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Antimicrobial Resistance in *Campylobacter jejuni* and *Campylobacter coli*

by

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To my wonderful children
Jenna and Joona

ABSTRACT

Mirva Lehtopolku
Antimicrobial Resistance in *Campylobacter jejuni* and *Campylobacter coli*

The Department of Medicine and the Department of Medical Microbiology and Immunology, University of Turku, Finland, and The Antimicrobial Resistance Unit, National Institute for Health and Welfare, Turku, Finland.
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Campylobacters are a common cause of bacterial gastroenteritis worldwide, with *Campylobacter jejuni* and *C. coli* being the most common species isolated in human infections. If antimicrobial treatment is required, the drugs of choice at the moment are the macrolides and fluoroquinolones. In this thesis, the *in vitro* resistance profiles of the *C. jejuni* and *C. coli* strains were evaluated with emphasis on multidrug resistance. The aim was also to evaluate the different resistance mechanisms against the macrolides. Further, the disk diffusion method was compared to agar dilution method and its repeatability was evaluated, since it has been widely used for the susceptibility testing of campylobacters.

The results of the present study showed that resistance to the fluoroquinolones is common in strains isolated from Finnish patients, but resistance to the macrolides is still rare. Multidrug resistance was associated with resistance to both ciprofloxacin and erythromycin. Among the available per oral drugs, least resistance was observed to co-amoxiclav. There was no resistance to the carbapenems. Sitafloxacin and tigecycline were *in vitro* highly effective towards *Campylobacter* species. A point mutation A2059G of the 23S rRNA gene was the main mechanism behind the macrolide resistance, whereas the efflux pumps did not seem to play an important role when a strain had A2059G mutation. A five amino acids insertion, which has not been described previously, in the ribosomal protein L22 of one highly-resistant *C. jejuni* strain without mutation in the 23S rRNA gene was also detected. Concerning the disk diffusion method, there was variation in the repeatability

In conclusion, macrolides still appear to be the first-choice alternative for suspected *Campylobacter* enteritis. The *in vitro* susceptibilities found suggest that co-amoxiclav might be a candidate for clinical trials on campylobacteriosis, but in life-threatening situations, a carbapenem may be the drug of choice. More studies are needed on whether the disk diffusion test method could be improved or whether all susceptibilities of campylobacters should be done using a MIC based method.

Keywords: *Campylobacter*, antimicrobial resistance, fluoroquinolone, macrolide, multidrug resistance, macrolide resistance mechanisms, antimicrobial susceptibility testing, disk diffusion testing

TIIVISTELMÄ

Mirva Lehtopolku
Mikrobilääkeresistenssi *Campylobacter jejunilla* ja *Campylobacter colilla*

Sisätautien klinikka ja Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto, ja Mikrobilääkeresistenssiyksikkö, Terveyden ja hyvinvoinnin laitos, Turku.
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Kampylobakteeri on yleinen ripulin aiheuttaja maailmanlaajuisesti. *Campylobacter jejuni* ja *C. coli* ovat tavallisimmat ihmisnäytteistä eristetyt lajit. Makrolidi- tai fluorokinoloniryhmän mikrobilääkkeet ovat olleet ensisijaisia vaihtoehtoja silloin, kun mikrobilääkehoito on tarpeen. Tässä väitöskirjatyössä tutkittiin *C. jejunin* ja *C. colin* mikrobilääkeresistenssiä keskittyen erityisesti moniresistenssiin. Lisäksi tutkimuskohteena olivat makrolidiresistenssin mekanismit. Kampylobakteereilla herkkyystestaus tehdään useimmiten kiekkoherkkyysmenetelmällä. Tavoitteena oli myös selvittää kiekkoherkkyysmääritysten toistettavuutta ja luotettavuutta maljalaimennosmenetelmään verrattuna.

Tutkimustulokset osoittivat, että fluorokinoloniresistenssi on Suomessa yleistä erityisesti ulkomailta peräisin olevissa kampylobakteerikannoissa, kun taas makrolidiresistenssi on yhä harvinaista. Sekä fluorokinoloni- että makroliresistenssiin liittyi moniresistenssi. Suun kautta annosteltavista lääkkeistä vähiten resistenssiä havaittiin amoksisilliini-klavulaanihappo -yhdistelmää kohtaan. Tigesykliini ja sitafloksasiini olivat *in vitro* -tulosten mukaan hyvin tehokkaita myös muille lääkkeille resistenttejä kampylobakteerikantoja kohtaan. Karbapeneemeille ei havaittu resistenssiä. A2059G mutaatio oli yleisin makrolidiresistenssiä aiheuttava mutaatio. Sen sijaan efflux-pumpuilla ei vaikuttanut olevan suurta merkitystä resistenssiin, kun kannalla oli 23S rRNA -geenin mutaatio. Yhdestä resistentistä kannasta löytyi uusi insertio ribosomaalisesta proteiinista L22. Kiekkotestauksen toistettavuudessa todettiin merkittävää vaihtelua toistettavuudessa.

Koska fluorokinoloniresistenssi on yleistä, makrolidiryhmän mikrobilääke on tällä hetkellä kampylobakteeri-infektion ensisijainen hoitovaihtoehto. Moniresistenttien kampylobakteeri-infektioiden mikrobilääkevaihtoehdot ovat vähäiset. Amoksisilliiniklavulaanihappo vaikuttaa lupaavalta *in vitro* -tulosten pohjalta, mutta hengenvaarallisissa tilanteissa karbapeneemi vaikuttaa tehokkaimmalta vaihtoehdolta. Lisäselvitykset ovat tarpeen joko kiekkomenetelmän parantamiseksi tai sen arvioimiseksi pitäisikö sitä käyttää vain seulontamenetelmänä.

Avainsanat: kampylobakteeri, mikrobilääkeresistenssi, fluorokinoloni, makrolidi, moniresistenssi, makrolidiresistenssimekanismit, mikrobilääkeherkkyystestaus, kiekkoherkkyystesti

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ABBREVIATIONS

23S	major component of the prokaryotic ribosomal subunit 50S
50S	larger subunit of prokaryotic 70S ribosome
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute (former NCCLS)
CmeABC	multidrug efflux pump, consisting of the three components CmeA, CmeB, and CmeC.
co-amoxiclav	amoxicillin and clavulanic acid were used in a 2:1 (weight/weight) ratio. Values indicate the concentration of amoxicillin
DNA	deoxiribonucleic acid
DSM(Z)	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)
EUCAST	European Committee for Antimicrobial Susceptibility Testing
gyrA	DNA gyrase gene encoding the GyrA subunit of the gyrase enzyme
gyrB	DNA gyrase gene encoding the GyrB subunit of the gyrase enzyme
I	intermediately resistant bacteria, antimicrobial activity is associated with an indeterminate or uncertain therapeutic effect
L4	50S ribosomal protein in *Campylobacter*
L22	50S ribosomal protein in *Campylobacter*
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration required to inhibit the growth of 50% of organisms
MIC_{90}	minimum inhibitory concentration required to inhibit the growth of 90% of organisms
MDR	Multidrug-resistant is considered to be resistance to three or more antimicrobial groups. Groups were as follows: (i) quinolones, (ii) macrolides, telithromycin and clindamycin, (iii) tetracycline and tigecycline, (iv) β-lactams, (v) gentamycin and (vi) chloramphenicol
oqxAB	gene encoding efflux pumps, which can extrude quinolones
qepA	gene encoding efflux pumps, which can extrude quinolones

parC	bacterial gene encoding parC subunit of the topoisomerase IV enzyme
parE	bacterial gene encoding parE subunit of the topoisomerase IV enzyme
PCR	polymerase chain reaction
PMQR	plasmid-mediated quinolone resistance
QRDR	quinolone resistance-determining region
R	resistant bacteria that might not be successfully treated with antimicrobial evaluated
rRNA	ribosomal ribonucleic acid
S	susceptible bacteria, can usually be successfully treated with the antimicrobial evaluated
THL	Terveyden ja hyvinvoinnin laitos (National Institute for Health and Welfare, former KTL)
aac(6')-Ib-cr	gene encoding aminoglycoside acetyltransferase variant, which can inactivate ciprofloxacin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals I-V. In addition, some unpublished results have also been included.

I Hakanen AJ, **Lehtopolku M**, Siitonen A, Huovinen P, Kotilainen P. Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995-2000. J Antimicrob Chemother 2003;52:1035-1039.

II **Lehtopolku M**, Hakanen AJ, Siitonen A, Huovinen P, Kotilainen P. *In vitro* activities of 11 fluoroquinolones against 226 *Campylobacter jejuni* strains isolated from Finnish patients with special reference to ciprofloxacin resistance. J Antimicrob Chemother 2005;56:1134-1138.

III **Lehtopolku M**, Nakari U-M, Kotilainen P, Huovinen P, Siitonen A, Hakanen AJ. Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: in vitro activities of 20 antimicrobial agents. Antimicrob Agents Chemother 2010;54(3):1232-1236.

IV **Lehtopolku M**, Kotilainen P, Haanperä-Heikkinen M, Nakari U-M, Hänninen M-L, Huovinen P, Siitonen A, Eerola E, Jalava J, Hakanen AJ. Ribosomal mutations as the main cause of macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. AAC in press.

V **Lehtopolku M**, Kotilainen P, Puukka P, Huovinen P, Nakari U-M, Siitonen A, Eerola E, Jalava J, Hakanen AJ. Comparison of the disk diffusion and the agar dilution method in susceptibility testing of 174 *Campylobacter* strains. Submitted.

1. INTRODUCTION

Campylobacter species are an important cause of mainly food-borne bacterial gastroenteritis in humans all over the world. In nature campylobacters are present in a large reservoir, including the intestinal tracts of domestic and production animals, and natural waters. In humans the main pathogens are *Campylobacter jejuni* and *C. coli*. In industrialized countries, the illness is most common in adults whereas in developing countries it affects mainly children and contributes to considerable childhood mortality. Infections due to campylobacters are often self-limiting and require no antimicrobial treatment (Belanger and Shryock 2007). However, antimicrobial therapy may be needed in severe and prolonged cases of enteritis, in septicemia or other invasive infections and in patients who are immunocompromised as well as in very young children and pregnant women (Allos 2001). Fluoroquinolones and macrolides are the most commonly used antimicrobial agents in the treatment of campylobacteriosis worldwide (de Saussure 2009; Hill, Ericsson et al. 2006).

Resistance to the fluoroquinolones has been increasing since the 1990's (Aarestrup and Engberg 2001). This renders the fluoroquinolones unsatisfactory in the empirical treatment of bacterial gastroenteritis in many parts of the world. Use of the fluoroquinolones should therefore be avoided in severe bacterial diarrhea in countries where fluoroquinolone-resistant strains are prevailing, or for tourists returning from those areas (Hakanen, Jousimies-Somer et al. 2003). For this reason, the macrolides are now considered to be the drugs of choice in the treatment of campylobacteriosis. Although there are reports of resistance also to this group of antimicrobial agents, the resistance has remained stable at a low level (Vlieghe, Jacobs et al. 2008).

Because of the resistance situation, antimicrobial susceptibility testing has become more important than ever in routine clinical practice. The slow growth and special growth requirement of campylobacters cause difficulties in the antimicrobial susceptibility testing. Although the agar dilution and broth dilution methods have been standardized for the susceptibility testing of campylobacters, clinical microbiology laboratories widely use the disk diffusion testing with varying protocols. Since the susceptibility testing is rather time consuming, a faster way of detecting *Campylobacter* resistance in clinical laboratories is also needed.

This thesis concentrates on the drug susceptibilities of *C. jejuni* and *C. coli* strains with special emphasis on the fluoroquinolone and macrolide resistance as well as multidrug resistance. Based on *in vitro* susceptibilities, efforts have been made to delineate the different possibilities for the treatment of infections caused by multidrug-resistant *Campylobacter* strains. Macrolide resistance mechanisms were analyzed by pyrosequencing and sequencing. It is important to provide reliable resistance information for the clinicians' use. To investigate the currently employed susceptibility testing methods, the widely used diffusion testing was compared to the agar dilution method. In addition, the repeatability of the disk diffusion method in assessing the macrolide susceptibility of *C. jejuni* and *C. coli* strains was examined.

2. REVIEW OF THE LITERATURE

2.1. The genus *Campylobacter*

2.1.1. The history of *Campylobacter*

Campylobacters are now a leading cause of bacterial gastroenteritis in Finland and worldwide (www3.ktl.fi/stat/ (last visited 25.7.2011); Friedman, Neimann et al. 2000; Ruiz-Palacios 2007). This was not the case a few decades ago, when campylobacters were considered to be opportunistic human pathogens.

The first reports of *Campylobacter* were probably made in the 1880s, when Theodor Escherich for the first time observed spiral-shaped bacteria in the colons of diarrheic infants who had died. In 1913, John MacFadyean and Stewert Stocman recognized a "vibrio" causing abortions in sheep. In 1919, Smith and Taylor isolated the same kind of organism causing vibrionic abortion in cattle. This organism was then named *Vibrio fetus*, and is now called *Campylobacter fetus*. In the 1930s and 1940s, veterinarians also recognized bacteria, in cattle called *Vibrio jejuni* and in swine *Vibrio coli*, as causes of enteric infection and diarrhea. The emergence of the species has been outlined in a number of reviews in detail Butzler 2004; Skirrow 2006).

In humans, a *V. jejuni*-like organism was isolated from blood samples of patients with diarrhea in 1938. In 1947, venereally transmitted "vibrio" strains were found as the cause of death of the fetus and infectious infertility. Those "vibrio" strains were then accorded a subspecies status, being nowadays named *C. fetus* subsp. *venerealis*. Ten years later, Elizabeth King proposed that there are actually two different types of vibrios causing enteric illness, *V. fetus*, and related vibrios now called *Campylobacter jejuni* and *Campylobacter coli* (Butzler 2004; Skirrow 2006).

In 1963, the genus *Campylobacter* was proposed to be different from *Vibrio* spp. Even though these bacteria were recognized and could be found in microscopy, culturing failed until the year 1972. Veterinary microbiologists played an important role in the development of the specific culturing methods. Martin Skirrow described a rather simple technique for the culturing of campylobacters in 1977: blood agar containing vancomycin, polymyxin and trimethoprim and incubation at 43 °C in a microaerobic atmosphere. The use of this new technique led to the insight that campylobacters cause infections worldwide and have to be considered a significant health problem (Moore, Corcoran et al. 2005). The first campylobacteriosis in Finland was reported in 1978 (Kosunen 1978).

2.1.2. Classification and taxonomy

Campylobacters are Gram-negative curved S-shaped rods. Their length varies between 0.5 μm and 5 μm and their width between 0.2 μm and 0.8 μm. They can have a flagellum at one or both ends of the bacteria and can therefore be motile. Cells grow

in microaerobic conditions consisting of 5% O_2, 10% CO_2 and 85% N_2. The optimal growth temperature for campylobacters is 37°C, except for *C. jejuni*, which grows better at higher temperatures like at 42-43°C. Campylobacters multiply more slowly than the usual enteric normal flora pathogens. Because of this, campylobacters need a longer incubation time, typically 48 h. (Nachamkin 1999)

Campylobacters were originally named "*Vibrio*" species. In 1963 Sebald and Véron named *Vibrio fetus* and *Vibrio bubulus* as *Campylobacter fetus* and *Campylobacter bubulus*, respectively, and thereby formed a new genus called *Campylobacter* (Vandamme 2000).

Campylobacters belong to the family *Campylobacteriaceae* together with the *Arcobacter* (Fitzgerald and Nachamkin 2007; Lastovica and Allos 2008; Vandamme, Falsen et al. 1989; Vandamme, Daneshvar et al. 1995). There are several different *Campylobacter* species in this family: *C. jejuni* subsp. *jejuni*, *C. coli*, *C. fetus*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, *C. jejuni* subsp. *doylei, C. sputorum* biovar paraureolyticus, *C. curvus*, *C. concisus*, *C. insulaenigrae*, *C. helveticus*, *C. mucosalis*, *C. hominis*, *C. lanienae*, *C. rectus*, *C. showae*, and *C. gracilis*. In addition, there are several new species proposed during recent years: *C. avium* sp. nov., *C. canadensis* sp. nov., *C. cuniculorum* sp. nov., *C. peloridis* sp. nov., *C. subantarcticus* sp. nov., *C. troglodytis* sp. nov., *C. ureolyticus* comb. nov., and *C. volucris sp.* nov. (Inglis, Hoar et al. 2007; Inglis, Boras et al. 2011; Rossi, Debruyne et al. 2009; Stoddard, Miller et al. 2007; Vandamme 2000; Zanoni, Debruyne et al. 2009). *C. hyolei* is now included in *C. coli*, although it has a higher 16S rRNA sequence similarity to *C. jejuni* (Alderton, Korolik et al. 1995). According to Saénz et al., *C. jejuni* is the most frequently isolated species from poultry (81%) and humans (84%), and *C. coli* is the most frequently isolated species from pigs (100%) (Saenz, Zarazaga et al. 2000). *C. jejuni* and *C. coli* are the most common species causing infections in humans: while *C. jejuni* causes 90-95% of the human cases, *C. coli* causes approximately 5-10% of the diagnosed cases. Also other species can cause human infections (Table 1).

2.1.3. Epidemiology

Campylobacters are zoonotic bacteria, and they are commonly present in the intestinal tract of the domestic and wild animals (Mataragas, Skandamis et al. 2008; Oporto, Esteban et al. 2007). Campylobacters are present in nature in a large reservoir and they can be found in birds, pigs, cows, poultry, dogs, cats, hamsters, shellfish, molluscs, seals, reptiles, reindeer, and rabbits. The risk factors for human infections are considered to be handling or eating poultry meat, eating raw or undercooked meat, drinking unpasteurized milk or untreated water, and traveling (Neimann, Engberg et al. 2003; Rodrigues; Cowden et al. 2001). Also swimming in natural waters and contact with domestic animals are believed to be risk factors for infection (Schönberg-Norio, Takkinen et al. 2004). Sometimes occupation also exposes to campylobacteriosis. The organism does not replicate in food, which prevents large foodborne outbreaks. In Finland a number of waterborne outbreaks caused by campylobacters have been reported (Kuusi, Nuorti et al. 2005). Several waterborne outbreaks of campylobacteriosis have also been reported from abroad, e. g. from

Table 1. Sources of *Campylobacter* infections and features of associated human disease. Sources: Blaser and Engberg 2008; Lastovica and Allos 2008.

Campylobacter species	Usual source of infection	Associated human disease
C. jejuni	Poultry	Enteritis, septicemia, skin and soft tissue infections, carditis, meningitis, colitis, toxic megacolon, intestinal hemorrhage, abscesses, hepatitis, pancreatitis, abortion, late-onset complications, post-infectious sequelae
C. coli	Pigs	
C. concisus	Humans	Periodontal disease, enteritis, septicemia
C. fetus subsp. *fetus*	Cattle, sheep	Septicemia, enteritis, abortion, meningitis
C. fetus subsp. *venerealis*	Cattle	Septicemia (rare)
C. hyointestinalis subps. *hyointestinalis*	Pigs, cattle, hamsters	Enteritis, septicemia
C. jejuni subps. doylei	Humans	Enteritis, septicemia
C. lari	Cats, dogs, chickens, monkeys, seals, mussels, oysters	Enteritis, septicemia
C. sputorum	Humans, cattle, pigs, sheep	Abscesses
C. upsaliensis	Cats, dogs, ducks, monkeys	Enteritis, septicemia

the United States (Vogt, Sours et al. 1982), Sweden (Mentzing 1981) and Denmark (Engberg, Gerner-Smid et al. 1998).

Although campylobacteriosis is largely considered to be a mainly foodborne infection, there is mounting evidence for other routes of transmission including direct animal contact and handling of raw food (Kapperud, Espeland et al. 2003; Neimann, Engberg et al. 2003; Potter, Kaneene et al. 2003). Human-to-human spread has also been observed, although at low frequencies (Musher and Musher 2004). Reduction of infections caused by campylobacters can be achieved by modifying kitchen hygiene practices and preparation of poultry and, in addition, by modifying animal contact patterns. Also improving the hygienic quality of drinking water and dairy products as well as reducing the prevalence of campylobacters in poultry are important methods for reducing *Campylobacter*-induced infections (Kapperud, Espeland et al. 2003).

Campylobacters are a common cause of bacterial gastroenteritis worldwide. In industrialized countries, campylobacters most commonly affect adults mainly because of traveling. On the contrary, in the tropical developing countries, campylobacteriosis is more prevalent among young children (Allos 2001). Community-based studies estimate the incidences of campylobacteriosis in tropical developing countries to be between 40,000 and 60,000 notifications/100,000 population for children between 0 and 5 years

of age (Coker, Isokpehi et al. 2002; Oberhelman, Gilman et al. 2003). In developed countries, the same number is significantly lower being 300 notifications/100,000 population (Coker, Isokpehi et al. 2002).

The Foodborne Diseases Active Surveillance Network (FoodNet) of the CDC's Emerging Infections Program produces reports of the United States of America for all laboratory-confirmed infections with enteric pathogens commonly transmitted through food. According to the 2009 report, a total of 17,468 laboratory-confirmed cases of enteric infections were identified (Centers for Disease Control and Prevention, CDC, 2010). For *Campylobacter* species there were 6,033 reported infections, and the incidence per 100,000 population was 13.0 per year (FoodNet). The same numbers for *Salmonella* were 7,039 and 15.2, respectively. Shah et al. (Shah, DuPont et al. 2009) examined 51 published studies from 1973 to the present day on travelers' diarrhea to look for regional differences in the pathogens identified. In that study, *Campylobacter* species were more often found in Asia as compared to Latin America and Africa, and it was also noteworthy that ciprofloxacin-resistant *Campylobacter* strains seemed to be commonly encountered in Asia. In Australia, during the year 2009, the OzFoodNet sites reported 27,037 notifications of nine diseases or clinical conditions that are commonly transmitted by food. The most frequently notified infections were caused by *Campylobacter* species with 15,973 notifications. As a comparison, there were 9,533 notified infections by *Salmonella* species during 2008 (OzFoodNet Working Group 2009).

In Finland, the National Institute for Health and Welfare (THL) produces a yearly report of diagnosed *Campylobacter* infections (http://www3.ktl.fi/stat/ (last visited 25.7.2011)). In 1995, 2,197 infections were reported, whereas in 2009, 4,050 infections were reported. From 1999 onwards, campylobacteriosis has been the most common bacterial enteric infection in Finland (Figure 1). During the last few years, there have been over 4,000 infections yearly and a seasonal peak during the summer months (Figure 2).

2.1.4. Diagnostics, isolation, identification and species determination

A campylobacteriosis diagnosis can be made direct microscopy examination of Gram-stained fecal sample, or by isolation of the organism after culturing the stool sample (Allos and Blaser 2005). A number of commercial immunoassays are also available for detecting *Campylobacter* species straight from the feces, but their specificities and sensitivities vary (Tissari and Rautelin 2007; Tolcin, LaSalvia et al. 2000). Occasionally also blood samples are taken, but the total amount of blood culture-positive samples is not known. For research purposes, also PCR-based methods are available for detecting campylobacters straight from stool samples.

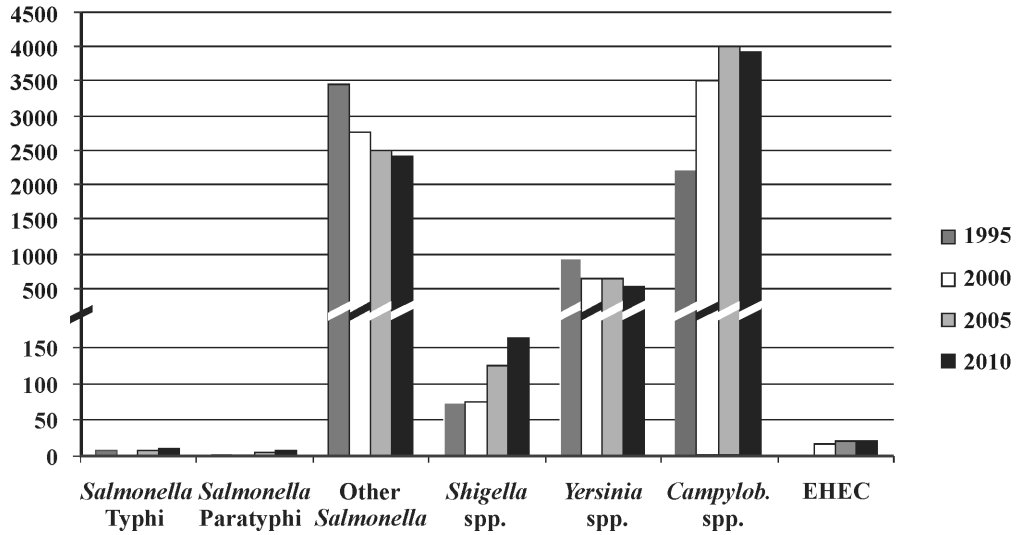

Figure 1. The annual incidence of bacterial gastroenteritis during four selected years presented according to causative bacteria in Finland. Campylobacters have been the most common bacteria causing gastroenteritis during the last years. Source; National Infectious Diseases Register of Finland (http://www3.ktl.fi/stat/ (last visited 25.7.2011)).

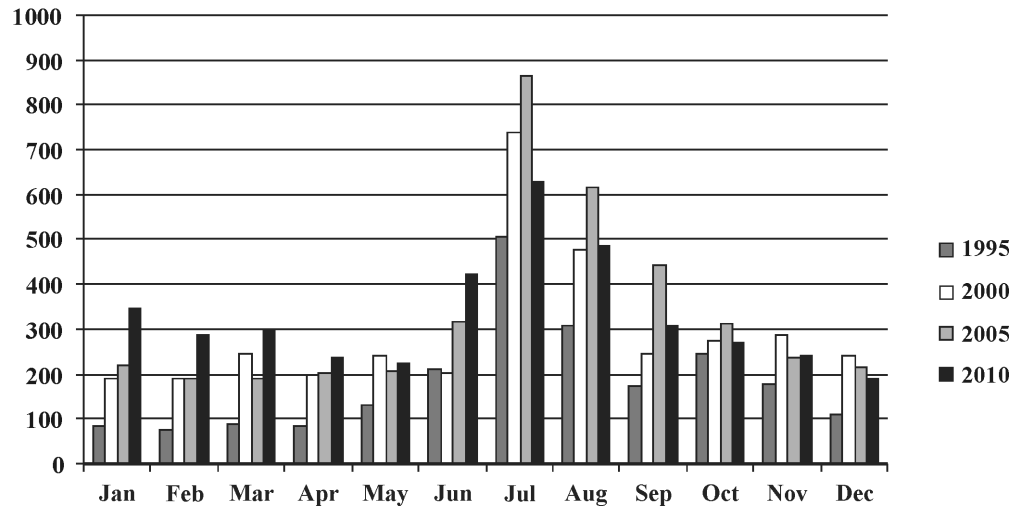

Figure 2. The incidence of campylobacter cases reported monthly in Finland. A seasonal peak can be seen during the summer months in Finland. Source; National Infectious Diseases Register of Finland (http://www3.ktl.fi/stat/ (last visited 25.7.2011)).

Because of the slow growth and specific growth conditions of campylobacters, also isolation from fecal samples needs selective techniques. Isolation is possible by using selective media, of which almost all contain antimicrobial agents, or by using a membrane filter system and growth onto a nonselective growth agar medium (Engberg, On et al. 2000). A blood-free charcoal cefoperazone deoxycholate agar (CCDA), cefoperazone-amphotericin-teicoplanin medium, and Skirrow's medium,

which contains *Campylobacter* agar base, 5% horse blood, vancomycin, trimethoprim, and polymyxin B are a few examples of the selective media used in isolation of *Campylobacter* species. The growth time is usually two to four days in microaerobic atmosphere at 43±1 ºC. The small size of campylobacters enables also the use of filtration through 0.65 micrometer filters onto a surface of nonselective, antibiotic free blood-agar plates (Lastovica 2006), which is a more preferable method for isolation. Campylobacters can cause systemic illness, and in those cases they can be isolated also from blood samples.

Serologic tests can also be used for detecting infections caused by campylobacters. During the first weeks of infection, IgA group *Campylobacter*-specific antibodies are present in both serum and feces. Later, serum IgG, IgM and IgA levels increase. They are not suitable for the diagnostics of an acute infection, but they might be useful in epidemiological investigations (Taylor, Williamson et al. 2004). Serology can also be used in order to determine causative agents for reactive arthritis.

In routine clinical microbiology laboratories, species identification is mainly performed by phenotypic methods. In *Campylobacter* species, a hippurate hydrolysis test is used to differentiate between *C. jejuni* strains. Of other species, *C. jejuni* is able to hydrolyze hippurate. *C. coli* strains are considered to be hippurate-negative, although false-positive hippurate test results have also been reported. Hippurate-negative isolates are classified as *Campylobacter* species, since there are also reports on hippurate-negative *C. jejuni* strains and they cannot be classified as *C. coli* strains without further investigations (Jensen, Andersen et al. 2005; Steinhauserova, Ceskova et al. 2001; Wainø, Bang et al. 2003).

According to Jensen et al. (2005), the prevalence of hippurate-negative *C. jejuni* strains was 28%, when the results were confirmed by rt-PCR. They also reported that 33% of their strains had a variable result in hippurate-hydrolysis although the tests were made according to the standard method. Totten et al. (1987) and Wainø et al. (2003) have previously reported that hippurate-negative *C. jejuni* represented 1.6% and 13.4% of their strains, respectively. Nakari et al. (2008) have standardized the hippurate hydrolysis test for *Campylobacter* species, and according to their results, *C. jejuni* strains can be identified reliably using this test. They found that the use of standardized cell suspension turbidity limits eliminated all false-positive reactions. Nevertheless, there are still hippurate-negative isolates, which need other determinations for species identification.

Species determination is important for reliable epidemiological data. In addition, correct species determination is important clinically, since *C. coli* strains are reported to be resistant to different antimicrobial agents more often than *C. jejuni* strains (Engberg, Aarestrup et al. 2001; Schönberg-Norio, Hänninen et al. 2006). There are also observations implying that resistance may vary depending on whether the strains are isolated from clinical human infections, from food of animal origin or from feces of healthy poultry or pigs. For example, a study performed by Saénz et al. (2000) reported that in *C. coli* strains isolated from pigs resistance frequencies to erythromycin (81.1%), ampicillin (65.7%), gentamycin (22.2%), and amikacin (21.6%) were higher compared to those isolated from humans (34.5%, 29.3%, 8.6%, and 0%, respectively).

A variety of assays exist for confirming *Campylobacter* to the genus and species level. These include genetic (PCR) methods, immunochemical chemotaxonomic fatty acid profiling, protein one-dimensional gel electrophoresis methods and, recently, microarray-based methods (Jensen, Andersen et al. 2005; Mandrell, Harden et al. 2005; Nayak, Stewart et al. 2005; Rönner, Engvall et al. 2004).

2.1.5. Clinical features and treatment of infections

Campylobacteriosis is an acute diarrheal illness resembling other acute bacterial infections of the intestinal tract. All campylobacters can cause gastroenteritis, but in some cases the infection can be asymptomatic. Asymptomatic infections can be due to, for example, a small amount of the bacteria, low virulence factors, or immunity against campylobacters. After exposure, the symptoms usually develop within two to four days, but the incubation period varies from one to seven days (Allos and Blaser 2005). In some cases, a quite small amount of bacteria can cause illness, even 500-1000 cells can be enough as infective dose (Black, Levine et al. 1988). However, there is also evidence that even after an ingestion of large numbers of campylobacters, clinical illness evolves in only a small proportion of subjects (Skirrow 1990). One cause for this can be low pH in the stomach, which can destroy even a large amount of campylobacters (Black, Levine et al. 1988). It is also of note that the use of drugs neutralizing the stomach's pH (proton pump inhibitors) or chronic intestinal illnesses are associated with campylobacter infections (Doorduyn, Van Den Brandhof et al. 2010).

Acute gastroenteritis is the most typical clinical picture of *C. jejuni* infection. It is usually a mild and self-limiting disease requiring no antimicrobial treatment. In most cases, the symptoms last for a week; in only 10-20% of the patients, the symptoms last longer (Allos and Blaser 2005). The most common symptoms are diarrhea, vomiting and stomach pain. Diarrhea can be massive, watery, or even bloody. Other symptoms include fever, headache, muscle pain, and nausea, which can begin at the same time as the gastroenteritis, or 12 to 24 hours before. Infections may also mimic symptoms of acute colitis. In some cases, stomach pain may be the only symptom of an infection, being therefore falsely diagnosed as appendicitis. The diagnosis is made from stool samples. (Allos and Blaser 2005) Extraintestinal manifestations are rare: e.g. septicemia, skin and soft tissue infection, carditis, infective endocarditis, and meningitis (Arai, Kitano et al. 2007; Kerstens, Endtz et al. 1992; Kotilainen, Lehtopolku et al. 2006; Monselise, Blickstein et al. 2004). Complications are also rare but includ bacteremia, appendicitis, and colitis or toxic mega colon (Allos and Blaser 2005). A fatal outcome in campylobacteriosis is rare, and it is usually confined with elderly patients or patients with another serious illness. According to a study made by Pacanowski et al. independent risk factors associated with death were cancer and isolated fever for patients with *C. fetus* bacteremia, the absence of treatment with appropriate antibiotics, and the prescription of third-generation cephalosporins for bacteremia due to other *Campylobacter* species (Pacanowski, Lalande et al. 2008). It is also of note, that according to the same study, 88% of their elderly and immunocompromised patients who were not treated with appropriate antibiotics died within 30 days after of illness (Pacanowski, Lalande et al. 2008).

Post-infectious sequelae include Guillan-Barré syndrome and reactive arthritis. They are considered to be immunological consequences of campylobacteriosis and occur especially in HLA-B27 positive patients (Schönberg-Norio, Mattila et al. 2010; Tam, Rodrigues et al. 2006). The Guillan-Barré syndrome typically gives symptoms within two to three weeks after infection. In Europe, North and South America, Japan and Australia, *C. jejuni* infection is preceding Guillain-Barrè syndrome in 20-50% of these Guillain-Barrè patients (Jakobs, van Belkum et al. 2008). On the other hand, reactive arthritis incidence is reported to be 1-5% after campylobacteriosis (Pope, Krizova et al. 2007). In Finland, after *C. jejuni* outbreak, 2.6% of patient developed reactive arthritis (Hannu, Kauppi et al. 2004). Other late-onset complications include Reiter's syndrome and carditis (Rees, Soudain et al. 1995; Uzoigwe 2005). Carditis can occur at the same time as the enteritis or as a post-infectious sequelae (Uzoigwe 2005). Post-infectious irritable bowel syndrome has also been reported after *Campylobacter* infection (Spiller 2007).

Depending on the species, campylobacters can cause different types of illness. Both *C. jejuni* and *C. coli* typically cause gastroenteritis and both of them also cause illness mainly in previously healthy people of all age groups. *Campylobacter fetus* subsp. *fetus,* however, usually causes opportunistic systemic infections in immunocompromised patients. On the other hand, *C. fetus* can cause disease also in previously healthy individuals.

Campylobacter infections typically cause self-limiting gastroenteritis and therefore the most important treatment is to avoid dehydration. Antimicrobial treatment is needed only in the most severe and persisting infections. In addition, certain groups of patients should also be treated with an antimicrobial agent, including very young children, pregnant women and old patients as well as immunocompromised patients (Allos 2001; Pacanowski, Lalande et al. 2008). Despite of the recommendations of starting antimicrobial treatment only in persisting infections in previously healthy patients, early clinical studies showed that the best effect of the antimicrobials is obtained by starting the drugs as soon as possible after the beginning of the symptoms. The later the treatment is started, the less effective it was in the clinical experiment (Anders, Lauer et al. 1982). For the empiric treatment of a patient with acute severe gastroenteritis, fluoroquinolones, for example ciprofloxacin and levofloxacin, are recommended (The Sanford Guide to Antimicrobial Therapy 2011). Macrolides (for example azithromycin) are recommended for the treatment of enteritis caused by campylobacters despite of the origin of the acquired infection, especially infections from areas where fluoroquinolone resistance is prevailing (Hakanen, Jousimies-Somer et al. 2003; Hill, Ericsson et al. 2006; The Sanford Guide to Antimicrobial Therapy 2011). Tetracyclines and amoxicillin can also be used (Moore, Corcoran et al. 2005). In severe cases the drug of choice is carbapenem (Fernandez-Cruz, Munoz et al. 2010; Kerstens, Endtz et al. 1992; Lau, Woo et al. 2002).

2.2. Antimicrobial resistance

Campylobacters are naturally susceptible to several antimicrobial agents including the macrolides, fluoroquinolones, tetracyclines, aminoglycosides, nitrofurans and clindamycin. Moderate susceptibility is reported originally to chloramphenicol, cefotaxime, ceftazidime and cefpirome. Intrinsic resistance in *C. jejuni* and *C. coli* is described against the penicillins and most of the cephalosporins as well as trimethoprim, sulfamethoxazole, rifampicin and vancomycin. (Fitzgerald, Whichard et al. 2008; Fliegelman, Petrak et al. 1985; McNulty 1987; Vanhoof, Goossens et al. 1982; Walder 1979)

2.2.1. Fluoroquinolone resistance in *Campylobacter* species

2.2.1.1. Classification of fluoroquinolones

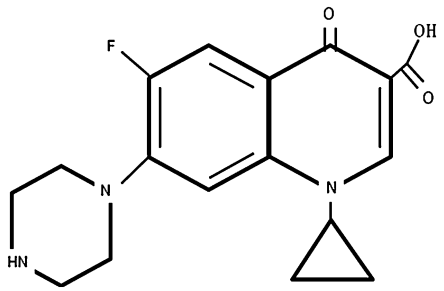

Figure 3. The structure of ciprofloxacin is based on the dual ring basic structure to which additional substitutions are attached. Figure modified from Andersson and MacGowan 2003.

The quinolones are synthetic compounds, which have a dual ring as a basic structure (Figure 3). Their development started during the 1960s, when nalidixic acid was discovered. Nowadays there are several compounds in this group and these antimicrobial agents are used widely, because of their broad-spectrum activity and good absorption after oral dosage.

The quinolones are divided into four groups according to their spectrum of activity (Table 2) (Andriole 2003; Van Bambeke, Michot et al. 2005). Nalidixic acid is a member of the first quinolones (first group). Newer members of this family have a fluorine substitution and are therefore called fluoroquinolones, as well as additional substitutions which give them enhanced potency against Gram-negative (2. group) and Gram-positive (3. group) bacteria and also against anaerobic bacteria (4. group). Newer compounds are active also against the etiological agents of atypical bacterial pneumonia, i.e. *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophila*. Fluoroquinolones can be used in the treatment of several infectious diseases for example urinary tract infections, bacterial prostatitis, gastrointestinal infections, sexually transmitted diseases and pelvic infections, respiratory infections, skin and soft tissue infections, bone and joint infections according to a causative bacteria (Andriole 2003; Hooper 2005; Reeves 1997; Van Bambeke, Michot et al. 2005).

Table 2. Classification of different groups of fluoroquinolones based on their antimicrobial spectrum.

	Antimicrobial spectrum	Antimicrobial agents (e. g.)
1st group	*Enterobacteriaceae*	Cinoxacin Nalidixic acid Oxolinic acid
2nd group	**In addition:** Pseudomonas aeruginosa, many Gram-positive cocci, *Neisseria* spp.	Ciprofloxacin[a] Norfloxacin[a] Ofloxacin[a] Enrofloxacin[b]
3rd group	**In addition:** *Streptococcus pneumonia,* some other Gram-positive cocci	Gatifloxacin Gemifloxacin Levofloxacin[a] Sparfloxacin Trovafloxacin
4th group	**In addition:** enhanced activity against anaerobes	Moxifloxacin[a] Sitafloxacin

[a]Currently available for clinical use in Finland.

[b]Available for animal use in Finland.

Sources: Reeves 1997; Andriole 2003; Van Bambeke, Michot et al. 2005.

2.2.1.2. Mechanisms of action of fluoroquinolones

The quinolones inhibit the DNA synthesis of bacteria, and are therefore bactericidal (Hooper 2001; Yao and Moellering 2003). The targets of quinolone action are two large bacterial enzymes, DNA gyrase and topoisomerase IV. DNA gyrase has two A and two B subunits, which are encoded by the *gyrA* and *gyrB* genes, respectively (Drlica and Zhao 1997; Wang 1996). Also topoisomerase IV has two pairs of subunits encoded by *parC* and *parE* (Kato, Nishimura et al. 1990). DNA gyrase and topoisomerase IV act mutually in bacterial DNA replication, transcription, recombination and repairing of DNA (Jacoby 2005). Fluoroquinolones bind to these enzymes and block the DNA synthesis. These actions cause cell death; apparently this complex acts as a sort of cellular poison (Hooper 2005; Jacoby 2005).

2.2.1.3. Fluoroquinolone resistance mechanisms

In Gram-negative bacteria, the mechanisms causing resistance to the fluoroquinolones are as follows: target mutations (topoisomerase genes), lowering outer membrane permeability or increasing efflux activity, target protection mediated by the *qnr* gene (Jacoby 2005) and the newest one an inactivating enzyme (Strahilevitz, Jacoby et al. 2009). The most important plasmid-mediated quinolone resistance (PMQR) mechanisms are the qnr genes. This group of genes produce proteins that reduce susceptibility to the quinolones by protecting the complex of DNA with either DNA gyrase or topoisomerase IV enzymes from the inhibitory effect of the quinolones (Strahilevitz, Jacoby et al. 2009). Also two additional PMQR mechanisms are reported: *aac(6')-Ib-cr* encodes an aminoglycoside acetyltransferase variant which can inactivate ciprofloxacin, and *oqxAB* and *qepA* encode efflux pumps, which can extrude quinolones (Strahilevitz, Jacoby et

al. 2009). To date plasmid-mediated quinolone-resistance determinants, such as *qnr*, *aac(6')-Ib-cr* and *qepA*, have not been reported in *Campylobacter* species.

In *Campylobacter* species, the resistance to the fluoroquinolones is mainly caused by chromosomal mutations in the QRDR of the *gyrA* gene coding the gyrA subunit of the DNA gyrase. Resistance causing modifications in the GyrB subunit have not been reported in campylobacters (Payot, Cloeckaert et al. 2002; Piddock, Ricci et al. 2003). It also seems that in *Campylobacter* species, the Topoisomerase IV encoded by *parC*/*parE* genes is absent (Bachoual, Dubreuil et al. 2000; Payot, Bolla et al. 2006). There are several different single GyrA modifications reported to be associated with fluoroquinolone resistance in *Campylobacter* species: Thr86Ile, Asp90Asn, Thr 86Lys, Thr86Ala, Thr86Val and Asp90Tyr. Thr86Ile has been the most common mutation found. Also the following double mutations have been reported to be connected with fluoroquinolone resistance: Thr86Ile-Pro104Ser and Thr86Ile-Asp90Asn (Payot, Bolla et al. 2006).

The CmeABC multidrug efflux pump has been described as being the major efflux mechanism and causing antimicrobial resistance to a wide variety of antimicrobials including the fluoroquinolones and macrolides (Lin, Michel et al. 2002; Pumbwe and Piddock 2002). The CmeABC multidrug efflux pump is the most common efflux system in *C. jejuni*, and consists of three components: the cmeA periplasmic protein, the cmeB inner membrane transporter, and the cmeC outer membrane channel protein (Lin, Michel et al. 2002). The CmeABC efflux pump works in synergy with GyrA mutations in causing fluoroquinolone resistance in campylobacters (Luo, Shahin et al. 2003). There is also evidence that the efflux pump is needed for the growth of campylobacters in cecal extract, and also for the *in vivo* colonization of poultry by campylobacters (Lin, Sahin et al. 2003). When the efflux pump is blocked, the minimum inhibitory concentration (MIC) values for ciprofloxacin are reduced to the level of susceptible strains even with mutations in the GyrA (Luo, Sahin et al. 2003). A recent study by Guo et al. (Guo, Lin et al. 2010) showed that functional CmeABC homologs can be identified in five *Campylobacter* species including *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus*. Additional pump systems have also been described, but their role in the development of resistance appears to be less important.

2.2.1.4. Epidemiology of fluoroquinolone resistance

In campylobacters, resistance to the fluoroquinolones was first reported in the late 1980s (Allos 2001). Since then, fluoroquinolone resistance has been increasing. It has been observed that resistance appeared at the same time as the introduction of these agents in animal production and veterinary medicine (Aarestrup and Engberg 2001). Nowadays, worldwide fluoroquinolone resistance is common. For example in Thailand and India, 80% and 77% of *Campylobacter* isolates, respectively, have been reported to be resistant to the fluoroquinolones (Hoge, Gambel et al. 1998; Jain, Sinha et al. 2005). A high incidence of resistance has also been observed in the United Arab Emirates (85.4%) (Sonnevend, Rotimi et al. 2006), and South Africa (91%) (Bester and Essack 2008). In China, very high resistance rates to ciprofloxacin have been reported in *C. coli* strains isolated from swine: 95.8-99% of the strains were classified as resistant (Qin, Wu et al. 2011). In Spain,

the emergence of fluoroquinolone resistance was evaluated between 1993 and 2003 and a statistically significant increase was observed for ampicillin, nalidixic acid, and ciprofloxacin (Ruiz, Marco et al. 2007). In that study, resistance rates for ciprofloxacin and nalidixic acid were 46.7% and 52.2%, respectively. There are also opposite observations. For example in Norway, no fluoroquinolone-resistant *Campylobacter* isolates have been detected (Norström, Johnsen et al. 2007), and in Grenada, a ciprofloxacin resistance rate of only 9.4% has been observed (Hariharan, Sharma et al. 2009). A recent study from Denmark showed that resistance rates to ciprofloxacin, nalidixic acid and tetracycline were significantly higher in travel-associated infections compared to infections acquired domestically, and that the occurrence of resistance increased during the years 2006 and 2007 (Skjøt-Rasmussen, Ethelberg et al. 2009).

In Finland, ciprofloxacin resistance in campylobacters has emerged after the 1980s; until then no ciprofloxacin-resistant *Campylobacter* strains were observed (Rautelin, Renkonen et al. 1991). According to the same study, already 9% of the strains were ciprofloxacin-resistant in 1990. After this, the rate of ciprofloxacin resistance has clearly increased, and between 1998 and 2000, the majority of strains isolated from Finnish patients after travel to Spain or Thailand (70% and 79%, respectively) were resistant to ciprofloxacin (Hakanen, Jousimies-Somer et al. 2003). According to a recent report, domestic *Campylobacter* strains in Finland are still mainly susceptible to the fluoroquinolones (Schönberg-Norio, Hänninen et al. 2006).

2.2.1.5. Factors influencing fluoroquinolone resistance

Since campylobacteriosis is considered to be a zoonosis, the presence of resistant strains in the food chain also has an influence on human infections. One of the main factors influencing fluoroquinolone resistance is considered to be mainly due to the use of antimicrobial agents in animal production. In the early 1990s, when enrofloxacin was taken into use in animal production in Asia and in Europe, fluoroquinolone resistance started to increase among human isolates at the same time (Endtz, Ruijs et al. 1991). In the United Kingdom (UK) and in the USA, the same phenomenon was observed after the approval of the use of fluoroquinolones in veterinary medicine (Nachamkin, Ung et al. 2002; Sam, Lyons et al. 1999).

In many countries where fluoroquinolone use in animal production is low, the incidence of fluoroquinolone-resistant strains has remained moderate or low. For example in Australia, where the use of fluoroquinolones in animal production is prohibited, *Campylobacter* strains isolated from pigs are mainly ciprofloxacin-susceptible (Hart, Heuzenroeder et al. 2004). In Finland, the findings are similar: the use of fluoroquinolones is limited in the animal production, and the resistance to fluoroquinolones among domestic *Campylobacter* isolates has remained low and constant (Myllyniemi, Koppinen et al. 2005; Schönberg-Norio, Hänninen et al. 2006). Similar results have also been reported from Sweden (Sjögren, Lindblom et al. 1997). In Denmark, the use of fluoroquinolones in animal husbandry has been restricted since 2003. A recent study from Denmark reported significantly higher resistance to ciprofloxacin, nalidixic acid and tetracycline in *C. jejuni* from imported poultry meat compared to Danish poultry meat (Skjøt -Rasmussen, Ethelberg et al. 2009).

The use of fluoroquinolones for infections other than gastroenteritis as well as self-medication are often causes of resistance in the developing countries (Coker, Isokpehi et al. 2002). For this reason, traveling to developing countries has been implied to be a risk factor for gaining an infection caused by a resistant *Campylobacter* strain. In industrialized countries, one reason behind the fluoroquinolone resistance might also be their inadequate clinical use in the treatment of human infections. Patients treated with fluoroquinolones were later found to have bacteria resistant to these antimicrobial agents (Bacon, Alm et al. 2000). The rapid evolvement of resistance has been detected also in poultry and pigs (Adler-Mosca, Luthy-Hottenstein et al. 1991; Farnell, Donoghue et al. 2005; Delsol, Sunderland et al. 2004). It is also noteworthy that fluoroquinolone-resistant campylobacters do not necessarily lose fitness and can effectively compete with fluoroquinolone-susceptible strains also in the absence of antimicrobial agents (Luo, Pereira et al. 2005). There is also evidence that GyrA mutations in campylobacters even may cause enhanced fitness (Luo, Pereira et al. 2005). Thus, when antibiotic pressure is taken away, fluoroquinolone resistance remains (Pedersen and Wedderkopp 2003; Price, Lackey et al. 2007). Therefore, it is hard to get rid of fluoroquinolone resistant strains by decreasing the use of fluoroquinolones in veterinary medicine.

2.2.2. Macrolide resistance in *Campylobacter* species

2.2.2.1. Classification of macrolides

The macrolides are mostly produced by *Streptomyces* and related bacteria. Erythromycin is a natural product found in *Streptomyces erythreus* (now called *Saccharopolyspora erythraea*) found in 1952, and is the first macrolide compound isolated (Figure 4).

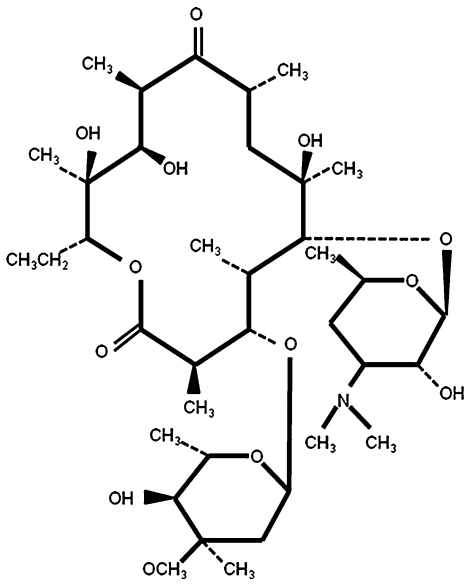

Figure 4. The structure of erythromycin. Figure modified from Douthwaite and Champney 2001

All macrolides share a macrolactone ring, and according to this ring structure, macrolides can be divided into either 14-, 15-, or 16-membered compounds (Table 3) (Bryskier and Butzler 2005). Macrolides can be derived from natural products or be semisynthetic.

The macrolides are widely used antimicrobial agents and considered to be safe and effective drugs. Their antimicrobial spectrum includes most of the Gram-positive organisms, *Neisseria* spp., *Bordatella pertussis* and the Gram-negative *Moraxella catarrhalis* as well as both Gram-positive and Gram-negative anaerobes. *Haemophilus* spp. are, according to EUCAST (European Committee on Antimicrobial Susceptibility Testing), intermediately susceptible to macrolides (http://www.eucast.org/ (last visited 25.7.2011)). The semisynthetic compounds do not give a substantially better activity than erythromycin A against

staphylococci and streptococci and the activity against enterococci is poor. However, these semisynthetic compounds provide important activity against many intracellular pathogens including e. g. *Chlamydia trachomatis*, *Chlamydophila pneumoniae*, and *Legionella* spp. (Bryskier and Butzler 2005) The ketolides are semisynthetic derivates of erythromycin and have a better activity against Gram-positive cocci than erythromycin A (Bryskier and Butzler 2005). Telithromycin is the most active compound, with activity also against most erythromycin A-resistant strains..

Table 3. Classification of the different groups of macrolides.

	14-membered ring	15-membered ring (Azalides)	16-membered ring	Ketolides (14-membered ring)
Natural products	Erythromycin A[a] Oleandomycin		Josamycin Kitasamycin (leukomycin) Midecamycin Spiramycin[a]	
Semi-synthetic compounds	Clarithromycin[a] Dinithromycin Flurithromycin Roxithromycin[a]	Azithromycin[a,b]	Miocamycin Rokitamycin Tylosin[c]	Telithromycin[a]

Table modified from Bryskier et al. 2003.
[a]Currently available for clinical use in Finland.
[b]Azithromycin has activity also against *Salmonella* spp. (Gunell, Kotilainen et al. 2010)
[c]On market for animal use in Finland.

2.2.2.2. Mechanisms of action of macrolides

Macrolides interrupt proteins synthesis in bacterial ribosome. The bacterial ribosome consists of the large 50S subunit and the small 30S subunits. The large subunit consists of the proteins L1-L36 and two rRNA molecules called 23S and 5S rRNAs. The small subunits, on the other hand, consist of one ribosomal RNA molecule called 16S rRNA and ribosomal proteins S1-S21.

Macrolides inhibit protein synthesis by targeting the 50S subunit of the bacterial ribosome and inhibit bacterial RNA-dependent protein synthesis (Poehlsgaard and Douthwaite 2005; Yao and Moellering 2003). According to structural studies, the 23S rRNA nucleotides Ala2058 and Ala2059 act as key contact sites for macrolide binding. The binding of the macrolide antimicrobial leads to conformational changes in the ribosome and subsequent termination of the elongation of the peptide chain (Pfister, Jenni et al. 2004).

2.2.2.3. Macrolide resistance mechanisms

In *Campylobacter* species, target modifications and active efflux are the main causes of macrolide resistance (Gibreel and Taylor 2006). Ribosomal target modifications causing macrolide resistance can be due to a point mutation in the 23S rRNA gene, and post-translational modifications in the ribosomal proteins L4 and L22 might also influence

macrolide resistance. Enzyme-mediated methylation can cause macrolide resistance, but in campylobacters it has only been reported in *Campylobacter rectus* (Roe, Weinberg et al. 1995). Mutations at the positions 2058 or 2059 of the 23S rRNA gene cause high-level resistance to the macrolides (Gibreel, Kos et al. 2003; Vacher, Menard et al. 2003). *Campylobacter* species contain three copies of this 23S rRNA gene, and mutations can be either homozygous having all three alleles mutated or heterozygous having possibly one or two of the alleles mutated. There are reports of macrolide-resistant *Campylobacter* strains with two mutated copies of the 23S rRNA gene (Gibreel and Taylor 2006). However, there are no reports of macrolide-resistant *Campylobacter* strains containing only one mutated copy of the 23S rRNA gene (Gibreel, Kos et al. 2005; Ladely, Meinersmann et al. 2009; Payot, Avrain et al. 2004). Resistance may also be caused by modifications of the ribosomal proteins L4 and L22. Several modifications have been reported in *Campylobacter* species and it is possible that they might be associated with low-level resistance to the macrolides. The exact role of these L4 and L22 modifications (mutations, insertions, deletions) is still under investigation (Cagliero, Mouline et al. 2006; Caldwell, Wang et al. 2008; Corcoran, Quinn et al. 2006; Payot, Avrain et al. 2004).

Efflux is another common mechanism causing macrolide resistance. In *Campylobacter* species, at least eight different efflux systems have been reported. Efflux pumps can also protect campylobacters against other factors, e. g. dyes and detergents (Lin, Michel et al. 2002). The CmeABC multidrug efflux pump is one of the efflux pumps reported in *Campylobacter* species, and it mediates resistance to variety of antimicrobial agents (Lin, Michel et al. 2002). This multidrug-efflux pump, in addition to being considered a significant player in intrinsic resistance, might also be needed for maintaining the acquired resistance to erythromycin in campylobacters (Lin, Michel et al. 2002; Lin, Yan et al. 2007). It might work in synergy with specific mutations, but even in the absence of any other factor affecting resistance (Cagliero, Mouline et al. 2006; Payot, Avrain et al. 2004). There is data suggesting that interplay between efflux activity and mutations in the 23S rRNA gene contribute to high-level macrolide resistance in some *Campylobacter* strains (Corcoran, Quinn et al. 2006).

2.2.2.4. Epidemiology of macrolide resistance

The macrolide resistance among campylobacters has remained at a low and stable level for a long time. However, there is also evidence from some parts of the world that resistance rates to erythromycin and other macrolides in *Campylobacter* species are slowly increasing (Bae, Kaya et al. 2005; Vlieghe, Jacobs et al. 2008). Since fluoroquinolone resistance is common, the macrolides have become important in the treatment of campylobacteriosis. This also influences to the development of macrolide resistance.

According to a study performed by Kassa et al. (2007) in Ethiopia in 2004 on 186 *Campylobacter* strains isolated from food animals, no resistance to ciprofloxacin was observed, but instead the resistance to erythromycin was 0.7% in their *C. jejuni* strains and 3.9% in their *C. coli* strains. Chen et al. (2010) studied antimicrobial resistance in poultry in 2008 in China by analyzing the *in vitro* susceptibilities of 275 *Campylobacter* isolates obtained from 767 poultry fecal samples; the resistance rates to erythromycin, azithromycin and clindamycin were 8.9%, 26.7%, and 13.9%, respectively, for the *C. jejuni* isolates, and 100%, 98.1%, and 100%, respectively, for the *C. coli* isolates. They

also reported high gentamycin resistance, even up to 92.3%, in their *C. coli* isolates. In another study from China, high resistance numbers were also reported in *C. coli* strains isolated from swine: 37.9-54.7% of the strains were resistant to erythromycin (Qin, Wu et al. 2011). Nevertheless, several countries still report a low level of erythromycin resistance in human clinical samples (Table 4).

2.2.2.5. Factors influencing macrolide resistance

Use of macrolides in animal production as therapeutic or growth-promoting agents has been considered to be one important factor in the selection of erythromycin-resistant *Campylobacter* strains. Ladely et al. (Ladely, Harrison et al. 2007) studied the effect of tylosin given to poultry at sub-therapeutic and therapeutic concentrations. They observed that after tylosin administration, the overall erythromycin resistance rate among *C. coli* isolates was at a higher frequency than among *C. jejuni* isolates (70.8% vs. 36.8%; $P < 0.01$). They also noticed that in *Campylobacter* species, the frequency of erythromycin resistance was higher when tylosin was administered at sub-therapeutic than at therapeutic concentrations (62.7%, 11.4%; $P < 0.001$) (Ladely, Harrison et al. 2007). Juntunen et al. (Juntunen, Heiska et al. 2010) studied the effects of tylosin treatment of pigs and observed that it selected high-level resistance to erythromycin, as well as resistance to ciprofloxacin and nalidixic acid. In addition, resistance to streptomycin also increased in *C. coli* isolates within a few days. When tylosin treatment was stopped, resistance to at least one antimicrobial was significantly lower when tested seven months later (Juntunen, Heiska et al. 2010). Lin et al. (2007) studied the frequency of spontaneous mutations to an erythromycin resistant phenotype and found that both *C. jejuni* and *C. coli* have extremely low rates of spontaneous mutations under *in vitro* culture conditions mutation emerging frequency being between 3×10^9 and $\leq 10^{10}$ as measured by a single-step selection. Moreover, acquisition of erythromycin resistance in *Campylobacter* species is a stepwise process and requires prolonged exposure in contrast to the rapidly envolving fluoroquinolone resistance (Table 5). Hao et al. (2009) have shown that erythromycin-resistant *Campylobacter* strains display a fitness disadvantage when compared with susceptible *Campylobacter* strains. They have speculated that the fitness reduction may lead to a low frequency of macrolide resistance in clinical isolates.

2.2.3. Resistance to other antimicrobial agents in *Campylobacter* species

2.2.3.1. β-lactams: Co-amoxiclav and carbapenems

Campylobacters are considered to be resistant to β-lactam antimicrobial agents, principally the penicillins and cephaloporins. Yet, campylobacters are only moderately susceptible to cefotaxime, ceftazidime, and cefpirome (Van der Auwera and Scorneaux 1985). There is, however, very little experience of their clinical use in the treatment of infections caused by *Campylobacter* species. In pediatric patients with acute diarrhea in Israel, Leibovitz et al. compared oral ciprofloxacin therapy to intramuscular ceftriaxone therapy in 95 patients treated with ciprofloxacin and 106 patients treated with ceftriaxone, and according to their results ceftriaxone was as safe and effective as oral ciprofloxacin (Leibovitz, Janco et al. 2000). However, of these patients, only 7 patients treated with ciprofloxacin and 6 patients treated with ceftriaxone, had an infection caused by campylobacter.

Table 4. Ciprofloxacin and erythromycin resistance among *C. coli* and *C. jejuni* from human clinical samples in different countries.

Country	Year	n	% resistant Erythromycin		% resistant Ciprofloxacin		Reference
			C. coli	*C. jejuni*	*C. coli*	*C. jejuni*	
Poland	2000-2007	251	0	0.4	51.3	49.5	Rozynek, Dzierzanowska-Fangrat et al. 2009
Germany	2006-2008	112	0	0	42.8	52.3	Valenza, Frosch et al. 2010
Iran	2004-2005	34	0	3.4	80	58.6	Feizabadi, Dolatabadi et al. 2007
Chile	2002-2007	73	ND	0	ND	32.4	Garcia, Valenzuela et al. 2009
Switzerland	2008	136	ND	0.7	ND	37.5	Kittl, Kuhnert et al. 2011
Japan	1996-2009	62	0		62.9		Yabe, Higuchi et al. 2010

Table 5. Comparison of fluoroquinolone and macrolide resistance mechanisms of *Campylobacter* species.

	Fluoroquinolone	Macrolide	Reference
Resistance mechanisms	- Modification in the GyrA subunit - Efflux pumps	- 23S rRNA gene mutations - Efflux pumps - L4 and L22 ribosomal modifications	Gibreel, Kos et al. 2005; Corcoran, Quinn et al. 2006; Piddock, Ricci et al. 2003; Pumbwe and Piddock 2002
Mutation frequency	High	Low	
Incidence of resistance	High	Low	
Fitness cost after mutation	Low	High	

Carbapenems are an exception to the general β- lactam resistance, and are considered to be effective also in the treatment of campylobacteriosis. In general, β-lactam antimicrobials bind to penicillin binding proteins and disrupt peptidoglycan cross-linking during bacterial cell wall formation. This leads to cellular swelling and finally to cell death (Martin and Kaye 2004). In *Campylobacter* species, resistance to this group of antimicrobials is caused by β-lactamases, which are frequently observed (Taylor and Courvalin 1988; Reviewed by Li, Mehrotra et al. 2007). An example of these β-lactamases is OXA-61, a novel enzyme causing resistance to β-lactam antimicrobials (Alfredson and Korolik 2005). Also alterations in the membrane structure or in porin proteins (Page, Huyer et al. 1989) and the efflux pump system can cause resistance to this antimicrobial group. It is also of note that *Campylobacter* porins are small and cation selective, and therefore influence on susceptibility to β-lactam antimicrobial agents.

Co-amoxiclav is a mixture of potassium clavulanate and amoxicillin trihydrate or amoxicillin sodium. The β-lactamase inhibitor restores activity against β-lactamase-producing strains. Campylobacters are primarily resistant to penicillins, but there are studies indicating that co-amoxiclav has good *in vitro* activity against *Campylobacter* species (Gaudreau and Gilbert 2003; Rodriguez-Avial, Rodriguez-Avial et al. 2006; Ruiz, Marco et al. 2007). Pigrau et al. (1997) have reported antimicrobial susceptibilities of *Campylobacter* species isolated from 58 patients with campylobacter-bacteremia and underlying illness. According to their results, resistance to co-amoxiclav was only 4%; thus, they suggested that it could be an effective alternative for antimicrobial therapy.

There is only limited data on the use of carbapenems for the treatment of campylobacteriosis, however. In adults, there are a few reports of good clinical outcome in severely immunocompromised patients who have recovered from severe or relapsing campylobacteriosis with the use of carbapenems (Burch, Saeed et al. 1999; Kerstens, Endtz et al. 1992; Monselise, Blickstein et al. 2004). There are also *in vitro* studies in which the carbapenems have had the lowest MIC values against *Campylobacter* strains (Clarke and Zemcov 1989; Yabe, Higuchi et al. 2010).

2.2.3.2. Tetracycline and tigecycline

Tetracyclines are a widely used group of broad-spectrum antimicrobial agents which are essentially bacteriostatic. They are natural products isolated from *Streptomyces* spp. and their semisynthetic derivates (Chopra 2003). They bind to the 30S subunit of the bacterial ribosome and inhibit peptide elongation (Connell, Trieber et al. 2003). Tetracyclines can be used in the treatment of campylobacteriosis, except in children under nine years of age (Moore, Corcoran et al. 2005). However, tetracycline resistance has emerged also among *Campylobacter* species. For example, Gibreel *et al*. reported that of 203 clinical *C. jejuni* strains of human origin isolated between 1999 and 2002, 101 isolates (50%) were resistant to tetracycline. Ruiz *et al*. reported in their study that tetracycline resistance occurred in up to 42.4% of the *Campylobacter* strains causing travelers' diarrhea during the period 1993 to 2003 (Ruiz, Marco et al. 2007).

For bacteria, there are four different mechanisms for achieving resistance to tetracycline: efflux of tetracycline, modification of tetracycline, ribosomal protection or mutation of the 16S rRNA. In *Campylobacter* spp. the most common tetracycline resistance mechanism

is a plasmid-mediated ribosomal protecting protein Tet(O) encoded by the *tet*(O) gene (Connell, Trieber et al. 2003; Gibreel, Tracz et al. 2004; Taylor 1986). Resistance can also be caused by Tet(M), another ribosomal protecting protein, and the efflux system (Pumbwe and Piddock 2002), although Tet(M) has not been reposted in *Campylobacter* spp. Tet(O) and Tet(M) can both dislodge tetracycline from the ribosome, and thereby inhibit the effect of the tetracyclines. Ribosomal protecting proteins and efflux pumps can also work synergistically and cause high-level tetracycline resistance (Gibreel, Wetsch et al. 2007).

Tigecycline is rather a new drug, which is derived from minocycline. In Finland it has been available for clinical use from the year 2006. Like tetracyclines, tigecycline binds to the 30S subunit of bacterial ribosome, and inhibits protein synthesis. Tigecycline is less effected of tetracycline resistance, because it has an enhanced affinity to its binding sites as compared to the tetracyclines. Tigecycline has an excellent activity e.g. in the treatment of complicated skin and soft tissue infection (Breedt, Teras et al. 2005). It circulates primarily as unchanged drug and its major route of elimination is through feces. Also biliary excretion and perhaps even an enterohepatic circulation may be involved (Agwuh and MacGowan 2006). Rodriguez-Avial et al. (2006) have studied the *in vitro* activity of tigecycline against *Campylobacter* species. Compared to the other antimicrobials studied (erythromycin, clindamycin and co-amoxiclav), tigecycline had the lowest MIC values.

2.2.4. Multidrug resistance in *Campylobacter* species

Multidrug resistance (MDR) is defined as resistance to three or more groups of antimicrobial agents. MDR in *Campylobacter* species has so far been quite rare. However, it has increased, posing a serious risk of treatment failures. Prasad et al. (1994) have reported that 2.2% of their *Campylobacter* strains were multidrug resistant in North India between 1989 and 1993. In the year 2005, the same group reported that MDR had increased to 30.6% among their *C. jejuni* and *C. coli* strains collected during the year 2002 (Jain, Sinha et al. 2005). The most frequent combination of resistance was ampicillin together with tetracycline and ciprofloxacin, comprising 14.9% of their strains. Chen et al.(2010) also reported a high incidence of MDR in China in 2008; over 90% of their *C. jejuni* strains and all of their *C. coli* strains were resistant to multiple antimicrobial agents. Another recent study from China reported a high incidence of multidrug-resistant *C. coli* strains while 76.8% of the strains displayed 19 different MDR patterns (Qin, Wu et al. 2011). These resistance frequencies were higher than those reported previously from the UK (3.8%) (Randall, Ridley et al. 2003), France (37%) (Payot, Avrain et al. 2004; Payot, Dridi et al. 2004), Canada (29.7%) (Varela, Friendship et al. 2007) and Korea (56.1%) (Shin and Lee 2010).

The emergence of MDR also seems to be connected with the slowly increasing macrolide resistance (Hoge, Gambel et al. 1998). The increase of these multidrug-resistant *Campylobacter* strains may reflect the overuse of different antimicrobial agents in veterinary medicine and, especially, in poultry production. Similar results have been reported also from swine production, especially concerning a high resistance rates in *C. coli* strains (Qin, Wu et al. 2011). Once MDR is prevalent, removal of the antibiotic

selection pressure may not readily cause reduction of the antimicrobial resistance (Chen, Naren et al. 2010).

There are very few treatment alternatives of campylobacteriosis caused by multidrug-resistant strains. Thus, MDR might elicit the possibility of clinical treatment failures in cases of severe *Campylobacter* infection, since only a limited amount of experience and information exists on the antimicrobial treatment of infections caused by multidrug-resistant *Campylobacter* strains.

2.3. Detection of antimicrobial resistance in *Campylobacter* species

In *Campylobacter* species resistance to fluoroquinolones is widespread, and also resistance to macrolides has been increasing in some countries. Routine susceptibility testing is therefore needed for adequate antimicrobial therapy. Information on resistance is also needed for efficient monitoring of the antimicrobial resistance situation. Several laboratory methods have been applied to susceptibility testing of *Campylobacter* species although only a few methods have been standardized. Therefore, different laboratories might use different susceptibility testing methods, but also different breakpoints to define susceptibility and resistance.

2.3.1. Antimicrobial susceptibility testing

Several laboratory methods including agar dilution, broth microdilution, disk diffusion, and strip tests such as E-test have been applied for susceptibility testing of *Campylobacter* species.

The agar dilution, broth dilution, and E-test are MIC-based methods, and give an Minimal Inhibitory Concentration (MIC) value as a result. The MIC value is the lowest concentration that is able to inhibit the growth of bacteria. In clinical use, the MIC values should be compared to the dosage and also to the achieved levels of antimicrobial agent in the tissues. The agar dilution and broth dilution methods have been standardized by the CLSI (Clinical and Laboratory Standards Institute, former NCCLS) (CLSI 2006; CLSI 2008; CLSI 2009). The agar dilution method needs a large amount of manual handling; thus, it is not convenient for the testing of only a few isolates at a time. Similarily, the broth dilution method needs also a rather large amount of manual handling. Alternatively, there are commercially prepared antimicrobial panels for broth dilution method also available, but this is a more expensive alternative. A third method for determining the MIC value is a strip test (e.g. E-test) which uses diffusion of antimicrobial agent from a coated plastic strip placed onto the surface of an inoculated agar plate. The E-test is convenient for the testing of even small numbers of isolates, being an easier method than the agar or broth dilution methods. However, the E-test method has not been standardized for susceptibility testing of campylobacters. There are several studies comparing E-test and agar dilution (Ge, Bodeis et al. 2002; Varela, Friendship et al. 2008; Valdivieso-Garcia, Imgrund et al. 2009), with somewhat controversial results.

The disk diffusion method is as easy to perform as the E-test method. It is also based on diffusion of the antimicrobial agent in a specific concentration from the disks inpregnated

with antimicrobial compounds placed onto the surface of an inoculated agar plate. The disk diffusion does not give an MIC value as a result, but the possible resistance is determined based on the inhibition zone value. Disk diffusion has been standardized according to the CLSI, but it should only be used as a screening method for resistance in *Campylobacter* spp. According to CLSI, any appearance of an inhibition zone, requires an MIC determination for accurate categorization of susceptibility (CLSI 2006; CLSI 2008; CLSI 2009). There are several studies focusing on disk diffusion, and comparing it to other methods. The results have been varying though, since in some studies, both of the methods have been in line (Gaudreau, Girouard et al. 2007; Gaudreau, Girouard et al. 2008; Schönberg-Norio, Hänninen et al, 2006) while in other studies, clear differences have been observed (McGill, Kelly et al. 2009; van der Beek, Claas et al. 2010).

2.3.2. Antimicrobial breakpoints

The clinical breakpoint for the MIC-value of the antimicrobial agent and the bacterial pathogen is considered to be the threshold above which the pathogen causing infection is unlikely to respond clinically to treatment with that particular drug, and is classified as resistant. There are also determinations for the breakpoints of susceptible and intermediate; susceptible, where the antimicrobial activity is associated with the likelyhood of a therapeutic success; and intermediate, where the antimicrobial activity is associated with an indeterminate or uncertain therapeutic effect.

Differing from the clinical breakpoints, EUCAST is also using the term epidemiological cut-off value, which is a breakpoint meant for the detection of bacteria with resistance mechanisms and for monitoring the development of antimicrobial resistance. It is important to distinguish these two, since they are meant for different purposes of use. The clinical breakpoints are for clinical use, with the aim to guide antimicrobial treatment. The epidemiological cut-off value, on the other hand, is meant for harmonizing breakpoints in order to detect antimicrobial resistance with the aim to follow resistance rates (Kahlmeter, Brown et al. 2003).

The CLSI determines breakpoints for several different bacterial species including *Campylobacter* species (Table 6) There are also susceptibility testing committees who have determined breakpoints, e.g. the BSAC (The British Society for Antimicrobial Chemotherapy) (Andrews 2008) and EUCAST (http://www.eucast.org/ (last visited 1.8.2011)). BSAC is now incorporated in the EUCAST. The information regarding the breakpoints shows variation between these different organizations (Table 6). For example, the CLSI has instructions concerning the disk diffusion method in susceptibility testing of campylobacters, but there are only screening criteria for resistance to erythromycin and ciprofloxacin. The BSAC on the other hand gives inhibition zone limits for resistant and susceptible strains. It also recommends that quinolone resistance is most reliably detected with nalidixic acid disks; strains with reduced susceptibility to the fluoroquinolones give no zone of inhibition with a 30 µg nalidixic acid disk. There is a need for homogeneous breakpoints in order to get appropriate resistance information worldwide. (Andrews 2008; Kahlmeter, Brown et al. 2003)

Table 6. In *Campylobacter* species there are differences between breakpoints of different suscebtibility testing commitees. Only for a limited number of antimicrobial agents there are currently determined breakpoints available.

Antimicrobial agent	MIC breakpoint for resistance (≥ µg/ml)				Disk diffusion inhibition zone for resistance (≤)					
					CLSI[f]		BSAC		EUCAST	
	CLSI[a]	BSAC[b]	EUCAST[c] *C. jejuni*[d]	EUCAST[c] *C. coli*[e]	mm	Disk content (µg)	mm	Disk content (µg)	mm	Disk content (µg)
Erythromycin	32	1	8	-	6	15	19	5	-	-
Ciprofloxacin	4	2	2	2	6	5	17	1	-	-
Tetracycline	16	-	-	-	-	-	-	-	-	-
Doxycycline	8	-	-	-	-	-	-	-	-	-

[a]CLSI 2009
[b]Andrews 2008
[c]http://www.eucast.org/
[d] Resistance breakpoints available also for levofloxacin and ofloxacin being ≥ 4 µg/ml and ≥ 2 µg/ml, respectively.
[e] Breakpoint also for sulfa-trimethoprim available (≥ 8 µg/ml).
[f] No zone around the disk indicates resistance, and appearance of any zone requires MIC determinations.

2.3.3. Genotypic methods for detecting resistance

2.3.3.1. Fluoroquinolone resistance

There are several genotypic methods available for the detection of resistance mechanisms in *Campylobacter* species. DNA **sequencing** is commonly used in the analysis of mutations causing fluoroquinolone resistance (Kinana, Cardinale et al. 2007). Mutations in GyrA gene have also been analyzed by mismatch amplification mutation assays (**MAMA**), which have proved to be reliable methods, when confirmed by sequencing (Sonnevend, Rotimi et al. 2006). Also PCR-based restriction fragment length polymorphism (**PCR-RFLP**) assay is used in *Campylobacter* species. It is a simple, rapid and reproducible method for identification of mutations mediating fluoroquinolone resistance (Alonso, Mateo et al. 2004). In addition, nonradioisotopic **SSCP** analysis and direct sequencing have been used in analyzing alterations in QRDR of gyrA in *C. jejuni* (Beckmann, Muller et al. 2004). Westin et al. have combined anchored *in situ* amplification on a **microelectronic chip array** to detect mutations in gyrA gene and also to discriminate between species (Westin, Miller et al. 2001).

Efflux pumps have been studied by a number of PCR-based methods, e.g. by a comparative (C)-reverse transcriptase polymerase chain reaction (**CRT-PCR**) (Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2004).

2.3.3.2. Macrolide resistance

For detecting the macrolide resistance mechanisms in *Campylobacter* spp. several methods are available. DNA **sequencing** still remains the 'golden standard' for the

identification of mutations causing macrolide resistance. During the last years, it has become a much easier protocol after automated procedures (Gibreel and Taylor 2006). New PCR-based methods have been developed and there are several methods described for *Campylobacter* species.

PCR and line probe assay (**PCR-LiPA**) was developed to detect the mutations associated with 23S rRNA of *C. jejuni* and *C. coli* (Niwa, Chuma et al. 2001). Based on the same method, a few years later a macrolide and quinolone line probe assay (**MQ-LiPA**) was developed for simultaneus observation of both a mutation in gyrA causing fluoroquinolone resistance and mutations in 23S rRNA causing macrolide resistance (Niwa, Chuma et al. 2003). Vacher et al. have reported a combined PCR-restriction fragment length polymorphism technique (**RFLP**) for detecting mutations in 23S rRNA by using the BsaI and BceAI enzymes (Vacher, Menard et al. 2003). A real-time fluorescence resonance energy transfer PCR **(FRET-PCR)** assay using a melting curve analysis in the 23S rRNA gene of *C. coli* and *C. jejuni* assay is reported to be more sensitive and more rapid than the other PCR assays since the entire procedure takes less than two hours (Vacher, Menard et al. 2005). Also a combined mismatch amplification mutation assay-PCR **(MAMA)** technique has been developed to detect mutations in the 23S rRNA gene (Alonso, Mateo et al. 2005). According to Haas et al. a fluorescence in situ hybridization **(FISH**) is a rapid and reliable detection method for mutation causing macrolide resistance in thermotolerant *Campylobacter* (Haas, Essig et al. 2008). **TaqMan probe-based real-time polymerase chain reaction** method presented by Hao et al. is rapid, sensitive, and accurate for analyzing mutated alleles in 23S rDNA associated with high-level macrolide resistance in *Campylobacter* spp. (Hao, Dai et al. 2009).

Pyrosequencing is a reliable method for the detection of 23S rRNA mutations and the number of the mutated alleles (Haanperä, Huovinen et al. 2005; Ren, Wang et al. 2011). In pyrosequencing, a sequencing primer is annealed to a single-stranded PCR product. When nucleotides incorporate by DNA polymerase, pyrophosphate is released and further processed by sulfurylase and luciferase. This reaction produces light in proportion to the amount of pyrophosphate. The light can be detected and presented as a pyrogram. From the pyrogram the peak heights can be measured, since the peaks are proportional to the number of nucleotides incorporated and the amino acid sequence detected.

Mutations in ribosomal proteins L4 and L22 have been analyzed by sequencing (Corcoran, Quinn et al. 2006; Caldwell, Wang et al. 2008; Ladely, Meinersmann et al. 2009).

3. AIMS OF THE STUDY

The purpose of the present study was to examine the antimicrobial susceptibility of *C. jejuni* and *C. coli* in Finland, to analyze the mechanisms behind the *Campylobacter* macrolide resistance, and to evaluate currently employed susceptibility testing methods for campylobacters.

The specific aims were:

1) To study the fluoroquinolone and macrolide resistance in *Campylobacter* species in Finland **(I, II, III)**

2) To analyze multidrug resistance in *Campylobacter* species and to identify agents potentially effective towards the multidrug-resistant *Campylobacter* strains **(I, III)**

3) To analyze the macrolide resistance causing mutations in *Campylobacter* species **(IV)**

4) To evaluate the use of the disk diffusion method in the antimicrobial susceptibility testing of *Campylobacter* species **(V)**

4. MATERIALS AND METHODS

4.1. Bacterial isolates (I-V)

Bacterial isolates that were used in the **Studies I-V** are shown in Table 7.

Table 7. Bacterial isolates used in the studies I to V.

Study	Species	Number of isolates	Isolation period	Origin
I	*C. jejuni*	376	1995 – 2000	354 of foreign origin 22 of domestic origin
II	*C. jejuni*	226	1995 – 2000	226 of foreign origin
III	220 *C. jejuni*, 18 *C. coli*	238	2003 – 2005	122 of foreign origin 92 of domestic origin 24 of unknown origin
IV	55 *C. jejuni*, 21 *C. coli*	76	2003 – 2008	38 of foreign origin 12 of domestic origin 26 of unknown origin
V	151 *C. jejuni*, 23 *C. coli*	174	2003 – 2008	79 of foreign origin 55 of domestic origin 40 of unknown origin

Study I. A total of 376 *C. jejuni* strains were included in the study. The strains were isolated in the laboratory of a private hospital in Helsinki, Finland, over two distinct time periods between 1995 and 2000. Between January 1995 and November 1997 a total of 216 consecutive strains, and between October 1998 and January 2000 a total of 160 consecutive strains were isolated.

Study II. A total of 226 *C. jejuni* strains were included in the study. All of the strains that grew after freezing and storage at -70 ºC from the **Study I** strains were included in this study. The strains of the study collection were collected between January 1995 and November 1997 (123 strains) and between October 1998 and January 2000 (103 strains).

Study III. The initial study collection included a total of 1808 *Campylobacter* strains isolated between 2003 and 2005 in the clinical microbiology laboratories of ten hospital districts in different parts of Finland and send to the Bacteriology unit of the National Public Health Institute (former Enteric Bacteria Laboratory of KTL). Laboratories send all of their Campylobacters strains forward during the collection period. In addition to the specimen, information on patient's travel history and date of the specimen were also send in a specific form. All isolates were screened for erythromycin susceptibility using an erythromycin disk (content 15 µg, BBL, Beckton Dickinson, Sparks, MD, USA) with the aim to distinguish the evidently macrolide-susceptible population

from the population that contained the macrolide-resistant strains. An inhibition zone diameter >23 mm around the erythromycin disk was chosen to indicate macrolide susceptibility, and all strains having a zone diameter ≤23 mm were included in this study. A total of 183 isolates exhibiting inhibition zone diameters ≤23 mm and 55 randomly selected *Campylobacter* species isolates exhibiting zone diameters >23 mm were included in the final study collection of 238 strains. Of these strains, 122 were of foreign origin, 92 were of domestic origin, and the origin was unknown for 24 strains. 220 strains were identified as *C. jejuni* and 18 strains were identified as *C. coli*. Of the *C. coli* strains, four strains were domestic, while 88 of the *C. jejuni* strains were domestic.

Study IV. The study collection consisted of 76 *Campylobacter* strains isolated between 2003 and 2005. Of these strains, 53 were selected from the **Study III** so that 33 erythromycin-resistant strains in that study and 20 of erythromycin-susceptible strains to serve as a control group were included. In addition, 23 strains collected locally in the area of the Turku University Hospital were included. The total number of erythromycin-resistant strains was 33.

Study V. A total of 174 *Campylobacter* strains were included consisting of 33 erythromycin-resistant strains used in the **Study IV** and a number of erythromycin-susceptible strains (141) collected during **Study III**.

4.2. Isolation and species determination (I-V)

Studies I-V. All *Campylobacter* strains were isolated from clinical human fecal samples from Finnish patients. The cultivation of stool samples and preliminary identification of the isolates were carried out by standard microbiological methods. The strains isolated from patients traveling abroad within two weeks preceding their symptoms were classified as foreign strains; all other strains were classified as domestic strains. The information concerning traveling history was obtained by special questionnaire send to the patients or given by the referring laboratory when delivering sample. Therefore there can be inaccuracy of determining the origin of the strain in some cases.

Studies I-II. The hippurate hydrolysis test was used for species determination. All hippurate-positive strains were classified as *C. jejuni.*

Study III, IV and V. Hippurate hydrolysis was tested for all strains and hippurate-positive strains were classified as *C. jejuni*. At the Bacteriology unit, National Institute for Health and Welfare, Helsinki, Finland former Enteric Bacteria Laboratory of KTL), all hippurate-negative isolates were confirmed by PCR as either *C. jejuni* or *C. coli* (Nakari, Puhakka et al. 2008).

4.3. The antimicrobial susceptibility testing (I-V)

4.3.1. Standard agar plate dilution method (I-III, V)

The MICs for the *Campylobacter* strains were determined according to the CLSI guidelines (CLSI 2009) by using the standard agar plate dilution method. A bacterial suspension equivalent with the turbidity of 0.5 McFarland standard was made, and 1 µl was transferred onto antimicrobial plates with a Denley Multipoint Inoculator (Denley Instruments Ltd., West Sussex, UK). The plates contained a series of doubling dilutions of each antimicrobial agent. Mueller-Hinton II agar (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) with 5% defibrinated sheep blood was used as a culture medium. The plates were incubated at 35 ± 1°C for 48 h in a microaerobic atmosphere (CampyPak, BBL). *C. jejuni* DSM 4688 (same as ATCC 33560 and NCTC 11351) was used as a control in susceptibility testing and also as a growth control strain. In addition, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 35218 were used as controls in susceptibility testing.

In **Study I**, susceptibilities were determined for ciprofloxacin (Bayer, Wuppertal, Germany); ampicillin, erythromycin, cefotaxime, clindamycin, co-amoxiclav, nalidixic acid, tetracycline, gentamicin and chloramphenicol (Sigma, Steinheim, Germany); azithromycin (Fluka, Buchs, Switzerland); and imipenem (MSD, United Kingdom).

In **Study II**, susceptibilities were determined for ciprofloxacin, moxifloxacin (Bayer, Wuppertal, Germany); clinafloxacin (Pfizer, Ann Arbor, MI, USA); enrofloxacin (Bayer, Elberfeld, Germany); gatifloxacin (Grunenthal BMBH, Aachen, Germany); gemifloxacin (GlaxoSmithKline, Worthing, United Kingdom), levofloxacin (Hoechst Marion Roussel, Romainville, France), lomefloxacin (Sigma, St. Luis, MO, USA); norfloxacin, ofloxacin (Sigma, Steinheim, Germany); and sitafloxacin (Daiichi Pharmaceutical, Tokyo, Japan).

In **Study III**, susceptibilities were determined for ampicillin, chloramphenicol, clarithromycin, clindamycin, co-amoxiclav, erythromycin, gentamicin, nalidixic acid, norfloxacin, ofloxacin, and tetracycline (Sigma, Steinheim, Germany); azithromycin, ciprofloxacin and levofloxacin (Fluka, Buchs, Switzerland); imipenem (MSD, United Kingdom); meropenem (AstraZeneca, Espoo, Finland); moxifloxacin, Bayer (Wuppertal, Germany); sitafloxacin (Daiichi Pharmaceutical, Tokyo, Japan); and telithromycin (Aventis Pharma, France).

4.3.2. MIC-breakpoints in the agar dilution method (I-V)

MIC breakpoints are MIC values, which divide bacteria into categories resistant, intermediate or susceptible. The CLSI has limited information and criteria for broth microdilution and disk diffusion susceptibility testing of campylobacters. The MIC breakpoints used to define resistance in the agar dilution method are therefore taken from different sources. In Table 8 the breakpoints used to define resistance in this thesis are presented.

Table 8. Breakpoints used in this thesis to define resistance.

Antimicrobial agent	Resistance breakpoints (μg/ml)	Source
Chloramphenicol	≥ 32	CLSI[a]
Ciprofloxacin	≥ 4	CLSI[a]
Cefotaxime	≥ 64	CLSI[a]
Gentamicin	≥ 16	CLSI[a]
Imipenem	≥ 16	CLSI[a]
Tetracycline	≥ 16	CLSI[a]
Levofloxacin	≥ 8	CLSI[a]
Meropenem	≥ 16	CLSI[a]
Norfloxacin	≥ 16	CLSI[a]
Ofloxacin	≥ 8	CLSI[a]
Ampicillin	≥ 32	CLSI[b]
Co-amoxiclav	≥ 32	CLSI[b]
Nalidixic acid	≥ 32	CLSI[b]
Azithromycin	≥ 4	(Kuschner, Trofa et al. 1995)[c]
Erythromycin	≥ 16	(Rautelin, Renkonen et al. 1991)[c]
Clindamycin	≥ 8	(Sjögren, Kaijser et al. 1992)[c]
Tigecycline	> 0.5	EUCAST[d]

[a] CLSI 2009 for non-*Enterobacteriaceae*
[b] CLSI 2009 for *Enterobacteriaceae*
[c]Resistance breakpoints were chosen based on earlier publications and histogram analyses in Study I.
[d]Tigecycline susceptibilities were determined by E-test, http://www.eucast.org/

4.3.3. The E-test method (III, V)

The MICs of tigecycline were determined by the E-test (Biodisk AB, Solna, Sweden) according to the manufacturer's instructions. In brief, after culturing the isolates by standard microbiological methods, inocula, prepared in NaCl at a density adjusted to a 1.0 McFarland turbidity standard, were delivered onto 5% sheep blood Mueller-Hinton agar plates. An E-test strip with a tigecycline concentration range from 0.016 to 256 μg/ml was applied onto each plate. The plates were incubated at 35°C for 48 h in a microaerobic atmosphere generated using special sachets (CampyGen, BBL). The MIC value was read at the point of intersection between the growth zone edge and the E-test strip. *C. jejuni* DSM 4688 was used as a control in susceptibility testing and also as a growth control strain. In addition, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 35218 were used as controls in susceptibility testing. The results were interpreted using the non-species related EUCAST (http://www.eucast.org/) breakpoints for susceptibility (MIC ≤0.25 μg/ml) and resistance (MIC >0.5 μg/ml).

4.3.4. Disk diffusion method (V)

The following antimicrobial disks were used: clarithromycin 15 μg, clindamycin 2 μg, erythromycin 15 μg, nalidixic acid 30 μg, spiramycin 100 μg, tetracycline 30 μg, azithromycin 15 μg, ciprofloxacin 5 μg, telithromycin 15 μg, Oxoid (UK), and tigecycline 15 μg (delivered by Wyeth, Vantaa, Finland). The disk diffusion tests

were made as follows: Inoculum prepared in sterile NaCl at a density adjusted to a 0.5 McFarland turbidity standard were spread onto 5% sheep blood Mueller-Hinton agar plates filled with an amount of agar giving a uniform depth of 4 ± 0.5 mm. Disks were added, and the plates incubated in a microaerobic atmosphere at 35 ± 1 °C for 48 h. A maximum of three disks at the same time were applied onto one agar plate to ensure the readability of even large inhibition zones. Tigecycline was always placed alone onto one agar plate. Therefore, four plates were made for each strain for the testing of ten different antimicrobials from the same inoculum at one measurement time. The inhibition zone was measured after incubation by hand and by the same reader. All disk diffusion results were determined several times according to the same instructions for each strain; three to four times for erythromycin, and for the other antimicrobials two to four times. *C. jejuni* DSM 4688 and *C. coli* DSM 4689 (same as ATCC 33559 and NCTC 11366) were used as controls in susceptibility testing.

4.4. Detection of macrolide resistance mechanisms (IV)

4.4.1. Primers

The primers used for the PCR and sequencing of the 23S rRNA mutations and ribosomal protein L4 and L22 modifications are presented in Table 9. All primers were produced by Thermo Fisher Scientific (Vantaa, Finland).

Table 9. Primers used in PCR and sequencing of the 23S rRNA gene and the ribosomal proteins L4 and L22 (Study IV).

Target molecule	Use	Sequence (5' - 3')	Reference
23S rRNA	PCR	TAAGGTAGCGAAATTCCTTGTCG	(Haanperä, Huovinen et al. 2005)
23S rRNA	PCR	CGACCGCCCCAGTCAAACT[a]	(Haanperä, Huovinen et al. 2005)
23S rRNA	Pyrosequencing	CCGCGGCAAGACGG	(Haanperä, Huovinen et al. 2005)
L4	PCR / Sequencing (for)	GTAGTTAAAGGTGCAGTACCA	Study IV
L4	PCR / Sequencing (rev)	GCGAAGTTTGAATAACTACG	Study IV
L22	PCR / Sequencing (for)	GAATTTGCTCCAACACGC	Study IV
L22	PCR / Sequencing (rev)	ACCATCTTGATTCCCAGTTTC	Study IV

[a]This primer was biotinylated.

4.4.2. Pyrosequencing of the 23S rRNA mutations

Mutations at the positions 2058 and 2059 (*E. coli* numbering) of the 23S rRNA gene were analyzed by pyrosequencing. These mutations were screened in all of our erythromycin-resistant (erythromycin MIC ≥ 16 µg/ml) *Campylobacter* strains and, in addition, in 42 erythromycin-susceptible (erythromycin MIC < 16 µg/ml) *Campylobacter* strains. Pyrosequencing was performed using the primers (Table 9)

and protocols previously described by Haanperä et al. (Haanperä, Huovinen et al. 2005). In brief, *Campylobacter* isolates were cultured for 48 h in a microaerobic atmosphere. Bacterial suspensions were made in sterile water and lyzed by heating at 95° for 10 min. The PCR was done as previously described (Haanperä, Huovinen et al. 2005). The PSQ 96MA pyrosequencer (www.pyrosequencing.com; Qiagen, Hilden, Germany) was used for pyrosequencing, and the reactions were performed according to the manufacturer's instructions. *Streptococcus pyogenes* N1 4277 was used as a positive control in pyrosequencing.

4.4.3. Detection of modifications of the ribosomal proteins L4 and L22

50S ribosomal proteins L4 and L22 modifications might affect erythromycin resistance in *Campylobacter* species. The L4 and L22 gene sequences of all 33 erythromycin-resistant and 20 erythromycin-susceptible *Campylobacter* strains were determined. The primers used in PCR and sequencing are listed in Table 9. *Campylobacter* isolates were cultured for 48 h in a microaerobic atmosphere. Bacterial suspensions were made in sterile water and lyzed by heating at 95° for 10 min. The suspensions were further diluted 1 to 10 in sterile water and 5 µl was used as a template in PCR. The PCR mixture (50 µl) contained 0.2 µM of primers, 0.04 U/µl of AmpliTaqGold DNA polymerase, 1x buffer, 2 mM $MgCl_2$ (Applied Biosystems Inc, Foster City, CA, USA) and 0.2 mM nucleotides (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The reactions were performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) using the following cycling conditions: 94 °C for 10 min followed by 38 amplification cycles (30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C). The PCR products were purified using the High Pure PCR Purification Kit (Roche Applied Science, Basel, Switzerland). Sequencing reactions were prepared by ABI BigDye™ Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) and sequencing was performed with an Applied Biosystems 3730 DNA Analyser. *C. jejuni* DSM 4688 type strain (erythromycin-susceptible, wild-type ribosomal molecule) and *C. coli* DSM 4689 type strain (erythromycin-susceptible, wild-type ribosomal molecule) were used as control strains for susceptibility testing and as reference strains for ribosomal molecules.

4.4.4. Measurement of efflux pump activity

The efflux activity on erythromycin MICs was evaluated in 32 *Campylobacter* patient isolated strains and the control strains *C. jejuni* DSM 4688 and *C. coli* DSM 4689. The strains were selected to represent all the MIC classes. The MICs of the selected strains were determined as described above, except that the MICs of highly resistant strains were tested up to 512 µg/ml, in the presence or absence of the efflux pump inhibitors phenyl-arginyl-β-naphtylamide (PAβN, Sigma) or 1-(1-naphtylmethyl)-piperazine (NMP, Chess, Mannheim, Germany) at concentrations of 50 µg/ml and 100 µg/ml, respectively (12).

4.5. Data analysis (I-V)

4.5.1. Data analysis of susceptibility results (I-V)

The WHONET 5.4 computer program was used in the analysis of the susceptibility data (**I-III, V**). The WHONET is a software, which can be downloaded free of charge from the website http://www.whonet.org. It was developed by Thomas O'Brien and John Stelling at the WHO collaborating centre for the surveillance of antibiotic resistance (O'Brien TF and Stelling JM 1995).

4.5.2. Statistical analysis (III, V)

In **Study III**, comparisons of the susceptibility data between the erythromycin-susceptible and erythromycin-resistant *Campylobacter* strains as well as between the *C. jejuni* and *C. coli* strains were performed using Fisher's exact test. *P* values of less than 0.05 were considered significant.

In **Study V**, statistical analyzes were made using SPSS 17.0 and SAS for Windows 9.1. To describe the variability within the three or four disk diffusion test results, coefficients of variation (%) and maximum differences (mm) between the tests were determined for all strains. In the susceptible strains, repeated measures analysis of variance was used to compare the repetitions. Due to the very skewed distributions in of the resistant strains, pair-wise differences between the repetitions were tested by nonparametric Wilcoxon Rank Sum test. The Bonferroni method was used to adjust for the multiple comparisons. *P* values of less than 0.05 were considered significant.

4.5.3. Sequence analysis (IV)

The Vector NTI program (Invitrogen, Carlsbad, CA, USA) and bioEdit Sequence alignment Editor programs (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) were used for assembling, editing and analyzing DNA and protein sequences.

5. RESULTS

5.1. Antimicrobial susceptibility testing results by agar dilution (I-III)

5.1.1. Fluoroquinolone resistance (I-III)

During the first collection period, 1995- 2000, resistance to ciprofloxacin was observed in 46% (174/376) of the *Campylobacter* strains **(Study I)**. All of the strains collected during this period were *C. jejuni*, and 94% (354/376) of them were of foreign origin. Of the foreign strains, 172 (49%) were ciprofloxacin-resistant, compared with only two (9%) of the 22 domestic strains being resistant to ciprofloxacin ($P < 0.01$).

During the second collection period, 2003-2005, 45% (107/238) of the *Campylobacter* strains studied were ciprofloxacin-resistant (Table 10) (**Study III**). Included were both *C. jejuni* (n=220) and *C. coli* (n=18) strains. Four (22.2%) *C. coli* strains and 88 (40%) *C. jejuni* strains were domestic. The MIC_{50} and MIC_{90} values of ciprofloxacin were 0.5 and 32 µg/ml for all 238 *Campylobacter* strains. Of the 122 foreign strains, 71% (87/122) were resistant to ciprofloxacin, compared with 12% (11/92) of the domestic strains. Ciprofloxacin resistance was observed in 42% (92/220) and 83% (15/18) of the *C. jejuni* and *C. coli* strains, respectively.

The activities of various older and newer fluoroquinolones towards a subset of 226 *C. jejuni* strains, collected during 1995-2000, were evaluated **(Study II)**. Of these strains, 59.3% (134/226) were resistant to ciprofloxacin (MIC ≥ 4 µg/ml), with the MIC_{50} and MIC_{90} values 16 and 64 µg/ml, respectively. The corresponding MIC_{50} and MIC_{90} values were 4 and 32 µg/ml for levofloxacin, and 2 and 16 µg/ml for moxifloxacin, being lower than those of norfloxacin and ofloxacin. Sitafloxacin and clinafloxacin exhibited the lowest MIC_{50} and MIC_{90} values for all *C. jejuni* strains: 0.125 and 1 µg/ml for sitafloxacin and 0.5 and 2 µg/ml for clinafloxacin, respectively.

The MIC_{50} and MIC_{90} values for ciprofloxacin for the 91 ciprofloxacin-susceptible *C. jejuni* strains were 0.25 and 0.5 µg/ml, respectively (Table 11). The corresponding MIC_{50} and MIC_{90} values for levofloxacin were 0.25 and 0.5 µg/ml, and those for moxifloxacin 0.125 and 0.25 µg/ml. The MIC_{50} and MIC_{90} values for sitafloxacin and clinafloxacin for the ciprofloxacin-susceptible *C. jejuni* strains were 0.016 and 0.064 µg/ml, and 0.032 and 0.125 µg/ml, respectively.

For the 134 ciprofloxacin-resistant *C. jejuni* strains, the MIC_{50} and MIC_{90} values were 16 and 32 µg/ml for levofloxacin and 4 and 16 µg/ml for moxifloxacin (Table 10). Sitafloxacin and clinafloxacin exhibited the lowest MIC_{50} and MIC_{90} values for these ciprofloxacin-resistant *C. jejuni* strains: 0.25 and 1 µg/ml for sitafloxacin and 1 and 4 µg/ml for clinafloxacin, respectively.

Table 10. The antimicrobial susceptibilities of the *Campylobacter jejuni* and *C. coli* strains **(Study I, III),** and comparison of antimicrobial susceptibility between *C. jejuni* and *C. coli* strains **(Study III)**.

Antimicrobial agent	Resistance breakpoint (µg/ml)	Study I (1995-2000)	Study III[a] (2003-2005)		
		C. jejuni (n=376) Number (%) of resistant strains	*C. jejuni* (n=220) Number (%) of resistant strains	*C. coli* (n=20) Number (%) of resistant strains	*P* value (*C. jejuni* vs. *C. coli*)
Erythromycin	16	8 (2.1)	10 (4.5)	9(50)	<0.001
Azithromycin	4	6 (1.6)	11 (5)	7 (38.9)	<0.001
Clindamycin	8	8 (2.1)	11 (5)	9 (50)	<0.001
Nalidixic acid	32	176 (46.8)	91 (41.4)	15 (83.3)	<0.001
Ciprofloxacin	4	100 (46.3)	92 (42.4)	15 (83.3)	<0.001
Ampicillin	32	63 (16.8)	40 (18.2)	4 (22.2)	0.751
Co-amoxiclav[b]	32	1 (0.3)	42 (19.1)	0	0.757
Chloramphenicol	32	10 (2.7)	1 (0.5)	0	1.000
Imipenem	16	0	0	0	not applicable
Meropenem	16	ND	0	0	not applicable
Gentamicin	16	6 (1.6)	2 (0.9)	12 (66.7)	1.000
Tetracycline	16	99 (46.0)	61 (28.1)	0	0.002
Tigecycline	0.5	ND	0	0	not applicable

[a]For ciprofloxacin and tetracycline, data available for 216 strains. For tigecycline, data available for 211 strains. For meropenem, data were available for 189 strains. For imipenem data were available for 118 strains.
[b]Values indicate the concentration of amoxicillin. Amoxicillin and clavulanic acid were used in a 2:1 (w/w) ratio.

Table 11. The MICs of 11 fluoroquinolones for 226 *Campylobacter jejuni* strains collected from Finnish patients between 1995 and 2000[a].

	Ciprofloxacin-resistant strains[b] (n=134) MIC (µg/ml)			Ciprofloxacin-susceptible strains[c] (n=91) MIC (µg/ml)		
	Number of strains	MIC_{50}	MIC_{90}	Number of strains	MIC_{50}	MIC_{90}
Ciprofloxacin	134	32	128	91	0.25	0.5
Clinafloxacin	134	1	4	91	0.032	0.125
Gatifloxacin	133	4	16	91	0.125	0.5
Gemifloxacin	134	64	64	91	0.5	1
Levofloxacin	134	16	32	91	0.25	0.5
Lomefloxacin	134	>64	>64	91	1	2
Moxifloxacin	134	4	16	91	0.125	0.25
Norfloxacin	132	64	>64	89	1	4
Ofloxacin	133	16	64	91	0.5	1
Sitafloxacin	134	0.25	1	91	0.016	0.064

[a]One strain with a ciprofloxacin MIC of 2 µg/ml (intermediate) was included into the total amount of strains, but not presented in the table.
[b]MIC ≥ 4 µg/ml.
[c]MIC ≤ 1 µg/ml.

Scattergrams correlating the MICs of ciprofloxacin to those of norfloxacin, ofloxacin, levofloxacin, moxifloxacin, clinafloxacin, and sitafloxacin for the 226 *C. jejuni* strains are presented in **Study II,** and those of sitafloxacin and moxifloxacin here in Figure 5. For the ciprofloxacin-resistant strains, the MIC values of norfloxacin were similar to or higher than those of ciprofloxacin; and the MIC values of ofloxacin similar to, or one dilution step lower, than those of ciprofloxacin. For levofloxacin and moxifloxacin, the MIC values of ciprofloxacin-resistant strains were one to three dilution steps lower than those of ciprofloxacin. The MIC values of clinafloxacin were four dilutions steps lower than those of ciprofloxacin and the MIC values of sitafloxacin five to seven dilution steps lower than those of ciprofloxacin.

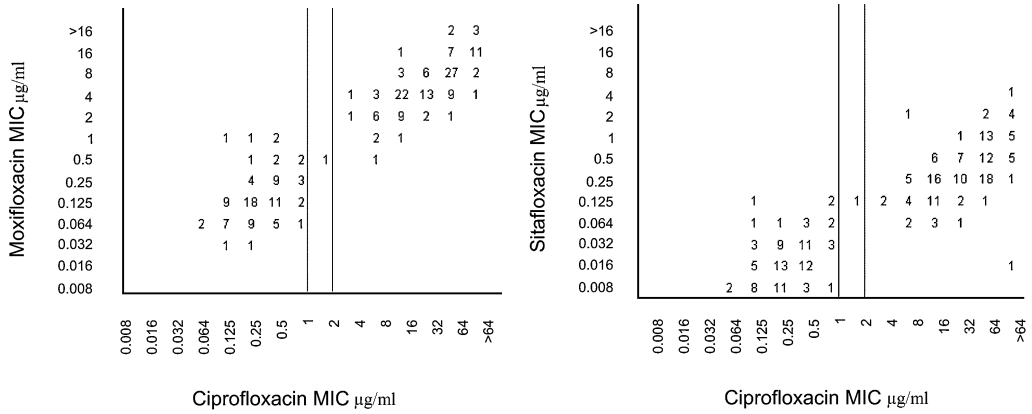

Figure 5. Scattergrams of the 226 *C. jejuni* strains correlating the MICs of ciprofloxacin to those of moxifloxacin and sitafloxacin **(Study II)**.

5.1.2. Macrolide resistance (I, III)

Resistance to the macrolides was rare: during the period 1995-2000, 2% (8/376) of the *Campylobacter* strains were resistant to erythromycin (**Study I**). Similarly, only 2% (6/376) of the strains were resistant to azithromycin or clindamycin.

During the period 2003-2005, 19 *Campylobacter* strains were confirmed to be erythromycin-resistant based on MIC determinations among the initial study collection of 1808 isolates (**Study III**). Based on these data, the frequency of erythromycin resistance of the *Campylobacter* strains during that period was 1.1%. The MIC_{50} and MIC_{90} of erythromycin for the 19 erythromycin-resistant strains were >128 µg/ml. The MIC_{50} and MIC_{90} values were 32 and >128 µg/ml for telithromycin, and 64 and 64 µg/ml for clindamycin, these being lower than those for azithromycin and clarithromycin. Among the 238 *Campylobacter* strains, the rates of azithromycin and clindamycin resistance were 8% and 8%, respectively. Of the 19 erythromycin-resistant strains, 17 were foreign, one was domestic and for one strain, the origin was unknown. Ten resistant strains were identified as *C. jejuni* and nine strains were identified as *C. coli*. Erythromycin resistance was significantly more common among *C. coli* strains than among *C. jejuni* strains ($P < 0.01$) (Table 10).

From the initial study collection of 1808 isolates, 262 were examined with the aim to establish the adequacy of the erythromycin screening system. Included were all 202 isolates exhibiting inhibition zone diameters ≤23 mm in erythromycin screening and 60 randomly selected isolates with erythromycin zone diameters >23 mm, which served as erythromycin-susceptible control strains. Of these 262 isolates, 24 did not grow after freezing and storage, resulting in a final collection of 238 *Campylobacter* spp. strains. Of the 183 *Campylobacter* spp. strains with inhibition zone diameters ≤23 mm in the erythromycin disk screening, 19 were classified by the agar plate dilution method as erythromycin-resistant (MIC ≥16 μg/ml) and 164 as erythromycin-susceptible (MIC <16 μg/ml). All of the 55 strains with inhibition zone diameters >23 mm were classified by the agar plate dilution method as erythromycin-susceptible. Thus, the final study collection consisted of 219 erythromycin-susceptible and 19 erythromycin-resistant *Campylobacter* strains. The finding that all 19 strains classified as erythromycin-resistant based on MIC determination had erythromycin inhibition zone diameters ≤20 mm, while all strains exhibiting zone diameters ≥21 mm were classified by MIC determination as erythromycin-susceptible confirmed the fitness of the initial screening system.

5.1.3. Susceptibilities of other antimicrobial agents (I, III)

Testing of the susceptibilities of the strains to several other antimicrobial groups showed that among the 376 *C. jejuni* strains collected during 1995-2000, resistance to tetracycline and ampicillin were most common being 46% and 17%, respectively (**Study I**). Resistance to gentamycin was observed in 2% of the strains studied, and one (0.3%) isolate was resistant to co-amoxiclav. Of these *C. jejuni* strains, 2% were resistant to cefotaxime, but the proportion of intermediately cefotaxime-resistant strains was 40%. No resistance to imipenem was observed.

During the period 2003-2005, resistance to tetracycline, ampicillin, and gentamycin was observed in 34%, 19%, and 0.8% of the C. *jejuni* and C. *coli* strains studied, respectively (**Study III**). The resistance to co-amoxiclav was 19%. No resistance to imipenem was observed. Tigecycline exhibited the lowest MIC values against the studied *C. jejuni* and *C. coli* strains, with all of the strains susceptible.

When the antimicrobial susceptibilities were analyzed separately for the ciprofloxacin-susceptible and ciprofloxacin-resistant strains, 68% and 25% of the 174 ciprofloxacin-resistant *C. jejuni* strains collected between 1995-2000 were resistant to tetracycline and ampicillin, respectively (**Study I**). When compared to the ciprofloxacin-susceptible strains, resistance to tetracycline and ampicillin were clearly lower being 27% and 9%, respectively ($P < 0.01$). Resistance to erythromycin, clindamycin, gentamycin, or cefotaxime was observed in 3% of the ciprofloxacin-resistant strains. However, the proportion of intermediately cefotaxime-resistant isolates was up to 48%. One (0.6%) strain was resistant to co-amoxiclav and none were resistant to imipenem.

Among the 107 ciprofloxacin-resistant *C. jejuni* and *C. coli* strains collected 2003-2005, resistance to tetracycline was most common, rising to 63% (67/107). Of these ciprofloxacin-resistant strains, 17% (18/107) were resistant also to erythromycin and clindamycin, and 16% (17/107) were resistant to azithromycin. Resistance to co-amoxiclav and ampicillin were observed in 32% (34/107) and 30% (32/107) of the strains, respectively. Of the 130

ciprofloxacin-susceptible strains, one strain was resistant to erythromycin. Resistance to clindamycin was observed in 1.5% (2/130), and resistance to azithromycin in 0.8% (1/130) of the strains. Resistance to co-amoxiclav and ampicillin were 8.5% (11/130) for both of these antimicrobial agents. Of the ciprofloxacin-susceptible strains, only 4.6 % (6/130) were resistant to tetracycline.

Analyzing the antimicrobial susceptibilities separately for the erythromycin-resistant and erythromycin–susceptible *C. jejuni* and *C. coli* strains collected between 2003-2005 showed that for the 19 erythromycin-resistant strains, the MIC_{50} and MIC_{90} values were 16 and > 32 µg/ml for ciprofloxacin, 4 and 64 µg/ml for co-amoxiclav, and 8 and 128 µg/ml for ampicillin, respectively (**Study III**). Imipenem, meropenem, gentamycin, sitafloxacin, and tigecycline exhibited the lowest MIC_{50} and MIC_{90} values. Determined by E-test, the MIC_{50} and MIC_{90} values of tigecycline were 0.008 and 0.023 µg/ml for the erythromycin-resistant strains, and 0.008 and 0.032 µg/ml for the erythromycin-susceptible strains, respectively.

5.1.4. Multidrug-resistance (I, III)

In this thesis MDR was defined as resistance to three or more antimicrobial groups. The groups were as follows: (i) quinolones, (ii) macrolides, telithromycin and clindamycin, (iii) tetracycline and tigecycline, (iv) β-lactams, (v) gentamycin, and (vi) chloramphenicol.

Among the 376 *C. jejuni* strains collected during 1995-2000, multidrug-resistance was detected in 22% (**Study I**). Because of the high number of intermediately cefotaxime-resistant isolates, cefotaxime was excluded from the multidrug-resistance profile analysis. While 33% of the ciprofloxacin-resistant isolates had three or more additional resistance properties, only 12% of the susceptible isolates were resistant to three or more antimicrobial agents ($P < 0.01$). All of the eight erythromycin-resistant *C. jejuni* isolates were multidrug-resistant, 75% of them being resistant also to ciprofloxacin. None of these strains were resistant to co-amoxiclav or imipenem.

Of all 238 *Campylobacter* strains collected during 2003-2005, 16.8% (40/238) were multiresistant. Of the 107 ciprofloxacin-resistant strains, 32.7% (35) were multidrug-resistant. All of the 19 erythromycin-resistant strains were multidrug-resistant, 94.7% (18) of them being resistant also to ciprofloxacin. As compared to the erythromycin-susceptible *C. jejuni* and *C. coli* strains, the erythromycin-resistant strains were significantly more often resistant to several antimicrobial agents (Table 10).

5.2. Macrolide resistance mechanisms (IV)

5.2.1. Mutations at the 23S rRNA

A point mutation A to G at the position 2059 of the 23S rRNA gene was detected by pyroseqencing in 93.9% (31/33) of the erythromycin-resistant *Campylobacter* strains (Table 13). Of the highly erythromycin-resistant (MIC ≥ 128 µg/ml) *Campylobacter* strains 96.9% (31/32) had this same mutation (Table 11). Based on the pyrosequencing results, 93.5% (29/31) of the mutated strains had all three alleles of the 23S rRNA gene

mutated. The remaining two strains (one *C. jejuni* and one *C. coli*) had two mutated 23S rRNA copies, and both of these strains had erythromycin MICs > 128 μg/ml. There were three erythromycin-resistant strains (two *C. jejuni* and one *C. coli*) with the MICs > 128 μg/ml, 64 μg/ml, and 16 μg/ml, respectively, which had a wild-type 23S rRNA sequence. The A2059G mutation was not observed in any of the erythromycin-susceptible strains. In addition, none of the 33 resistant and none of the 42 erythromycin-susceptible strains had a point mutation at the position 2058 of the 23S rRNA gene.

Table 12. Erythromycin MIC values and ribosomal mutations for 76 *C. jejuni* and *C. coli* strains studied.

Erythromycin MIC value (μg/ml)	Analyzed strains (n)	23S rRNA	
		A2059G[a] (n)	Wild type (n)
≥128	32	31[b]	1
64	1	0	1
16	1	0	1
8	9	0	9
4	8	0	8
2	9	0	9
1	10	0	10
0.5	6	0	6

[a]A to G mutation at the position 2059 (*Escherichia coli* numbering).
[b]Two strains had two copies of the mutated allele, the rest of the strains had three mutated copies of the 23S rRNA gene.

5.2.2. Modifications of the ribosomal proteins L4 and L22

Sequencing of the L4 and L22 ribosomal protein genes was performed for all 34 erythromycin-resistant as well as for 20 erythromycin-susceptible *C. jejuni* and *C. coli* strains. The amino acid sequences of the *C. jejuni* and *C. coli* strains were compared to those of *C. jejuni* DSM 4688 and *C. coli* DSM 4689 reference strains, respectively, and several different amino acid substitutions and their combinations were revealed (Figures 6 and 7). Several amino acid deletions in the ribosomal protein L22 were observed in a number of strains.

As compared to the erythromycin-susceptible *C. jejuni* DSM 4688 reference strain, certain substitutions in the ribosomal protein L22 were present in highly erythromycin-resistant *C. jejuni* strains, in the erythromycin-susceptible *C. coli* DSM 4689 reference strain and in 15 *C. coli* strains with erythromycin MICs between 8 μg/ml and > 128 μg/ml. These substitutions were the following: A74G, E111A, T114A, A130T, A132V, and A141V.

One highly erythromycin-resistant (MIC ≥ 128 μg/ml) *C. jejuni* strain without mutations in the 23S rRNA gene (number 1, Figure 7) had an amino acid insertion between positions 73 and 74 in the ribosomal protein L22. This insertion was not detected in any other strain. Another *C. jejuni* strain, without mutations in the 23S rRNA gene, but with

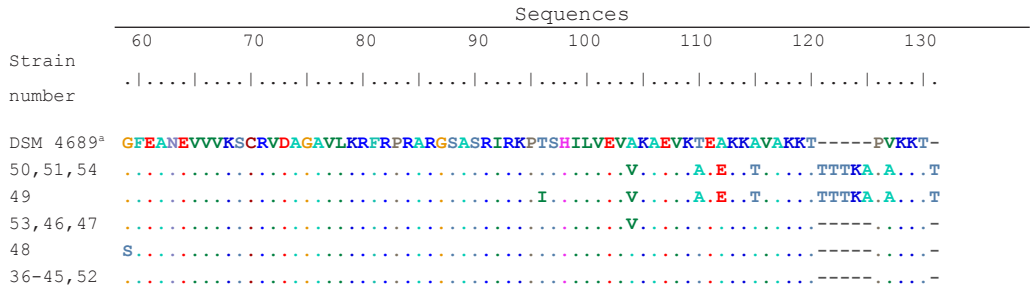

Figure 6. The L22 protein sequences and found mutations of the *Campylobacter coli* strains as compared to the *C. coli* type strain. [a]*C. coli* DSM 4689 type strain. (-) Indicates deleted amino acids. (.) Indicates the same amino acid as in the type strain.

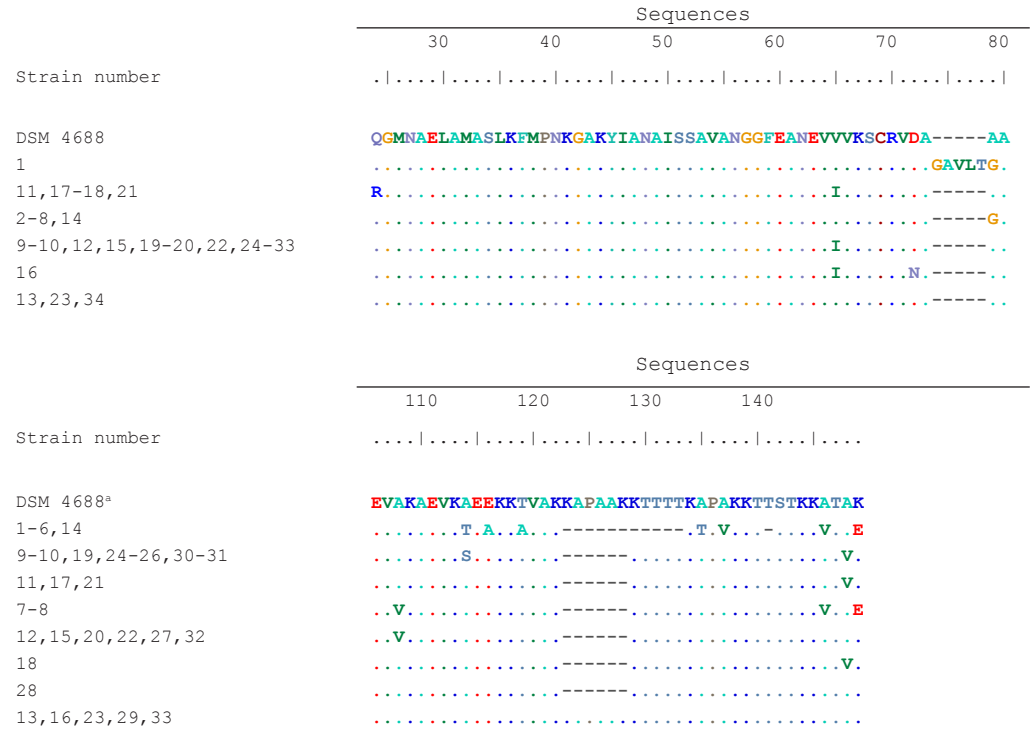

Figure 7. The L22 protein sequences and found mutations of the *Campylobacter jejuni* strains as compared to the *C. jejuni* type strain. [a]*C. jejuni* DSM 4688 type strain. (-) Indicates deleted amino acids. (.) Indicates the same amino acid as in the type strain.

an erythromycin MIC of 64 μg/ml, showed D72N substitution in the ribosomal protein L22. The same substitution was not found in any other strain.

Only a few amino acid substitutions were found in the ribosomal protein L4, and no differences in these substitutions were observed between the erythromycin-resistant and susceptible *Campylobacter* strains. In addition, four strains exhibiting no L4 substitutions were observed.

5.2.3. Efflux pump activity

The effect of efflux inhibitors PAβN and NMP in 32 *Campylobacter* strains was evaluated. The MICs of both susceptible- and highly-resistant *Campylobacter* strains remained similar in the presence or absence of efflux pump inhibitors PAβN and NMP, except for one *C. coli* (Figure 6, strain number 51). This strain exhibited a two-fold decrease in the MIC value (512 μg/ml → 128 μg/ml) when NMP was used to inhibit efflux pumps. PAβN did not change the MIC value.

5.3. Antimicrobial susceptibility testing results by disk diffusion (V)

In this study, the disk diffusion method was compared to the agar dilution method, and resistance and susceptibility were determined according to the agar dilution method. There were 33 erythromycin-resistant (MIC ≥ 16 μg/ml) and 87 ciprofloxacin-resistant (MIC ≥ 4 μg/ml) *Campylobacter* strains determined by the agar dilution. A total of 141 strains were erythromycin-susceptible (MIC < 16 μg/ml), and 87 strains were ciprofloxacin-susceptible (MIC < 4 μg/ml) (Table 12). The MIC determinations were made twice for each strain, with identical susceptibility results.

Table 13. The number of the resistant and susceptible *Campylobacter* strains.

	Number of resistant strains	Number of susceptible strains
Erythromycin	33	141
Azithromycin	31	143
Clindamycin	33	141
Ciprofloxacin	87	87
Nalidixic acid	86	87
Tetracycline	65	107
Tigecycline	0	174

For all but three erythromycin-resistant strains disk diffusion tests were performed four times. For these remaining three strains disk diffusion tests were performed three times. The number of total measurements for the erythromycin-resistant strains was therefore 129 measurements with an erythromycin disk. Inhibition zone variation was between 6 mm (i. e. no inhibition zone) and 44 mm. A total of 13 (10.1 %) measurements were over 6 mm. Of the 33 erythromycin-resistant strains, 24 strains had an erythromycin inhibition zone of 6 mm in disk diffusion in all of the repeated measurements. Seven of the erythromycin-resistant strains with MICs ≥ 128 μg/ml had inhibition zones between 6 and 44 mm, and two of these resistant strains (MICs 16 and 64 μg/ml) had 22-42mm inhibition zones in the repeated measurements. For the 141 erythromycin-susceptible (MIC <16 μg/

ml) isolates, there were a total of 477 measurements performed. For these susceptible strains. the inhibition zone for erythromycin varied between 6 and 61 mm, and ten (2.1 %) measurements of these were equal to or less than 20 mm, and two (0.42%) were 6 mm.

There were 87 ciprofloxacin-resistant (MIC ≥ 4 μg/ml) *Campylobacter* strains. For 57 of these, disk diffusion tests were performed four times. For the remaining 30 strains disk diffusion tests were performed two to three times. The total number of measurements was therefore 316 measurements for the ciprofloxacin disk. Inhibition zone variation was between 6 and 60 mm. Of the ciprofloxacin-resistant strains, 47 (54%) had an inhibition zone of 6 mm in all of the repeated measurements. For 40 (46%) strains, the inhibition zone varied from 6 to 60 mm. In 11 measurements, the inhibition zone was over 10 mm, and for 6 ciprofloxacin-resistant strains (MICs between 4 and 32 μg/ml) inhibition zones were equal to or over 20 mm in all of the repeated measurements. For the 87 ciprofloxacin-susceptible isolates, there were a total of 267 measurements. For these susceptible strains, the inhibition zone for ciprofloxacin varied between 6 and 66 mm, and a 6-mm inhibition zone was observed in nine (3.4%) measurements.

According to the results of the disk diffusion method, the results for azithromycin and nalidixic acid were similar to those of erythromycin and ciprofloxacin, respectively. There were 31 strains resistant to azithromycin (MIC ≥ 4 μg/ml), and inhibition zones varied between 6 and 57 mm. Inhibition zone variation was between 6 and 66 mm for the 143 azithromycin-susceptible strains. For 33 clindamycin-resistant (MIC ≥ 8 μg/ml) and 65 tetracycline-resistant (MIC ≥ 16 μg/ml) strains, inhibition zones varied between 6 and 58 mm and 6 and 62 mm, respectively. For 141 clindamycin-susceptible and 107 tetracycline-susceptible strains, inhibition zone variation was between 6 and 60 mm and 6 and 70 mm, respectively. For nalidixic acid, the inhibition zone variation for 86 resistant strains (MIC ≥ 32 μg/ml) was between 6 and 44mm, and for the 88 susceptible strains between 6 and 56 mm. For tigecycline, all strains were classified as susceptible, and inhibition zone variation was between 6 and 86 mm.

The coefficient of variation (CV) was determined for resistant and susceptible strains for all measurements of six antimicrobial compounds (azithromycin, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, tetracycline). For all antimicrobial agents even large values of variation coefficient were observed in both susceptible and resistant strains. When the different repetition times were evaluated, significant pair-wise differences were observed for all antimicrobials expect for erythromycin- and ciprofloxacin-resistant strains. Concerning the variation in the erythromycin disk diffusion method, there was only small (CV less than 5%) variation for 53 strains and substantial (CV greater than or equal to 15%) variation for 36 strains. For ciprofloxacin, small variation was found in 64 strains and substantial variation in 52 strains. 17 strains showed substantial variation for both erythromycin and ciprofloxacin. For all of the strains susceptible to these antimicrobial agents, the mean of maximum difference between two different measurements was over 8 mm. Even for the resistant strains, mean values of maximum difference of over 4 mm were found for ciprofloxacin, nalidixic acid and tetracycline. The mean values of coefficient of variation for all antimicrobial agents were over 10%. When the different repetitions were evaluated, significant differences were observed for all antimicrobial agents and for all strains except for the macrolide-resistant strains regarding erythromycin and azithromycin.

6. DISCUSSION

6.1. Fluoroquinolone resistance in *Campylobacter* species (I-III)

No ciprofloxacin-resistant *Campylobacter* strains were detected in Finland in the 1980's. In the 1990's, however, Rautelin *et al*. (Rautelin, Renkonen et al. 1991) showed that 9% of the *Campylobacter* strains isolated from Finnish patients were ciprofloxacin-resistant. Although no efforts were made in that study to determine the origin of all ciprofloxacin-resistant *C. jejuni* isolates, the authors speculated that the majority, if not all, of their resistant strains were derived from abroad. The present thesis supports that speculation, since ciprofloxacin resistance was significantly more common among the foreign isolates than among the domestic ones in Finland. Among the foreign *C. jejuni* isolates included in the present study, the resistance rate against the fluoroquinolones was high (49%), which suggests that most international holiday destinations popular among Finns now belong to areas where fluoroquinolone-resistant strains are prevailing. There are similar findings in previous studies, where ciprofloxacin resistance has been significantly more common among the foreign *Campylobacter* strains in Finland (Hakanen, Jousimies-Somer et al. 2003; Schönberg-Norio, Hänninen et al. 2006). Only 9% of the domestic *Campylobacter* strains included in this thesis were resistant to ciprofloxacin. This low rate of ciprofloxacin resistance among the domestic strains is also supported by other papers (Schönberg-Norio, Hänninen et al. 2006). Since at least 80% of the clinical strains in Finland are acquired abroad, fluoroquinolones are of limited usefulness in the treatment of campylobacteriosis in Finland (Rautelin, Renkonen et al. 1991), despite the low rate of ciprofloxacin resistance among the domestic *C. jejuni* isolates. Since the domestic isolates are a minority of all strains, this might be the cause for the discrepancy between the numbers of foreign and domestic isolates included in **Study I** where consecutive *C. jejuni* isolates were collected at one hospital.

It was shown in this work that among the fluoroquinolones presently on market in Finland, levofloxacin and moxifloxacin exhibited the lowest *in vitro* MIC values towards the 226 *C. jejuni* strains studied. These compounds also had a better *in vitro* activity than the others against the ciprofloxacin-resistant *C. jejuni* strains. However, according to these results, sitafloxacin and clinafloxacin had the best *in vitro* activities against the *C. jejuni* species, exhibiting the lowest MIC_{50} and MIC_{90} values for all of the tested *C. jejuni* strains and also for the 134 ciprofloxacin-resistant *C. jejuni* strains. For these ciprofloxacin-resistant *C. jejuni* strains, the MIC_{50} and MIC_{90} values were four to seven dilution steps lower than those of ciprofloxacin.

Earlier data on the efficacy of sitafloxacin and clinafloxacin towards *C. jejuni* strains are limited. In fact, the *in vitro* activity of sitafloxacin has been evalueted in only one previous study. According to those results, sitafloxacin had increased activity compared to ciprofloxacin against 39 clinical *Campylobacter* isolates (Tomayko, Korten et al. 1994). Also, clinafloxacin was found to be somewhat more active than ciprofloxacin against 18 *C. jejuni* isolates (Bauernfeind 1997). However, at present, clinafloxacin has no clinical relevance, since its development has been suspended. In contrast, sitafloxacin

has been approved in Japan for the treatment of respiratory and urinary tract infections (Anderson 2008). The encouraging results of the present study suggest that the clinical efficacy of sitafloxacin should be evaluated also in campylobacteriosis.

A number of previous studies have focused on the *in vitro* efficacy of sitafloxacin on microbes other than campylobacters. In these studies, sitafloxacin has exhibited a better activity than the other available fluoroquinolones towards e.g. several enterobacterial species, including ciprofloxacin-resistant strains (Milatovic, Schmitz et al. 2000). According to Deguchi *et al.* (1997a), sitafloxacin exhibited improved activity against quinolone-resistant *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates with alterations in *GyrA* and *ParC* proteins. In another study, Deguchi *et al*. (1997b) have reported that the activity of sitafloxacin against quinolone-resistant clinical isolates of *Neisseria gonorrhoeae* bearing mutant DNA gyrases was significantly greater than that of the other fluoroquinolones tested. These findings have led the authors of these papers to postulate that sitafloxacin might be a potentially useful antimicrobial agent for the treatment of bacterial infections caused by strains resistant to other fluoroquinolones. In a previous study of our group, the QRDR of the *gyrA* gene was sequenced from 115 of the 134 *C. jejuni* strains resistant to ciprofloxacin. The same strains were included in the present work indicating that the ciprofloxacin-resistant strains studied here exhibited a point mutation at the codon 86, substituting isoleucine for threonine (Hakanen, Jalava et al. 2002). Based on these data, the mutation was the cause of ciprofloxacin resistance in the majority, if not all, of the *C. jejuni* strains analyzed here. Thus, it is possible that sitafloxacin does not have adequate clinical activity against these ciprofloxacin-resistant strains with mutations in their *gyrA*. Yet, it is possible that clinical efficacy does exist considering the low MIC values of sitafloxacin. This question cannot be answered on the basis of *in vitro* studies. Therefore, clinical trials to treat enteritis caused by ciprofloxacin-resistant *C. jejuni* with sitafloxacin may be valuable. It is somewhat disappointing that earlier results indicate that the use of sitafloxacin in Caucasians should be limited because of its potential for phototoxicity (Anderson 2008).

6.2. Macrolide resistance in *Campylobacter* species (I-III)

Macrolides are currently the first-choice antimicrobials for the empirical treatment of suspected campylobacter enteritis in many countries (Kuschner, Trofa et al. 1995; Li, Chiu et al. 1998; Saenz, Zarazaga et al. 2000). The present study provides information on the macrolide resistance frequency in *Campylobacter* species in Finland between 1995 and 2000 as well as between 2003 and 2005. During the first period, macrolide resistance was relatively uncommon, with only 2% of all isolates and 3% of ciprofloxacin-resistant isolates classified as macrolide-resistant. During the second period, the finding that 19 of the 1808 *Campylobacter* isolates initially included in the study proved to be erythromycin-resistant by MIC determination indicates that the frequency of macrolide resistance was even slightly lower, 1.1%. We trust this material to be representative of the macrolide resistance situation in our country between 2003 and 2005, as these isolates comprised about one fifth of all campylobacters recovered from Finnish patients throughout the study. We also trust that our screening approach was appropriate, since the adequacy of the methodology was verified by comparing the results of the screening

tests to the MIC determinations, with the finding that all isolates with inhibition zone diameters ≥21 mm were defined as susceptible by agar dilution. Based on this, we have identified at least a great majority, if not all, of the macrolide-resistant strains present in the initial study population. Due to the low resistance rate, macrolides still appear to be the best treatment alternative in suspected *Campylobacter* enteritis also in Finland, if antimicrobial therapy is needed.

In some other countries, higher macrolide resistance rates have been reported. According to an earlier survey, the rates of erythromycin resistance among *C. jejuni* were 17% in both Spain and Taiwan (Li, Chiu et al. 1998; Saenz, Zarazaga et al. 2000). According to Isenbarger *et al*. (Isenbarger, Hoge et al. 2002), the azithromycin resistance rate in Thailand was only 6%, but all azithromycin-resistant isolates were also fluoroquinolone-resistant. A similar finding has been made by another group, on U.S. military personnel contracting acute diarrhea in Thailand in the 1990s; all of their *Campylobacter* isolates were susceptible to azithromycin, but 52% were resistant to ciprofloxacin (Kuschner, Trofa et al. 1995). In the present thesis, 75% of the erythromycin-resistant isolates were also ciprofloxacin-resistant during the first collection period, and all of them were considered to be multidrug-resistant. Based on *in vitro* results, no apparent benefits are gained by the use of newer macrolides or ketolides in the treatment of erythromycin-resistant *C. jejuni* infections, since here all isolates with elevated erythromycin MICs exhibited elevated MICs of clarithromycin and telithromycin as well.

Among the *C. jejuni* strains collected during the first study period, 1995-2000, only one isolate resistant to co-amoxiclav was found, and none were resistant to imipenem. These *in vitro* susceptibilities suggested that co-amoxiclav might be a useful antimicrobial agent in the treatment of the enteritis caused by multidrug-resistant *C. jejuni*, and if the situation is life-threatening, a carbapenem may be the drug of choice. However, during the second collection period, 2003-2005, the resistance to co-amoxiclav was clearly higher: 31.6%. This is unfortunate, since in enteric infections, per oral administration of antimicrobials is usually preferable to the intravenous route. The results of the present study show that no per oral antimicrobial agent currently available in Finland reliably covers the macrolide-resistant C*ampylobacter* strains. All but one erythromycin-resistant strain were here resistant to ciprofloxacin, the majority of them being resistant also to telithromycin and tetracycline. Considering the resistance figures of the first collection period, co-amoxiclav might still offer the best peroral treatment alternative. Nevertheless, it must be kept in mind that in the second collection period as many as one third of the strains were resistant to co-amoxiclav. Moreover, very little data exist on the clinical use of co-amoxiclav or carbapenems (Kerstens, Endtz et al. 1992) or of the other β-lactams (Leibovitz, Janco et al. 2000) in campylobacteriosis. Among the fluoroquinolones, sitafloxacin exhibited the lowest MIC values against the macrolide-resistant isolates. However, sitafloxacin is not presently on market, thus being of no clinical consequence. In addition, all our macrolide-resistant strains were susceptible to chloramphenicol, which is no longer on market in Finland for systemic use.

Of the 238 *Campylobacter* strains identified to species level, 7.6% were identified as *C. coli*, but of the macrolide-resistant strains almost half (47.4%) were identified as *C. coli*. This is consistent with earlier studies showing that *C. coli* strains are more often

macrolide-resistant than *C. jejuni* (Engberg, Aarestrup et al. 2001; Schönberg-Norio, Hänninen et al. 2006).

6.3. Multidrug resistance in *Campylobacter* species (I, III)

So far, the multiresistance of *C. jejuni* has not led to severe consequences, since campylobacter infections are often self-limiting and no antimicrobial treatment is required. However, in severe or persisting cases of enteritis, in invasive infections and in immunocompromised patients as well as in pregnant women, very young and old patients, antimicrobial therapy is needed (Allos 2001). There is little knowledge regarding antimicrobial agents that could be used in serious infections, if caused by these multidrug-resistant *Campylobacter* strains. Since campylobacteriosis rarely causes extraintestinal manifestations, data on antimicrobial therapy is based only on anecdotal case reports (Arai, Kitano et al. 2007; Monselise, Blickstein et al. 2004; Okada, Kitazawa et al. 2008) and small retrospective case series (Kerstens, Endtz et al. 1992; Lau, Woo et al. 2002). Thus, the appropriate protocols of antimicrobial treatment for bacteremic infections, whether caused by susceptible or resistant strains, have not been established for campylobacters. Therefore efforts should be made to delineate alternative drugs for the treatment of *Campylobacter* infections caused by these ciprofloxacin- and macrolide-resistant strains. Successful outcomes have been reported especially with the carbapenems (Arai, Kitano et al. 2007; Kerstens, Endtz et al. 1992; Lau, Woo et al. 2002; Monselise, Blickstein et al. 2004). In previous studies, this antimicrobial group has also shown excellent *in vitro* activities against *Campylobacter* species (Tajada, Gomez-Graces et al. 1996).

In this thesis, ciprofloxacin resistance of *C. jejuni* strains was significantly associated with resistance to three or more antimicrobial groups. Correspondingly, the macrolide-resistant *Campylobacter* strains were also uniformly multidrug-resistant. MDR is problematic, but there are still a number of drugs effective against these fluoroquinolone-resistant and multidrug-resistant *C. jejuni* strains. All macrolide-resistant strains were susceptible to meropenem and imipenem. Among the older antimicrobial agents, gentamycin was effective, and it may be effective in septicemia and other systemic infections in conjunction with carbapenem antibiotics. A cautionary note, however: gentamycin is not suitable for use e.g. in meningitis or during pregnancy. It is noteworthy that here also tigecycline was highly active against all *Campylobacter* strains including the macrolide- and multidrug-resistant strains. This is evidently an important finding since there is a limited number of other agents potentially effective in the treatment of multidrug-resistant campylobacteriosis.

The *in vitro* activity of tigecycline against *Campylobacter* strains has been analyzed in one previous study (Rodriguez-Avial, Rodriguez-Avial et al. 2006). In that study, tigecycline exhibited the lowest MIC values against resistant *Campylobacter* strains. Clinically, tigecycline has shown excellent activity e.g. in the treatment of complicated skin and soft tissue infections, which is known to be one manifestation of extraintestinal campylobacteriosis (Breedt, Teras et al. 2005; Kerstens, Endtz et al. 1992; Monselise, Blickstein et al. 2004). Tigecycline circulates primarily as unchanged drug and its

major route of elimination is through the feces, likely via biliary excretion (Agwuh and MacGowan 2006; Muralidharan, Micalizzi et al. 2005). On this basis, it seems reasonable to assume that tigecycline might be effective even for patients with gastroenteritis caused by multidrug-resistant campylobacteriosis.

6.4. Macrolide resistance mechanisms (IV)

In this thesis, a point mutation A to G at the position 2059 of the 23S rRNA gene was found to be the main mechanism behind macrolide resistance among *Campylobacter* strains, as 91.2% of all erythromycin-resistant strains and 96.9% of the highly erythromycin-resistant strains were shown to posses such a mutation. These results corroborate previous findings by others (Caldwell, Wang et al. 2008; Gibreel, Kos et al. 2005; Ladely, Meinersmann et al. 2009; Vacher, Menard et al. 2003). For example, while investigating a collection of 23 macrolide-resistant *Campylobacter* isolates, Gibreel *et al.* (Gibreel, Kos et al. 2005) found that about 78% of their isolates exhibited a point mutation A to G at the position 2059 of the 23S rRNA gene and about 13% of the isolates had a mutation at *E. coli* equivalent base 2058. This mutation at the position 2058 has been described earlier by Vacher *et al.* (Vacher, Menard et al. 2003); the same mutation was present in two of the 22 macrolide-resistant *Campylobacter* isolates in their study. Mutations at the position 2058 of the 23S rRNA gene were not found in the strains included in the present work, which is also in line with prior reports of some other groups (Corcoran, Quinn et al. 2006; Payot, Avrain et al. 2004).

According to a few earlier reports (Ladely, Meinersmann et al. 2009), the number of the mutated alleles of the 23S rRNA gene has an effect on the level of *Campylobacter* macrolide resistance, whereas in some other studies (Gibreel, Kos et al. 2005; Payot, Avrain et al. 2004), no correlation between the level of resistance and the number of mutated alleles has been observed. In the majority of resistant *C. jejuni* isolates analyzed by Gibreel *et al* (Gibreel, Kos et al. 2005), mutations in the 23S rRNA gene were homozygous except in two cases where a mutation was found in two of the three copies of the target gene. Correspondingly, in the present work there were two highly erythromycin-resistant strains (one *C. jejuni* and one *C. coli*) having only two mutated copies of the 23S rRNA gene instead of all three. Thus, these results support the conception that the number of mutated alleles does not invariably have an influence on the level of macrolide resistance.

It is of note that in previous studies focusing on *Campylobacter* macrolide resistance mechanisms, mutations in the 23S rRNA gene have been analyzed by DNA sequencing. The present study is different in that the strains were analyzed by pyrosequencing, which is a rapid and low-cost method as compared to DNA sequencing. In an identified *Campylobacter* strain, 23S rRNA-based resistance mechanisms can be detected by pyrosequencing during one working day (Haanperä, Huovinen et al. 2005). It is noteworthy that also the number of mutated alleles was determined here by pyrosequencing. Evaluating the number of mutated alleles from the heights of the particular peaks corresponding to the mutant and wild-type sequences in the pyrograms is easy and rapid.

In addition to mutations at the ribosomal target sites, several efflux pumps contribute to *Campylobacter* resistance towards macrolides, as well as a wide range of other antimicrobials (Lin, Michel et al. 2002; Mamelli, Prouzet-Mauleon et al. 2005; Payot, Avrain et al. 2004; Pumbwe and Piddock 2002). It is commonly speculated that the efflux pumps and mutations in the ribosomal proteins L4 and L22 can cause low-level macrolide resistance, while high-level erythromycin resistance is mainly caused by a specific point mutation of the 23S rRNA gene (Vacher, Menard et al. 2003). One highly-resistant *C. jejuni* strain (number 1, Figure 7) not exhibiting such a mutation was found here. To assess potential L4- or L22 -associated resistance mechanisms, the L4 and L22 genes of all three erythromycin-resistant strains without 23S rRNA mutations were sequenced. In order to estimate the normal variation of these genes, the L4 and L22 genes of 31 highly erythromycin-resistant *Campylobacter* strains with mutation in the 23S rRNA gene and 20 erythromycin-susceptible *Campylobacter* strains were sequenced. The insertion demonstrated here between the positions 73 and 74 in the ribosomal protein L22 of the highly-resistant *C. jejuni* strain without mutations in the 23S rRNA gene was unique in this strain collection, and according to the best of my knowledge, has not been previously described. It is proposed that the insertion might be associated with macrolide resistance in this particular *C. jejuni* strain. In addition to single amino acid substitutions, insertions and deletions positioned in this area are known to be associated with macrolide resistance in many other bacterial species (Chittum and Champney 1994; Malbruny, Canu et al. 2002; Jones, Farrell et al. 2003; Doktor, Shortridge et al. 2004). The high erythromycin MIC value of this *C. jejuni* strain can be regarded as suggestive of some genetic resistance mechanism. Testing these strains for the presence of efflux pumps showed that neither the efflux pump inhibitor PAβN nor NMP had any consequent effect on the MIC values of the *Campylobacter* strains. There are, however, previous data suggesting that interplay between efflux activity and mutations in the 23S rRNA gene may contribute to high-level macrolide resistance in some *Campylobacter* strains. One such strain described by Corcoran *et al.* (Corcoran, Quinn et al. 2006) was a clinical isolate of *C. jejuni* displaying high-level erythromycin resistance, in which the efflux pump inhibitor PAβN decreased the MIC values of erythromycin eight-fold (from 256 μg/ml to 32 μg/ml), though it did not restore susceptibility.

Sequencing of the ribosomal protein L22 revealed a D72N substitution in one of the two remaining *Campylobacter* strains without 23S rRNA mutations and exhibiting low-level erythromycin resistance. The substitution, also positioned close to the C-terminus region, was detected in this strain only. Further studies are needed to assess whether this modification might contribute to macrolide resistance.

A number of previous studies have reported modifications in the ribosomal proteins L4 and L22 (Cagliero, Mouline et al. 2006; Caldwell, Wang et al. 2008; Corcoran, Quinn et al. 2006). The role of these alterations in *Campylobacter* macrolide resistance is still unclear; evidently, they do not lead to high-level resistance. Several amino acid substitutions in the ribosomal protein L22 were observed also in the present study, and they appeared to be more common in the erythromycin-resistant *Campylobacter* strains as compared to the susceptible strains. In contrast, only a few amino acid substitutions were observed in the ribosomal protein L4, with no obvious difference between the erythromycin-resistant and susceptible *Campylobacter* strains. So far, the significance

of the amino acid substitutions found here in the ribosomal proteins L4 and L22 remains unknown, and warrants further evaluation.

In a clinical setting, macrolides are the first-choice alternatives when antimicrobial therapy is needed for a patient with campylobacteriosis. To confirm their efficacy, routine susceptibility testing of the causative strain is an important tool in clinical practice. The detection of macrolide resistance in *Campylobacter* isolates is currently based on phenotypic methods performed after culture and isolation. This poses a problem since due to the slow growth of *Campylobacter* species, the procedure is time-consuming and costly. Thus, there is a need for a faster approach to detect macrolide resistance in campylobacters. It was shown in this study that macrolide resistance-associated mutations are found mainly in the 23S rRNA gene of *C. jejuni* and *C. coli* strains and can be easily detected by pyrosequencing. Furthermore, these mutations do not occur in macrolide-susceptible strains. Here, 91-97% of the erythromycin-resistant strains had an A to G mutation at the position 2059. This means that after a *Campylobacter* strain has been isolated from a clinical specimen and identified by conventional methods, the presence of the mutation can be confirmed, thus establishing resistance during the same day. It is also important that several samples can be analyzed at the same time.

6.5. Antimicrobial susceptibility testing by the disk diffusion (V)

One aim of this thesis was to evaluate the adequacy of the disk diffusion method in comparison with the agar dilution method for determining the efficacy of important antimicrobial compounds towards *Campylobacter* spp. In so doing, there were differences in the results between these two methods, and significant differences were also found for several antimicrobial agents, when the disk diffusion test results were repeated and the results obtained at different measurement times compared. However, no significant differences in repeatability were observed among the erythromycin- and azithromycin-resistant strains. The reason for the better performance regarding these macrolide-resistance strains is unclear. It might be due to the rather small amount of macrolide-resistant strains, and therefore further studies are needed to evaluate this finding with a larger number of *Campylobacter* strains.

For *Campylobacter* spp., the agar dilution and the broth microdilution methods have been standardized by CLSI (CLSI 2009) for antimicrobial susceptibility testing. The agar dilution method was chosen to be the reference method in this thesis. The MIC results were performed twice for each strain with identical susceptibility results. Both of the standardized methods, however, have their limitations. The use of agar dilution and broth microdilution are problematic for testing only a few isolates at a time and both methods need a large amount of manual handling. The E-test is convenient for testing small amounts of isolates, but on the other hand it is a more expensive alternative. Disk diffusion is a commonly used method in susceptibility testing of *Campylobacter* spp., even without standardization since a) it can be done easily even for a small amount of strains, b) it does not need much preparation beforehand, and c) it can be done to a significantly lower cost than any other method.

According to the CLSI instructions, disk diffusion should only be used as a screening method in *Campylobacter* spp. and antimicrobial susceptibility should be further confirmed by using an MIC-based method if there is any sign of inhibition zone (CLSI 2009; King 2001). In our study, there were 129 zone measurements for strains that were erythromycin-resistant according to the MIC determinations. Of these, 11% exceeded 6 mm, indicating a need for more accurate susceptibility determination to demonstrate erythromycin resistance. For ciprofloxacin-resistant strains, confirmation by an MIC based method was indicated even more frequently, since there were 312 measurements of which 26% exceeded 6mm leading to to a false diagnosis of ciprofloxacin susceptibility without an MIC-based susceptibility determination. Azithromycin and nalidixic acid exhibited similar results to those of erythromycin and ciprofloxacin, respectively. It is noteworthy that for tetracycline, even 66% of the measurements would have required MIC-based determinations of susceptibility if applying this 6mm rule. On the other hand, 0.42% and 3.4% of all measurements for erythromycin-susceptible and ciprofloxacin-susceptible strains were without inhibition zone, respectively, being falsely classified as resistant, since extra determinations would have been considered unnecessary if the CLSI guidelines would have been followed. Apart from the small number of false-resistant strains, our findings support the CLSI recommendation that disk diffusion method should only be used as a screening method for erythromycin and ciprofloxacin resistance, and that any sign of inhibition zone requires a MIC-based susceptibility determination.

These results indicate that there is a need for a standardized protocol for susceptibility testing in clinical laboratories for *Campylobacter* spp. The falsely resistant strains might cause excessive use of more toxic and possible even less effective antimicrobial agents for patients with campylobacteriosis. The most serious threat is when a patient has an invasive and serious infection. Infections with resistant strains have been reported in association with a five-fold increase of the risk of invasive illness or death (Coker, Isokpehi et al. 2002). In addition, the varying and unreliable results in antimicrobial susceptibility testing also lead to problems in accurate monitoring of the resistance. Infections with resistant strains have been reported in association with a five-fold increase of the risk of invasive illness or death (Coker, Isokpehi et al. 2002). Also for that reason, it is of importance to be able to correctly distinguish the resistant strains. Moreover, an adequate monitoring of *Campylobacter* spp. resistance is impossible without reliable susceptibility testing results.

Several previous papers have reported varying results on the efficacy and accuracy of the disk diffusion method and the E-test method when compared to the agar plate dilution or broth microdilution methods (Schönberg-Norio, Hänninen et al. 2006; Gaudreau, Girouard et al. 2007; Gaudreau, Girouard et al. 2008; Varela, Friendship et al. 2008; McGill, Kelly et al. 2009; van der Beek, Claas et al. 2010). In these studies the disk diffusion tests were performed only once for each strain. Gaudreau *et al.* (Gaudreau, Girouard et al. 2007; Gaudreau, Girouard et al. 2008) have found the disk diffusion method to be a reliable, easy and inexpensive method for the testing of the susceptibility of *C. jejuni* to erythromycin, ciprofloxacin, and tetracycline. However, corroborating the present study, van der Beek *et al*. (van der Beek, Claas et al. 2010) reinvestigated 48 erythromycin-resistant *C. jejuni* and *C. coli* strains retrospectively to re-evaluate erythromycin resistance and only 11-14% of the *C. jejuni* strains and 67% of the *C.*

coli strains were erythromycin-resistant in this second analysis. In that study, the initial susceptibility testing was in most cases performed by disk diffusion and reinvestigation was carried out using broth microdilution. The authors conclude that routine determination of erythromycin resistance in *C. jejuni* and *C. coli* shows unacceptable interlaboratory variation. They also speculated on the possibility that differences could be caused by instability of the erythromycin resistance or by non-standardized susceptibility testing methods. Non-standardized susceptibility testing methods may be involved in the varying susceptibility results obtained by the disk diffusion method including i) varying protocols of the methods used, ii) long incubation time for campylobacters, iii) inaccuracy of the measurements between different times or between different persons measuring the inhibition zone, and iv) different methods for achieving microaerobic conditions during the incubation. Our results confirm the observation that when disk diffusion determinations are repeated, there is significant variation of the inhibition zone. In this study the same protocol for susceptibility testing was used in all of the repeated measurements, and the same person measured the inhibition zones every time to avoid variation do to these factors. It is also of note that between different measurement times, no rising trend during the repetitions was observed in the inhibition zone variation. Therefore instability of the erythromycin resistance seems not to be behind this variation.

In conclusion, it is a major concern that the disk diffusion method may not be a reliable tool for the susceptibility testing of *Campylobacter* species. Further studies are needed on whether the disk diffusion test method could be improved or whether all susceptibilities of campylobacters should be done using a MIC based method. Adequate monitoring of resistance is also impossible, if resistance is falsely reported due to unreliable susceptibility testing.

7. CONCLUSIONS

Among the fluoroquinolone compounds presently on the market in Finland, levofloxacin and moxifloxacin were the most effective drugs *in vitro* towards all 226 *C. jejuni* strains studied. Of the newer fluoroquinolones, sitafloxacin was highly effective *in vitro* towards *C. jejuni,* with low MIC values also against ciprofloxacin-resistant strains. Sitafloxacin might be a candidate for clinical trials on campylobacteriosis.

If the treatment is needed, macrolides still appear to be the first-choice alternative for suspected *Campylobacter* enteritis since fluoroquinolone resistance is common in Finland. The incidence of macrolide resistance among the *Campylobacter* strains was low in this thesis. *C. coli* strains were significantly more frequently macrolide-resistant as compared to *C. jejuni*. Based on our results, no perorally administered antimicrobial agent reliably covers the macrolide-resistant *Campylobacter* strains. Co-amoxiclav appears to offer the best per oral treatment alternative in these cases, with only one third of our isolates resistant.

MDR was found to be significantly associated with resistance to both ciprofloxacin and erythromycin. The *in vitro* susceptibilities found suggest that co-amoxiclav might be a candidate for clinical trials on enteritis caused by multidrug-resistant *Campylobacter* strain, and if the situation is life-threatening, a carbapenem may be the drug of choice. In addition to imipenem and meropenem, also tigecycline was highly effective *in vitro* against multidrug-resistant *Campylobacter* strains. The efficacy of tigecycline in the treatment of human campylobacteriosis should be evaluated in clinical trials.

A point mutation A to G at the position 2059 of the 23S rRNA gene was found to be the main mechanism behind macrolide resistance among our *Campylobacter* strains, corroborating previous studies. In the majority of the resistant strains, the mutations in the 23S rRNA gene were homozygous. Pyrosequencing is a rapid and low-cost method as compared to traditional DNA sequencing to detect these mutations. Sequencing revealed an amino acid insertion between positions 73 and 74 in the ribosomal protein L22 of one highly-resistant *C. jejuni* strain without mutations in the 23S rRNA gene. Further studies are needed to assess whether this insertion might contribute to *Campylobacter* macrolide resistance. The clinical applicability of pyrosequencing in rapid detection of macrolide resistance in *Campylobacter* isolates should also be evaluated.

It is a major concern that the disk diffusion method may not be a reliable tool for the susceptibility testing of *Campylobacter* species. Further studies are needed to assess whether the disk diffusion method could be improved or whether all susceptibilities of campylobacters should be only done using an MIC-based method.

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