 mg/l) in these *C. coli* isolates, as no previously described macrolide resistance-associated mutations were detected. Macrolide resistance-conferring mutations in the ribosomal protein L4 and L22 -encoding genes in *Campylobacter* spp. and other bacterial species have usually been located in the conserved region encoding amino acids 63-74 in L4 or leading to insertions or deletions of several amino acids in the C-terminal region of L22 (167, 171, 232-234). Even though we did detect several changes in both genes as compared with erythromycin-susceptible *C. jejuni* NCTC11168, the same genotypes were found in both susceptible and low-level resistant isolates. A more likely explanation for the observed low-level erythromycin resistance lies in the increased activity of the CmeABC efflux pump (168).

6.3 Associations of STR and ERY resistances in porcine *C. coli* and dynamics of STR resistance development after DANO and tylosin treatment

In a previous study, we observed an increase in the isolation rate of streptomycin-resistant *C. coli* following tylosin treatment of pigs at farm 1, where aminoglycosides had not been used in at least four years. In particular, occurrences of resistance to erythromycin, ciprofloxacin, nalidixic acid and streptomycin were found to be significantly higher among the isolates from treated pigs than from control animals (27). A common resistance mechanism for macrolides and quinolones is known to exist, namely the increased action of the CmeABC efflux pump, but nothing similar has been described for streptomycin. This led us to explore putative associations between resistance to streptomycin and erythromycin in *C. coli*. However, no obvious association between these resistance traits were detected, as the isolates did not become resistant to both after selection

with either agent *in vitro* and no changes in streptomycin MICs were detected after efflux pump inhibition. Further, at farm 3, even though resistance to streptomycin appeared already after weaning (25% of isolates were resistant), after danofloxacin treatment 82% of the isolates from the treated group were streptomycin-resistant, suggesting that the use of danofloxacin promoted their selection. However, tylosin treatment applied after danofloxacin treatment was not observed to further increase the rate of streptomycin-resistant *C. coli*. On the contrary, after tylosin treatment, the frequency of streptomycin-resistant isolates in the treated group returned to a level close to that observed already after weaning and before danofloxacin treatment. Our findings indicate that the streptomycin-resistant isolates persisted at weaning units and were transferred to new batches of weaned pigs and selected in pigs treated with danofloxacin.

In light of the above-characterized streptomycin, erythromycin and fluoroquinolone resistance mechanisms, no obvious connection between them can be seen, possibly indicating other epistatic mechanisms selecting for STR resistance alongside other resistance traits. For example, in a study conducted in *E. coli* in an antibiotic-free environment *in vitro*, specific combinations of point mutations in *rpsL* (including K43R) and *gyrA* alleles leading to a double-resistant phenotype were found to exhibit positive epistasis (235). The molecular targets and therefore resistance-conferring mutations of streptomycin, erythromycin and ciprofloxacin occur in different steps along the same pathway, e.g. protein synthesis, which could be one explanation of putative epistasis between these resistances.

It is noteworthy that both of these observations (statistically significant increase in streptomycin resistance in *C. coli* after tylosin (27) or danofloxacin treatment of pigs) were made in field conditions, which is the likely environment of resistance development in the food chain. These farm studies indicate that the dynamics of resistance development at farms are complex and can lead to multiresistance even when mechanistically unrelated antimicrobials are used.

6.4 Occurrence of resistance in canine *C. upsaliensis* and *C. jejuni*

Among the sampled dogs, all of the studied canine *C. upsaliensis* and *C. jejuni* isolates were, apart from streptomycin, highly susceptible to the antimicrobials investigated. The high streptomycin resistance rate detected in *C. upsaliensis* is concordant with findings of previous studies (5, 28). Also a low rate of quinolone resistance has been reported among *C. upsaliensis* from pets in several countries, including Belgium, Italy and Norway (28, 30, 236). No conclusions can, however, be drawn from this study on the prevalence of antimicrobial resistance in *Campylobacter* spp. among the dog population in Finland as only a small number of dogs belonging to a single breed was sampled.

7 CONCLUSIONS

1. Mutations in codons 43 and 88 of the *rpsL* gene encoding the ribosomal protein S12 were found in all streptomycin resistant *C. coli* isolates and variants (but not their susceptible parents) indicating an association with streptomycin resistance. Only mutations of the non-restrictive type were found in streptomycin-resistant isolates originating from pigs while *in vitro*-generated mutants exhibited both restrictive and non-restrictive mutations in *rpsL* and more diverse streptomycin MICs. This might be due to the slower growth rate of the isolates with restrictive *rpsL* mutations rendering them unable to survive competition *in vivo* as has been shown in other bacteria.

2. A novel resistance gene, *ant*-like gene A, was found to confer an intermediate level of streptomycin resistance in *C. coli*. Based on phylogenetic analysis, close homologues were not detected in any other bacterial species, except for *ant*-like gene B in *C. jejuni*, which, however, appeared not to be associated with streptomycin resistance. Based on our findings, it can be hypothesized that streptomycin resistance conferring *ant*-like gene A evolved from a common ancestral proto-resistance element in *Campylobacter* spp.

3. Almost 80% of the studied canine *C. upsaliensis* isolates showed streptomycin MICs of >4 mg/l, and mutations in *rpsL* codon 88 were found in all low-level streptomycin-resistant isolates, while *rpsL rsmG* double mutants exhibited a highly resistant phenotype. However, also streptomycin-susceptible *C. upsaliensis* isolates not harbouring any resistance-associated mutations in either gene were detected, indicating streptomycin resistance is not intrinsic for *C. upsaliensis*.

4. The presence of streptomycin resistance genes and mutations in *C. coli* and *C. upsaliensis* populations derived from hosts that are known not to be exposed to streptomycin for long periods of time provides further evidence that merely ceasing antimicrobial usage will not completely eradicate resistant strains and suggests a low fitness cost of streptomycin resistance in these *Campylobacter* species. Challenge with an antimicrobial belonging to a different class appeared to select also streptomycin-resistant *C. coli* isolates. The observed increase of resistant isolates could be a consequence of co-selection mediated by some, yet uncharacterized, epistatic interactions.

5. All ciprofloxacin-resistant *C. coli* isolates from pigs that had been administered danofloxacin were found to harbour the commonly reported C257T mutation in *gyrA*, leading to T86I substitution and a high level of ciprofloxacin resistance indicating that danofloxacin does not induce novel mutations in the QRDR of *gyrA* in *C. coli in vivo.* However, the same C257T mutation in *C. upsaliensis* leads to T86M substitution and resistance to nalidixic acid, but only decreased sensitivity or low level resistance to ciprofloxacin, implying that the most commonly described fluoroquinolone resistance associated mutation in *C. coli* and *C. jejuni* is not sufficient to cause high level fluoroquinolone resistance phenotype in *C. upsaliensis*.

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