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Helsinki, Finland

Severe *Campylobacter* infections in Finland

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the Auditorium XII, third floor in the main building of the University, Unioninkatu 34, Helsinki, on March 23 2012, at 12 noon.

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ISBN 978-952-10-7887-3 (paperback)

ISBN 978-952-10-7888-0 (PDF)

Helsinki University Printing House

Helsinki 2012

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Abbreviations

ATCC	American Type Culture Collection
<i>C. coli</i>	<i>Campylobacter coli</i>
<i>C. fetus</i>	<i>Campylobacter fetus</i>
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
CC	clonal complex
CDT	cytolethal distending toxin
CFU	colony forming unit
CiaB	<i>Campylobacter</i> invasion antigen B
CLSI	Clinical and Laboratory Standards Institute
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GBS	Guillain-Barré syndrome
GGT	γ -glutamyl transpeptidase
HL	heat labile
HS	heat stabile
IBS	irritable bowel syndrome
Ig	immunoglobulin
LOS	lipooligosaccharide
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
NIDR	National Infectious Disease Register
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PldA	Phospholipase A
QC	quality control
spp.	species
SPSS	Statistical Package for Social Sciences
ST	sequence type
TLR	Toll-like receptor

Abstract

Campylobacter jejuni and *C. coli* are among the most important bacterial enteropathogens worldwide, and the last decade *Campylobacter* infections in the European Union (EU) have shown a rising trend in incidence. The most commonly reported zoonosis in the EU, campylobacteriosis, can be transmitted to humans from a wide variety of wild birds, as well as from domestic and pet animals. Eating or handling raw or undercooked meat, particularly poultry, is considered the primary risk factor for *Campylobacter* infection, followed by other risk factors such as drinking unpasteurized milk or contaminated water, contact with domestic pets, and swimming in natural water sources.

The enteric disease caused by *C. jejuni* or *C. coli* is characterized by profuse watery diarrhea, fever, abdominal pain, nausea, and sometimes bloody stools. The disease usually lasts from a few days up to one week, and although *Campylobacter* infection usually resolves spontaneously without antimicrobial treatment, prolonged symptomatic periods and relapses do occur.

A complication that commonly follows and is attributed to *Campylobacter* infection is reactive arthritis, which occurs in approximately 1 to 10% of the patients. Closely associated with *C. jejuni* infection is also the more rarely encountered serious inflammatory polyneuropathy Guillain-Barré syndrome. Bacteremia due to *C. jejuni* or *C. coli* has been estimated to develop in 0.1 to 1% of patients with campylobacteriosis, and hematogenously spread disease is considered more common in older patients and those with severe underlying diseases.

The bacterial characteristics of *C. jejuni* or *C. coli* which could lead to a more severe course of disease, to bacteremia, or to post-infectious complications are not well understood. Certain putative virulence factors have been suggested to be of importance for either the invasiveness or the colonization potential of *C. jejuni*, some examples of which are the plasmid pVir, *Campylobacter* invasion antigen, and γ -glutamyl transpeptidase (GGT). Other examples of putative virulence factors include those suspected to affect the bacterium's iron uptake, survival or metabolism. Moreover, because of the seriousness of Guillain-Barré syndrome, development of post-infectious adverse immune responses has been subject to specific focus, and structural differences in the lipooligosaccharide in the outer membrane of *C. jejuni* have been under extensive study. The relevance of these bacterial factors to the actual clinical disease outcome has yet to be explained, however, possibly with the exception of the relatively clear association between sialylated lipooligosaccharides and the development of the Guillain-Barré syndrome. The primary goal of this PhD study was therefore to try to define which bacterial factors could lead to a more severe outcome of *Campylobacter* infection.

Two sets of patient data, along with the corresponding bacterial isolates of those patients, were included. The first was questionnaire-based, and collected from the Uusimaa region in southern Finland during July through December, 2006. The 192 patients included had been diagnosed as stool culture-positive for *C. jejuni* or *C. coli*, and all returned their questionnaires along with their informed consent. The second set of data was retrospective,

nationwide, and register-based, and included 76 patients diagnosed with *C. jejuni* or *C. coli* bacteremia during a 10-year period in Finland, 1998-2007.

The isolates from patients with enteritis were tested for antimicrobial susceptibility, the presence of putative virulence factor genes, and the production of GGT. Multilocus sequence typing (MLST) as well as determination of susceptibility to normal human serum was performed on the isolates from the patients with bacteremia. All *C. jejuni* isolates were tested for production of GGT, and in addition, the presence of the following putative virulence factor genes was tested by polymerase chain reaction (PCR) for all isolates; *ceuE*, *ciaB*, *cj0486*, and *virB11*, and additionally the gene cluster *cdtABC* and the genes *cgtB*, *pldA*, and *wlaN* for the enteritis isolates. For the bacteremia isolates, PCR analyses allowed determination of the MLST profiles and lipooligosaccharide (LOS) locus classes of the isolates.

Results of multivariate analyses revealed bacterial differences based on the origin of infection among the enteritis isolates, as domestically acquired infections were significantly associated with the production of GGT, while imported infections were significantly associated with *ceuE* and *cj0486*.

Susceptibilities of the bacterial isolates were tested for those antimicrobial agents most commonly used in the treatment of *Campylobacter* infections, and when these data were compared with the clinical information, some interesting findings emerged. First, among the patients with enteritis, bacterial resistance to ciprofloxacin did not seem to lead to more severe disease outcome. Instead, this was specifically attributed more to those isolates highly susceptible to ciprofloxacin. Second, the majority of the bacteremia isolates were susceptible to all tested antimicrobial agents. Third, among the patients with bacteremia, appropriate and empirical antimicrobial treatment affected neither patients' duration of hospitalization nor the mortality attributable to the infection, to any greater extent.

The *C. jejuni* and *C. coli* bacteremia patients were moderately young (median age, 46 years) and according to the Charlson weighted index of comorbidity, underlying diseases regarded as comorbidities significantly affecting disease outcome affected only 30%.

Among the bacteremia isolates, MLST revealed the clonal complexes ST-677, ST-45, and ST-21 to be the most prevalent, accounting for 48%, 16%, and 14%, of the isolates. All the isolates of the ST-677 complex originated from patients diagnosed with *C. jejuni* bacteremia during the seasonal peak in May through August; these isolates were significantly more serum resistant than were all other isolates, and all isolates of the ST-677 complex had nonsialylated LOS. Isolates of the ST-45 complex were associated with production of GGT and were significantly more serum sensitive than were all other isolates, and all the ST-45 complex isolates had nonsialylated LOS. Isolates of the ST-21 complex were associated with the gene *cj0486*, and all these isolates had sialylated LOS.

List of original publications

This thesis is based on the following publications:

- I. Feodoroff FB, Lauhio AR, Sarna SJ, Hänninen ML, Rautelin HI. Severe diarrhoea caused by highly ciprofloxacin-susceptible *Campylobacter* isolates. Clin Microbiol Infect. 2009;15:188-192.
- II. Feodoroff B, Ellström P, Hyytiäinen H, Sarna S, Hänninen ML, Rautelin H. *Campylobacter jejuni* isolates in Finnish patients differ according to the origin of infection. Gut Pathog. 2010;2:22.
- III. Feodoroff B, Lauhio A, Ellström P, Rautelin H. A nationwide study of *Campylobacter jejuni* and *Campylobacter coli* bacteremia in Finland over a 10-year period, 1998-2007, with special reference to clinical characteristics and antimicrobial susceptibility. Clin Infect Dis 2011;53:e99-e106.
- IV. Feodoroff B, de Haan CPA, Ellström P, Sarna S, Hänninen ML, Rautelin H. Clonal distribution and virulence properties of blood culture isolates of *Campylobacter jejuni* with special reference to clinical features. Submitted.

The publications are indicated in the text by their Roman numerals. The original articles have been reprinted with the permission from their copyright holders.

1. Introduction

In most parts of the world *Campylobacter* infection is common, with campylobacteriosis being the most frequently reported zoonosis in the European Union, as well as the most prevalent cause of bacterial enteritis in Europeans (EFSA, 2005; EFSA, 2009). Raw or undercooked meat, especially poultry, is considered the primary source of infection (Harris et al., 1986; Neal & Slack, 1995; Studahl & Andersson, 2000). In addition, contaminated drinking water (Schönberg-Norio et al., 2004), domestic pet contact (Kapperud et al., 1992; Friedman et al., 2004), and even swimming in natural sources of water (Schönberg-Norio et al., 2004) are important risk factors for *Campylobacter* infection.

The typical symptoms of *Campylobacter* enteritis include diarrhea, fever, abdominal cramps and pain, which sometimes may become so intense that it even mimics the pain during acute appendicitis (Blaser, 1997). Blood and mucus in the stools are also quite common, and some patients experience myalgia or nausea and sometimes, vomiting (Butzler & Skirrow, 1979). Bloody stools and vomiting have been regarded as more severe symptoms of *Campylobacter* infection (Gillespie et al., 2006).

Of the species of *Campylobacter*, *C. jejuni*, *C. coli* and *C. fetus* are of special clinical interest. *C. jejuni* is the most commonly isolated bacterial enteropathogen from stool samples of patients with diarrhea, and *C. coli* is the second most prevalent *Campylobacter* spp. isolated from enteritis patients. *C. fetus* is usually not isolated from fecal samples, but it causes extraintestinal infections, particularly in immunocompromised hosts, and it is sometimes isolated from blood (Lastovica & Allos, 2008).

Campylobacter bacteremia is uncommon, as it occurs in only approximately 0.1 to 1% of patients with *Campylobacter* enteritis (Skirrow et al., 1993; Samuel et al., 2004; Nielsen et al., 2010a), and is usually neither a septic nor a life-threatening condition in patients without significant underlying diseases (Pigrau et al., 1997; Tee & Mijch, 1998). Mortality attributable to the bacteremic episode has ranged from 4 to 16% in previous studies (Reed et al., 1996; Pigrau et al., 1997; Tee & Mijch, 1998; Pacanowski et al., 2008; Fernández-Cruz et al., 2010; Nielsen et al., 2010a). The distribution of *C. jejuni*, *C. fetus* and *C. coli* in bacteremia differs to some extent among studies, and the exact distribution of these species is unknown, as identification of the isolates by molecular methods is usually lacking.

Bacterial factors affecting the severity and outcome of *Campylobacter* infection are not well recognized. Some putative virulence factors may be of importance, including the cytolethal distending toxin (CDT) which causes cell cycle arrest (Lara-Tejero & Galán, 2001), γ -glutamyl transpeptidase (GGT), which plays a role in colonization in animal models (Hofreuter et al., 2006; Barnes et al., 2007), lipooligosaccharide (LOS), certain factors of which may enhance bacterial fitness (Habib et al., 2009), the fucose permease *cj0486* (Fearnley et al., 2008; Stahl et al., 2011), and the disputed plasmid pVir (Tracz et al., 2005; Louwen et al., 2006) which may affect the invasive capability of *C. jejuni*.

Treatment of *Campylobacter* infection with antimicrobial agents is common in many countries, although the overall effect of antimicrobial treatment seems limited (Ternhag et al., 2007).

In order to better understand which bacterial factors could be of importance for the outcome of *Campylobacter* infection or the severity of symptoms in the human host, it is important to combine well-defined clinical materials with analyses of the characteristics of the bacterial isolates. The aim of this PhD thesis was to study whether any specific factors exist which may lead to more severe *Campylobacter* infections.

2. Review of the literature

2.1 Historical aspects

Although the appropriate taxonomy of *Campylobacter* was introduced in the 1970's, bacteria belonging to this genus were actually first described by Theodor Escherich in 1886, a fact elegantly pointed out by Butzler along with a summary of some other important historical perspectives regarding the recognition of *Campylobacter* as a significant human pathogen (Butzler, 2004). In short, the genus name *Vibrio* was first suggested by Smith and Taylor in the beginning of the 20th century for these spirillum-like bacteria. In addition to the type species, *Vibrio fetus*, other catalase-positive “related vibrios” (King, 1957) were recognized as human pathogens in the 1950's. The cornerstones for recognizing the clinical importance of these “vibrios”, the term used for *Campylobacter* through the first half of the 20th century, were the isolation of “*Vibrio fetus*” from the blood of a pregnant woman with fever (Vinzent et al., 1947), the isolation of a “related vibrio” from human feces (Dekayser et al., 1972), and the development of a selective culture medium in order to simplify the isolation of *Campylobacter* from feces (Skirrow, 1977). These three important scientific achievements revealed that *Campylobacter* is a pathogenic genus which can be detected and isolated from both the blood and fecal samples of humans with a range of different clinical symptoms. After introduction of the term *Campylobacter*, Greek for ‘curved rod’ (Sebald & Véron, 1963; Véron & Chatelain, 1973), and once the technique needed for isolation from fecal samples was known (Skirrow, 1977), *Campylobacter* enteritis was recognized and described as an important human disease (Butzler & Skirrow, 1979; Blaser & Reller 1981).

Campylobacter has since been generally accepted as a clinically relevant zoonosis and an important human enteropathogen, with an incidence of campylobacteriosis estimated as higher than that of many other bacterial pathogens which cause febrile gastroenteritis.

2.2 Bacteriology

2.2.1 General description

Campylobacter spp. are Gram-negative, oxidase-positive, microaerophilic bacteria, which typically have a curved shape and are between 0.5 and 5 µm long and 0.2 to 0.8 µm wide (Snelling et al., 2005). Motility, essentially rapid, darting movements within the mucosal layer of the gastrointestinal tract, is enabled by either a single polar flagellum at one end, or a single flagellum at each end of the bacterium. *Campylobacter* spp. belong to the class of ϵ -proteobacteria, along with *Arcobacter* spp., *Helicobacter* spp., and *Wolinella* spp. In total, there are at least 18 species of *Campylobacter* (Humphrey et al., 2007), of which three are especially important human pathogens; *Campylobacter jejuni* and *C. coli* typically cause

enteritis, and *Campylobacter fetus* is commonly known to cause bloodstream infections (Guerrant et al., 1978; Schmidt et al., 1980; Francioli et al., 1985; Gazaigne et al., 2008).

C. jejuni and *C. coli* are thermophilic (optimal temperature for growth 42°C), whereas *C. fetus* is not generally able to survive at 42°C. *Campylobacter* spp. do not utilize carbohydrates, which limits the use of biochemical tests in differential diagnostics between species. *C. jejuni* is usually able to hydrolyze hippurate, while *C. coli* is hippurate-negative, and a hippurate hydrolysis test can be performed to differentiate between *C. jejuni* and *C. coli* isolates, although misidentification may occur due to the existence of *C. jejuni* strains which lack the ability to hydrolyze hippurate (Totten et al., 1987; Siemer et al., 2005). Two separate Finnish studies suggest that the species of at least all the hippurate-negative isolates should be confirmed with PCR (Rautelin et al., 1999; Nakari et al., 2008). It has also been suggested that *C. jejuni* evolution has actually been quite rapid, and that the separation into *C. coli* and *C. jejuni* may have occurred as recently as within the past 12 000 years (Wilson et al., 2009), and this further highlights the close relatedness of the two species. The genomes of several *C. jejuni* strains have been sequenced (Parkill et al., 2000; Fouts et al., 2005; Hofreuter et al., 2006; Lefébure & Stanhope, 2009).

Campylobacter will here be used to refer to *C. jejuni* and *C. coli*, unless otherwise stated.

2.2.2 Typing methods

Phenotyping methods

Before the development of reliable genotyping methods, phenotyping of *C. jejuni* and *C. coli* was usually performed by either of the two well-evaluated serotyping schemes: the Penner scheme (Penner & Hennessey, 1980) based on heat-stable (HS) antigens, and the Lior scheme (Lior et al., 1982), based on heat-labile (HL) antigens. The limitations of serotyping methods include high numbers of untypable strains and technical restrictions due to a lack of reagents and time-consuming and costly techniques.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a genotyping method in which certain restriction enzymes enable digestion and subsequently separation of comparatively large DNA fragments through a gel matrix with a specific kind of gel electrophoresis; it has been of practical use in epidemiological studies (Yan et al., 1991). PFGE allows creation of genetic fingerprint patterns for comparing genotypes and is more discriminatory than is multilocus sequence typing (MLST) (Thakur et al., 2009), although comparing PFGE profiles from various laboratories has proven difficult. Another problem is that PFGE sometimes fails to type isolates (Wassenaar & Newell, 2000). In Finland, certain PFGE types predominate in human and chicken isolates (Hänninen et al., 2000). PFGE has been used successfully in many outbreak studies (Lehner et al., 2000; Olsen et al., 2001, Hänninen et al., 2003).

Multilocus sequence typing

MLST is a genotyping method by which sequencing of seven housekeeping loci in the *C. jejuni* and *C. coli* genomes enable determination of the specific sequence type (ST) for a bacterial isolate (Maiden et al., 1998; Dingle et al., 2001). The seven housekeeping genes for the *C. jejuni* and *C. coli* MLST are *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*, and the combined allele profile of these genes determine the ST of the bacterium. ST clonal complexes (ST CCs) consist of STs with at least four alleles in common with the founder genotype. The primary advantage of this sequence-based method, compared to PFGE, is that the results of different laboratories can be easily compared, and MLST data from different study groups are accessible and comparable online (<http://pubmlst.org/campylobacter/>).

Other genotyping methods

Flagellin typing (*fla* typing) is based on restriction fragment length polymorphism (RFLP) analysis or sequencing of the flagellin gene locus (Nachamkin et al., 1993a), with both highly conserved and variable regions (Meinersmann et al., 1997). The lack of international standardization and comparability between laboratories, however, restricts this method's wider use (Wassenaar & Newell, 2000). More recently, sequence-based *fla* typing has become an additional part of MLST typing (Korczak et al., 2009).

Ribotyping is a genotyping method involving agarose gel electrophoresis of digested genomic DNA and Southern blot hybridization with a probe specific for rRNA genes. The major disadvantage of this technique is its lower discriminatory power, compared to that of other genotyping techniques.

Many other genotyping methods have been developed as well, but these methods are less widely used.

2.3 Pathogenesis and virulence factors

In order to successfully colonize the human intestinal tract, campylobacters need mechanisms for surviving in adverse environments and initiating chemotaxis, as well as structural and metabolic functions enabling bacterial motility (Nachamkin et al., 1993b), epithelial adhesion, cell invasion, iron acquisition, and toxin production (Ketley, 1997). Although several putative virulence and survival factors may be important for *Campylobacter* pathogenesis, the relevance of these particular genes and the proteins they encode for, considering the severity and outcome of campylobacteriosis, is generally poorly known.

The relation between the pVir plasmid in *C. jejuni* and the severity of infection can serve to demonstrate the complexity of putative virulence factors. It has been suggested that the pVir plasmid encodes for proteins of a type IV secretion system, and that mutation of the *virB11* gene in the plasmid results in reduced adherence and invasion potential *in vitro* as well as less severe symptoms *in vivo* (Bacon et al., 2000). Studies have shown both an association (Tracz

et al., 2005), and a lack of association (Louwen et al., 2006) between the pVir plasmid and bloody diarrhea in *C. jejuni* enteritis. pVir has typically only been detected in a minority of the strains however (Tracz et al., 2005; Louwen et al., 2006), and the gene *virB11* has in one study even been non-existent among *C. jejuni* strains (Talukder et al., 2008). This raises the question whether pVir can actually be considered an important virulence factor at all.

The motility of *Campylobacter* is enabled by the flagella at the end of the bacterium, allowing it to “move with quite astonishing speed up and down mucus stands” (Lee et al., 1986). The flagellar filament is composed of a major flagellin, FlaA, and a minor flagellin, FlaB, encoded by the genes *flaA* and *flaB* (Guerry et al., 1991; Alm et al., 1993). A special trait of ϵ -proteobacteria is that their flagella are glycosylated, and the flagella of *Campylobacter* have other functions in addition to enabling bacterial motility, such as the secretion of proteins which affect its virulence (Guerry, 2007). Recently, a link between *C. jejuni* flagella assembly and the biogenesis of lipooligosaccharide (LOS) on the outer core of *C. jejuni* emerged because the transferase Cj0256 modifies both these structures (Cullen & Trent, 2010).

Some putative virulence proteins are secreted from the flagellar export apparatus, for example *Campylobacter* invasion antigen B (CiaB), encoded by the *ciaB* gene (Konkel et al., 2004). *C. jejuni* isolates which lack the *ciaB* gene have non-invasive phenotypes (Konkel et al., 1999; Ziprin et al., 2001). On the other hand, a negative correlation has also been detected between *ciaB* and invasiveness (Fearnley et al., 2008).

Lipopolysaccharides may be necessary for both adhesion to and the invasion of epithelial cells (Fry et al., 2000). Some LOS structures in *C. jejuni* resemble human neuronal gangliosides. This molecular mimicry has been hypothesized to lead to autoimmune responses in the human host, including the most important neurological complication after *C. jejuni* infection, the Guillain-Barré syndrome (GBS) (Mishu et al., 1993; Rees et al., 1995). The putative virulence genes *cgtB* (Gilbert et al., 2000) and *wlaN* (Linton et al., 2000) are involved in the biosynthesis of LOS, and may encode for β -1, 3-galactosyltransferases with identical enzymatic activities (Linton et al., 2000; Gilbert et al., 2002). Evidence suggests that *C. jejuni* isolates with sialylated LOS are more invasive than nonsialylated isolates (Habib et al., 2009), and GBS-associated *C. jejuni* isolates have been associated with sialylated LOS (Koga et al., 2006).

The gene *cj0486* which encodes for a fucose permease (Stahl et al., 2011) may be associated with certain hyperinvasive *C. jejuni* strains (Fearnley et al., 2008). In addition, many other genes identified in the *C. jejuni* genome (Javed et al., 2010) are associated with metabolism and survival; their clinical relevance is unknown.

Phospholipase A (PldA), encoded by gene *pldA*, has been suggested to have a role in the lysis of erythrocytes by *C. coli*, and it has therefore been proposed to be a putative virulence factor of *Campylobacter* (Grant et al., 1997). Further, an enterochelin binding lipoprotein, CeuE, which enables uptake of ferric siderophore, produced by other enteric bacteria, has been detected in *C. coli* (Richardson & Park, 1995). The *Campylobacter* enterochelin uptake (*ceu*) operon, *ceuBCDE*, encodes for a periplasmic binding-protein-dependent (PBT) system,

which is thought to be important for iron uptake especially during tissue invasion (Richardson & Park, 1995; Ketley, 1997), and the gene *ceuE* encodes for CeuE, an important part of the PBT system.

Cytolethal distending toxin (CDT) is encoded by three genes, *cdtA*, *cdtB*, and *cdtC*, all of which have to function in order to enable the tripartite toxin to cause cell cycle arrest and distention of the cytoplasm in eukaryotic cells (Lara-Tejero & Galán, 2001). In a recent study from Bangladesh, CDT production was detected in almost all *C. jejuni* stool sample isolates (Talukder et al., 2008).

There is strong data that γ -glutamyl transpeptidase (GGT), encoded by the *ggt* gene, has a role in glutathione and glutamine metabolism in *C. jejuni* (Hofreuter et al., 2008). GGT activity has been shown to be required for persistent colonization of the avian gut (Barnes et al., 2007), and a *ggt* mutant has been shown to have lesser colonization potential compared to the wild-type *C. jejuni* 81-176 strain in a murine model (Hofreuter et al., 2006). The major limitation in both avian and murine models, when studying the importance of putative virulence factors, is that only colonization can be reliably studied, as *Campylobacter* infection in these animals is typically either asymptomatic or atypical (Haddad et al., 2010). The presence of *ggt* has been shown to be less common in bovine *C. jejuni* strains, compared to avian or human *C. jejuni* strains (Gonzalez et al., 2009).

Other proposed putative virulence factors include CheY (Yao et al., 1997), supposedly involved in bacterial chemotaxis and motility, PEB1 involved in adherence to, the invasion of, as well as colonization of HeLa cells (Pei et al., 1998), and the CadF protein has been proposed to be important for adhesion (Konkel et al., 1997). Alternate iron-acquisition systems have been described as well, for example *chuABCD*, and the FeoB protein, but the relevance of these systems for the uptake of iron by *Campylobacter* is still unclear. Possible virulence factors of *C. jejuni* and *C. coli* are shown in Table 1.

Table 1. Some putative virulence factors of *C. jejuni* and *C. coli*.

gene	virulence factor	function	reference
<i>cadF</i>	CadF	adhesion	Konkel et al., 1997
<i>cdtABC</i>	CDT	bacterial toxin	Johnson & Lior, 1988
<i>ceuBCDE</i>	PBT system	iron acquisition	Richardson & Park, 1995
<i>cheY</i>	CheY	chemotaxis	Yao et al, 1997
<i>chuA</i>	heme transport	iron acquisition	Ridley et al., 2006
<i>ciaB</i>	CiaB	invasion	Konkel et al., 1999
<i>cj0256</i>	transferase	modification of LOS and flagellae	Cullen & Trent, 2010
<i>cj0486</i>	putative sugar transporter	metabolism	Fearnley et al., 2008; Stahl et al., 2011
<i>iamA</i>	invasion-associated DNA marker	invasion	Carvalho et al., 2001
<i>cgfB, wlaN</i>	LOS	adhesion, invasion	Gilbert et al., 2000; Linton et al., 2000
<i>feoB</i>	FeoB	iron acquisition	Naikare et al., 2006
<i>flaA, flaB</i>	flagellum	motility, secretion	Guerry et al., 1991
<i>ggt</i>	GGT	metabolism, colonization	Hofreuter et al., 2006
<i>pldA</i>	PldA	erythrocytolysis	Grant et al., 1997
<i>virB11</i>	pVir plasmid	adhesion, invasion	Bacon et al, 2000

2.4 Epidemiology

2.4.1 General aspects

Campylobacter colonizes the intestinal tract of many different animals, including cattle, cats, dogs, poultry, sheep, swine, and many different species of wild birds, and although this colonization rarely causes any symptoms in the animal hosts, *C. jejuni* in particular is a considerable human pathogen, and the incidence of infections has been increasing in many industrialized nations (Olson et al., 2008). *C. jejuni* infection is common worldwide, with an estimated incidence rate of approximately 500 to 850 infections/100 000 in the USA (Samuel et al., 2004). This conforms to its estimated incidence in the UK, as the incidence calculated on the basis of reported human cases in 2004 was 84 infections/100,000 (EFSA, 2005), and an English study suggested that the actual incidence of *Campylobacter* infection may be almost 10-fold that, or higher (Wheeler et al., 1999). *Campylobacter* is the most commonly reported gastrointestinal pathogen in the European Union (EFSA, 2005; EFSA, 2009). It is also the most common zoonosis in the EU.

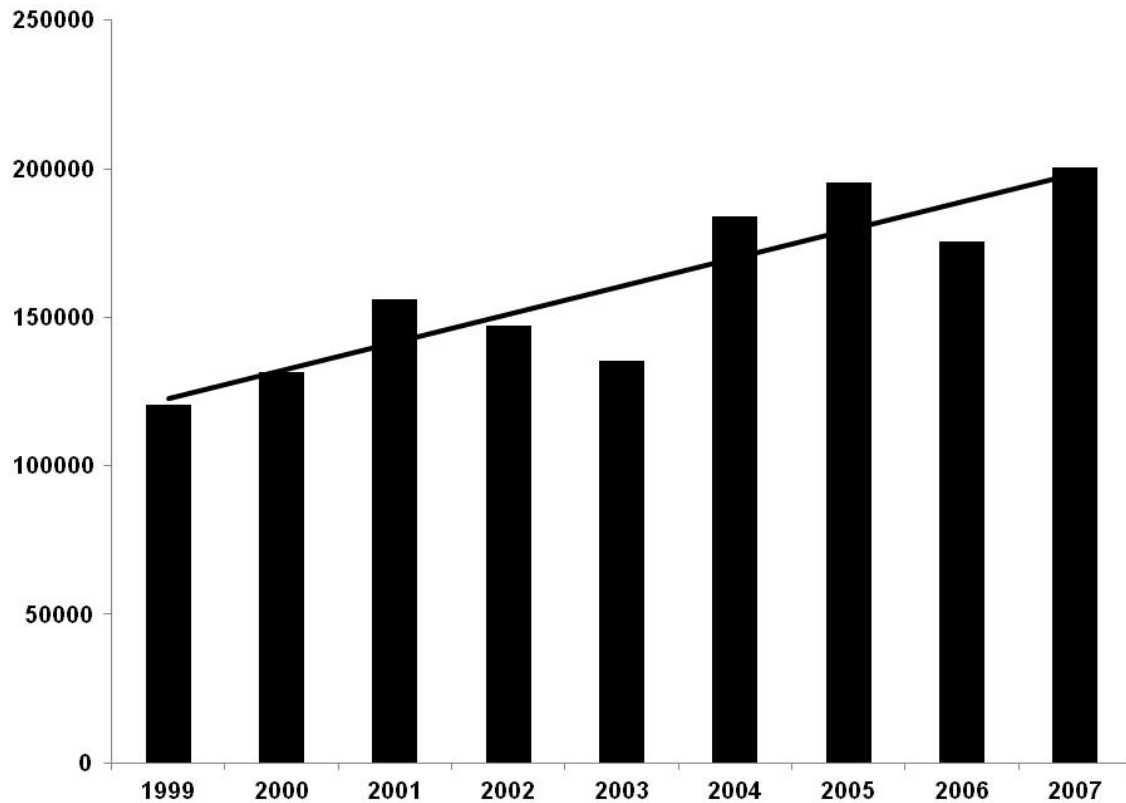


Figure 1 Reported EU cases of *Campylobacter* infection 1999-2007 (EFSA, 2009).

Small children and young adults are especially prone to *Campylobacter* infection (Blaser, 1997; Friedman et al., 2004; Samuel et al., 2004). Men are typically over-represented in patient groups (Butzler & Skirrow, 1979; Samuel et al., 2004; Strachan et al., 2008), and this trend can also be noted at the national level in Finland based on the annual surveillance statistics of the National Institute for Health and Welfare (www3.ktl.fi/stat/).

The seasonal variation of *Campylobacter* infection has been noted in many studies, as typically a peak in *Campylobacter* infection incidence occurs in developed countries in the northern hemisphere during June-August (Butzler & Skirrow, 1979; Rautelin & Hänninen, 2000; Samuel et al., 2004). This seasonal peak is also evident in the most recent EFSA report (EFSA, 2009).

2.4.2 Sources of infection

Eating or handling raw or inadequately cooked poultry meat is an important risk factor for campylobacteriosis (Harris et al., 1986; Neal & Slack, 1997; Studahl & Andersson, 2000). Eating seafood (Harris et al., 1986; Friedman et al., 2004), pork with bones (Studahl & Andersson, 2000), or eating either poultry or non-poultry meat at a restaurant (Friedman et al., 2004), eating at barbecues (Kapperud et al., 1992), eating chicken regularly or outside the

home only (Tam et al., 2009), as well as drinking unpasteurized milk (Studahl & Andersson, 2000; Friedman et al., 2004) are also documented sources of infection. Drinking water from dug wells has been a risk factor (Schönberg-Norio et al., 2004), and because many *Campylobacter* species are commensal organisms of birds, fecal contamination of surface water by birds can increase the risk for subsequent human infection (Broman et al., 2002). Further, domestic pet contact may be associated with increased risk for *Campylobacter* infection (Kapperud et al., 1992; Friedman et al., 2004); especially contact with a dog puppy seems to be associated with *Campylobacter* infection in infants (Tenkate & Stafford, 2001).

The infective dose of *Campylobacter* is extremely low; as few as 500 to 800 cells can cause infection (Robinson, 1981; Black et al., 1988). *Campylobacter* is capable of transition into a “viable, but nonculturable state” under adverse environmental conditions (Rollins & Colwell, 1986), swimming in natural sources of water is a risk factor for *Campylobacter* infection (Schönberg-Norio et al., 2004), and the pathogen can even survive on fresh produce long enough to indirectly cause infection in humans (Kärenlampi & Hänninen, 2004). A recent British study attributed the vast majority of *C. jejuni* infections in humans to chicken and cattle sources (Wilson et al., 2008).

2.4.3 Geographical aspects of *Campylobacter* infection

Considerable variation exists in the distribution of human *Campylobacter* infection incidence among European countries. For example, incidence rates for the Czech Republic versus Spain or Estonia suggest that rate-differences between European countries might even exceed 20-fold (EFSA, 2005). This can in part be explained by differences in their notification systems, such as improvements in the system showed in Austria in 2004 (EFSA, 2005). In Spain, only hospitalized cases are notifiable (EFSA, 2005), and some countries like Greece or Portugal have no surveillance systems at all (EFSA, 2009). Considerable variation exists in origin of confirmed *Campylobacter* infections as well, with the only countries in which imported cases of campylobacteriosis predominate over domestic cases are Finland, Sweden, and Norway (EFSA, 2009). Reported broiler-flock prevalences of *Campylobacter* have been consistently low in these three Scandinavian countries (EFSA 2005, EFSA 2009).

Many notable differences appear in *Campylobacter* epidemiology between developed and developing countries (Table 2). First, average age of the infected patient is lower in developing countries (Blaser et al., 1983; Blaser, 1997). Second, the incidence of *Campylobacter* infection, especially for infants, seems to be significantly higher, with campylobacteriosis even suggested as being a hyperendemic infection in developing countries (Coker et al., 2002). Third, in developing countries, after continuous or repeated infections, populations seem to develop some degree of immunity to campylobacteriosis (Blaser, 1997). Fourth, asymptomatic infections are known to occur in developing countries (Glass et al., 1983), but are very uncommon in developed countries (Blaser et al., 1979; Blaser et al., 1983).

Table 2. Comparison of characteristics of *C. jejuni* infections in developed and developing countries (adapted from Blaser, 1997).

Characteristic	Developed countries	Developing countries
“Infections/lifetime” (estimate according to Blaser)	Very few (“0-1”)	Numerous (“>5”)
Age group mostly affected	“Young adults”	“Children <2 years old”
Typical diarrhea	“Inflammatory diarrhea”	“Simple diarrhea”
“Widespread immunity among adults”	“Absent”	“Present”

2.4.4 Outbreaks

Campylobacter infections in humans are mostly sporadic, and those infected during *Campylobacter* outbreaks are few compared to the total number of patients diagnosed with campylobacteriosis. For example, a study which summarized findings from Communicable Disease Surveillance Centre reports noted that between 1992 and 1994, 706 patients were diagnosed with a *Campylobacter* infection, attributable to one of the 21 outbreaks in England and Wales with *Campylobacter* implicated as the causative organism (Frost et al., 2002). This would suggest that less than 0.5% of *Campylobacter* infections are related to outbreaks.

Outbreaks have usually been attributed to unpasteurized milk (Robinson & Jones, 1981; Lehner et al., 2000; Schildt et al., 2006) or tapwater, and during the last three decades several water-borne outbreaks have caused widespread infection in certain geographically distinct areas (Mentzing, 1981; Palmer et al., 1983; Engberg et al., 1998; Kuusi et al., 2004; Kuusi et al., 2005).

Water-borne outbreaks have typically been associated with groundwater plants, contaminated by *Campylobacter* due to heavy rainfalls (Hänninen et al., 2003). In addition, in Finnish outbreak studies, leaks or failure in well pipes or cross-connections between sewage- and drinking-water pipelines are probable reasons for water supply contamination (Rautelin et al., 1990; Laine et al., 2010).

2.5 Diagnostic methods

Four important types of diagnostic methods allow detection of *Campylobacter* infection: stool and blood culture, serum antibody detection, antigen detection from stools, and DNA-based methods.

Since the development of a selective growth medium for *Campylobacter* by Skirrow (1977), bacterial culture on different selective media, especially charcoal-based, has been an efficient method for detecting and isolating the important stool pathogens *C. jejuni* and *C. coli* (Endtz et al., 1991b; Kulkarni et al., 2002). Although filtration has been proposed as a cost-effective alternative to culture on selective media (Lastovica & le Roux, 2000), it still seems to be relatively insensitive and a more difficult method than either selective culture or PCR identification (Kulkarni et al., 2002).

Serodiagnosis of *Campylobacter* infection is enabled by use of an enzyme-linked immunosorbent assay (ELISA) to detect specific serum antibodies of the IgA, IgG, and IgM classes. To maximize the specificity of serological diagnosis, elevated titers of at least two immunoglobulin classes should probably be chosen for diagnosis of a recent *Campylobacter* infection (Taylor et al., 2004), although this typically results in a loss of sensitivity. In addition, paired serum samples showing at least four-fold antibody titer changes indicate an acute *Campylobacter* infection (Rautelin & Kosunen, 1987). IgG antibodies usually remain elevated for longer periods, up to several months in some patients (Kaldor et al., 1983; Rautelin & Kosunen, 1987), and determining the IgG titer alone is insufficient for diagnosis of an acute infection (Kaldor et al., 1983; Rautelin & Kosunen, 1987; Taylor et al., 2004). On the other hand, elevated antibody titers of IgA and IgM classes have commonly been detectable in patients with an active *Campylobacter* infection (Kaldor et al., 1983; Rautelin & Kosunen, 1987; Black et al., 1988; Strid et al., 2001).

Enzyme immunoassay-based stool antigen tests have been developed for rapid detection of *Campylobacter* infection, but clinical use of rapid antigen tests is limited because of low sensitivity (Tissari & Rautelin, 2007), and the need for later verification of negative test results (Hindiye et al., 2000; Tissari & Rautelin, 2007).

PCR assays enable rapid identification of *C. jejuni* and *C. coli* to species level (Linton et al., 1997; Denis et al., 1999; Gilbert et al., 2003), but the cost and workload of PCR assays, as well as the lack of a bacterial isolate for further analyses, have been pointed out as disadvantages, compared to culture techniques (Kulkarni et al., 2002).

Multiplex PCR assays allow the exact identification of several enteric pathogens at the same time. A study in which different PCR assays were compared for sensitivity and specificity found that most of the methods were reasonably trustworthy and performed equally (Debruyne et al., 2008). Recently, stool culture was shown to be clearly less sensitive to multiplex-PCR (Bessède et al., 2011).

In addition to these methods, recent results indicate that matrix-assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), available in a rapidly increasing number of clinical laboratories, should be a reliable and sensitive method for identifying *Campylobacter* isolates (Martiny et al., 2011).

2.6 Antimicrobial susceptibility

2.6.1 Antimicrobial susceptibility testing

Three commonly used methods are suitable for determining whether a bacterial isolate is susceptible or resistant to a certain antimicrobial agent: minimal inhibitory concentration (MIC) testing which can be performed by either an agar or a broth dilution susceptibility test, disk diffusion, and the combination of the disk diffusion method and the exact dilution method, the E-test.

The MIC value of an antimicrobial agent is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution-susceptibility test. If the E-test method is used, the corresponding value can be seen at the point where the zone of growth inhibition intersects the strip with a gradient of the antimicrobial agent tested (PDM Epsilon meter, AB Biodisk, Solna, Sweden). While an agar dilution method, using Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and *C. jejuni* ATCC 33560 as a quality-control strain (McDermott et al., 2004), has been developed for ciprofloxacin, doxycycline, erythromycin, gentamicin, and meropenem, and been recognized by the Clinical and Laboratory Standards Institute (CLSI) as the standard antimicrobial susceptibility testing method for thermophilic *Campylobacter* (CLSI, 2005), the less time-consuming and less expensive method of broth dilution is an alternative for determining MIC values (Luber et al., 2003). For broth dilution testing of *Campylobacter*, Mueller-Hinton broth with 2.5 to 5% lysed horse blood has been approved for determining MIC values (CLSI, 2006).

A disk diffusion method for determining antimicrobial susceptibility of *Campylobacter* involves use of Mueller-Hinton agar with 5% sheep blood inoculated with a direct colony suspension of the test organism - equivalent to a 0.5 McFarland standard - and paper discs containing specific concentrations of the antimicrobial agent are applied to the surface of the agar. After this, incubation for 24 to 48 hours at optimal temperature and atmosphere follows (CLSI, 2006). The antimicrobial agent diffuses into the agar, inhibiting the growth of bacteria which are susceptible to the antimicrobial agent, and this can be seen as a zone of inhibition around each disc.

In addition, study of plasmids or genes associated with resistance can aid in understanding of antimicrobial resistance mechanisms.

2.6.2 Antimicrobial susceptibility and resistance among *Campylobacter*

Macrolides and fluoroquinolones have been the primary drugs of choice for campylobacteriosis (Aarestrup & Engberg, 2001), but increasing antimicrobial resistance, especially to fluoroquinolones, is becoming an important health issue. The mechanisms by which *Campylobacter* develops resistance against fluoroquinolones and macrolides are well known. Fluoroquinolone resistance is attributed to mutations in genes encoding DNA gyrase

(*gyrA*), more specifically at positions Thr-86, Asp-90, and Ala-70 (Wang et al., 1993; Ge et al., 2005). Macrolide resistance is mostly considered to be due to mutations in the 23S rRNA gene (Gibreel et al., 2005). Another mechanism with a role in the development of macrolide resistance in *Campylobacter* is the *Campylobacter* multidrug efflux pump (CmeABC) (Cagliero et al., 2005; Gibreel et al., 2007); modifications in the ribosomal proteins L4 and L22 may also affect the susceptibility to macrolides (Cagliero et al., 2006).

In the beginning of the 1990's, an increase in quinolone resistance in human *Campylobacter* isolates in the Netherlands coincided with increased use of fluoroquinolones in both human and veterinary medicine (Endtz et al., 1991a). Shortly thereafter, in Finland, and later in other countries, many separate reports confirmed that quinolone resistance had increased significantly within only one decade (Rautelin et al., 1991; Reina et al., 1994; Murphy et al., 1996; Smith et al., 1999; Gupta et al., 2004). This, however, has not been the case among domestically acquired infections in Finland (Rautelin et al., 2003; Schönberg-Norio et al., 2006), where fluoroquinolones are not used in poultry production (Hänninen et al., 1999; Rautelin & Hänninen, 2000). In fact, few domestic *Campylobacter* isolates in Finland are resistant to fluoroquinolones, while the majority of isolates originating from countries with a high prevalence of quinolone resistance, for example Spain or Thailand, are resistant to fluoroquinolones (Rautelin & Hänninen, 2000).

Following the rapid emergence of fluoroquinolone resistance among *Campylobacter*, interest has increased in studying whether or not the decreased antimicrobial susceptibility affects the clinical outcome of disease. Results have been contradictory (Table 3).

Table 3. Impact of ciprofloxacin resistance on clinical outcome of *Campylobacter* infection.

Country	Time period	Isolates studied (N)	Ciprofloxacin resistance	Conclusion	Reference
Denmark	1996-2000	3471	22%	Quinolone resistance associated with increased risk for adverse events	Helms et al., 2005
USA	1998-1999	858	11%	Longer-lasting diarrhea among patients with ciprofloxacin-resistant isolates	Nelson et al., 2004
England and Wales	2000-2001	3489	19%	No difference in hospital admission or mean length of illness	The <i>Campylobacter</i> Sentinel Surveillance Scheme Collaborators, 2002
Re-analysis of studies from USA and UK	1998-2001			Infections caused by fluoroquinolone-resistant <i>Campylobacter</i> not more severe than those caused by susceptible isolates	Wassenaar et al., 2007
Australia	2001-2002	585	2%	Infection with ciprofloxacin-resistant <i>C. jejuni</i> not resulting in a more severe illness	Unicomb et al., 2006
Denmark	2001-2002	467	18%	Patients with quinolone-resistant <i>C. jejuni</i> infections with longer mean duration of illness	Engberg et al., 2004

Macrolide resistance is typically detected in *C. coli* strains, possibly because tylosin is a growth-promoter in pigs in some countries (Engberg et al., 2001; Gibreel et al., 2006). Interestingly, macrolide resistance is not usually detected in Finland (Rautelin et al., 2003), and the situation appears to be similar in Sweden (Rönner et al., 2004) and Japan (Gibreel et al., 2006).

When determining the therapeutic effect of an antimicrobial agent against a certain micro-organism, one problem is the lack of international standards and guidelines (Kahlmeter et al., 2003). The clinical breakpoints for ciprofloxacin and erythromycin are available for comparison from two major committees, the CLSI (CLSI, 2009) in the USA and the EUCAST (www.eucast.org) in Europe. For ciprofloxacin, a *Campylobacter* strain is considered resistant if the MIC-value is ≥ 4 $\mu\text{g/ml}$ according to CLSI guidelines, and for erythromycin, the breakpoint for resistance is ≥ 32 $\mu\text{g/ml}$. These breakpoints are somewhat higher than the EUCAST guidelines: for ciprofloxacin MIC-values ≥ 2 $\mu\text{g/ml}$, and for erythromycin MIC-values ≥ 8 $\mu\text{g/ml}$. In addition to clinical breakpoints, the EUCAST also

monitors resistance development, which is expressed with epidemiological cut-off values (Kahlmeter et al., 2003; www.eucast.org).

2.7 Clinical aspects of *Campylobacter* infection

2.7.1 Enteritis

The typical clinical signs after ingestion, and subsequent intestinal colonization of *C. jejuni* or *C. coli* indicate an inflammatory response in the affected human host; endoscopy may reveal macroscopically visible enteritis affecting the jejunum, ileum, and colon (Blaser et al., 1980; Blaser & Reller, 1981). The incubation period is normally 3 to 5 days but can sometimes be shorter or significantly longer, even up to 10 days (Butzler & Skirrow, 1979; Butzler, 2004). Nonspecific prodromal symptoms, for example headache, myalgia, and fever, may precede the diarrheal illness and last approximately one day (Blaser, 1997). Watery diarrhea, fever, and abdominal cramps and pain which sometimes mimic those of acute appendicitis, are common features of *Campylobacter* enteritis, and blood and leukocytes in the stools are relatively frequent, as well (Blaser, 1997; Rautelin & Hänninen, 2000; Butzler, 2004). The diarrheal phase usually lasts for a few days, and while patients often recover from the disease within a week, the symptoms can sometimes be prolonged or re-appear (Blaser, 1997; Rautelin & Hänninen, 2000; Butzler, 2004). The timing of clinical symptoms, in relation to stool culture and serology, is presented in Figure 2.

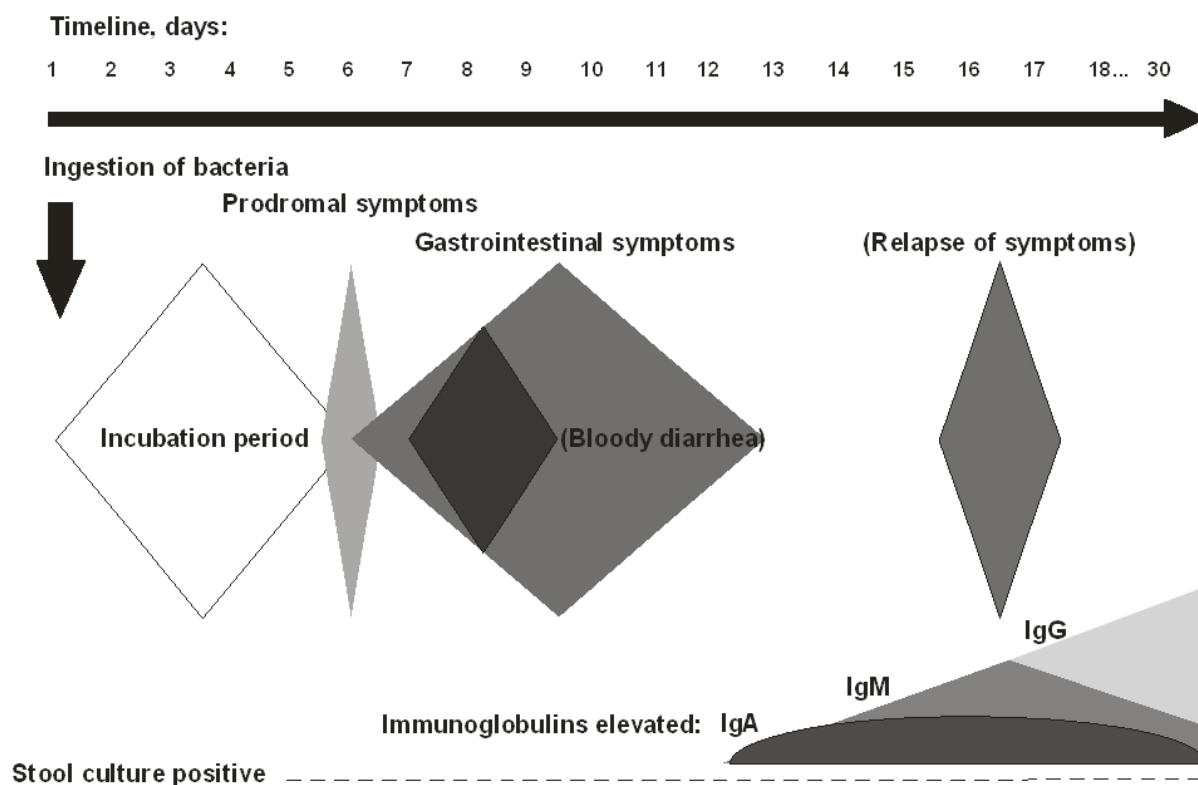


Figure 2 Timeline of an acute *Campylobacter* infection.

Patients with a clinical picture involving bloody stools or vomiting are likely to have a longer duration of illness and may require hospital treatment more often than others (Gillespie, et al., 2006).

2.7.2 Bacteremia

As noted by Butzler (2004), *Vibrio fetus* (*C. fetus*) was the first *Campylobacter* species to be detected and identified from human blood cultures; Vinzent *et al.* (1947) were able to isolate the pathogen from a pregnant woman well before the ‘related vibrios’ were recognized as human pathogens. However, it was later confirmed that *C. jejuni* and *C. coli* are also important pathogens causing *Campylobacter* bacteremia (Blaser et al., 1986; Skirrow et al., 1993). In fact, many studies have shown that the thermophilic *Campylobacter* species are isolated more often than *C. fetus* from human blood samples (Skirrow et al., 1993; Schønheyder et al., 1995; Nielsen et al., 2010a; Fernández-Cruz et al., 2010). Although *Campylobacter* are not commonly associated with invasiveness, it has been estimated that bacteremia can be detected in approximately 1% of patients with *Campylobacter* infection (Samuel et al., 2004). On the other hand, as blood cultures are usually taken only from those with febrile gastroenteritis who have been referred to, or seek hospital care, the actual incidence of *Campylobacter* bacteremia may be significantly higher.

Patients with *Campylobacter* bacteremia typically experience the same array of symptoms as do patients with uncomplicated enteritis: e.g. fever, malaise, and gastrointestinal symptoms, although diarrhea sometimes occurs in only about one third of patients (Pigrau et al., 1997; Pacanowski et al., 2008). Noteworthy also is that many *Campylobacter* bacteremia patients may have concomitant cellulitis, and although skin infection mostly seems to be attributed to *C. fetus* (Pigrau et al., 1997; Pacanowski et al., 2008; Gazonne et al., 2008), some report cellulitis as occurring concurrently with *C. jejuni* bacteremia (Tee & Mijch, 1998; Monselise et al., 2004).

Differences between clinical disease caused by *C. fetus* or by either *C. jejuni* or *C. coli* include the findings that *C. fetus* infection is not commonly associated with enteritis and diarrhea, and patients with *C. fetus* infection are often elderly or immunocompromised (Guerrant et al., 1978; Schmidt et al., 1980; Francioli et al., 1985; Gazonne et al., 2008). In recent European studies performed at hospitals in France and Spain, the mortality of *Campylobacter* bacteremia patients has been 10 to 15% (Pigrau et al., 1997; Pacanowski et al., 2008; Fernández-Cruz et al., 2010), while a Danish study found mortality to be as low as 4% (Nielsen et al., 2010a). *Campylobacter* bacteremia mortality data are presented in Table 4.

Table 4. Species distribution and mortality rates from *Campylobacter* bacteremia studies (adapted data from Fernández-Cruz et al., 2010).

Location	Study period	N of patients	<i>Campylobacter</i> species	Mortality	Reference
England and Wales	1981-1991	394	<i>C. jejuni/coli</i> 89%	NA	Skirrow et al., 1993
South Africa	1991-1994	19	<i>C. jejuni</i> 100%	16%	Reed et al., 1996
Australia	1985-1995	21	<i>C. jejuni</i> 100%	14%	Tee & Mijch, 1998
Barcelona, Spain	1979-1996	58	<i>C. jejuni/coli</i> 83%	10.5%	Pigrau et al., 1997
Paris, France	2000-2004	183	<i>C. jejuni/coli</i> 39%	15%	Pacanowski et al., 2008
Denmark	1995-2004	46	<i>C. jejuni/coli</i> 91%	4%	Nielsen et al., 2010a
Madrid, Spain	1985-2007	68	<i>C. jejuni/coli</i> 78%	15%	Fernández-Cruz et al., 2010

Well-characterized clinical materials for which *C. jejuni* bacteremia isolates are available are scarce, and therefore information regarding the possible differences in the virulence factor profiles of blood and stool isolates is limited. Nielsen et al found no association between the virulence genes *iam*, *cdtB*, *capA*, or *virB*, and *C. jejuni* blood isolates, as compared to stool culture isolates (Nielsen et al., 2010b).

2.7.3 Other extra-intestinal infections

In addition to bacteremia, other extra-intestinal infection foci for *C. jejuni/ coli* include cellulitis (Tee & Mijch, 1998; Monselise et al., 2004), cholecystitis (Blaser et al., 1986), meningitis (Blaser et al., 1986; Goossens et al., 1986), peritoneal infection, and abscesses, as well as urinary tract infection (Blaser et al., 1986). In addition, respiratory tract infections occur in immunocompromised patients with bacteremia (Tee & Mijch, 1998; Fernández-Cruz et al., 2010), although microbiological verification is lacking. Increased bacterial virulence, such as serum resistance, may need to occur before *Campylobacter* causes extraintestinal infection in normal, healthy human hosts (Blaser et al., 1986).

2.7.4 Guillain-Barré syndrome

As early as in 1982, two separate case reports suggested a link between an acute inflammatory polyneuropathy, termed GBS, and *C. jejuni* infection (Rhodes & Tattersfield, 1982; Molnar et al., 1982). GBS has since been shown to be a severe complication of *C.*

jejuni infection (Mishu et al., 1993; Rees et al., 1995), and the estimated risk for GBS after *Campylobacter* infection is approximately 1-3/10 000 cases of campylobacteriosis (McCarthy & Giesecke, 2001; Tam et al., 2006).

GBS is an autoimmune condition, in which the patients develop antibodies against gangliosides, most typically GM1, resulting in axonal degeneration through either demyelination or direct axonal damage. Carbohydrate mimicry between GM1 and *C. jejuni* LOS leading to the development of such cross-reactive antibodies has been shown to occur under experimental conditions in rabbits (Yuki et al., 2004). Some strains of *C. jejuni* have sialylated LOS (Parker et al., 2005). These sialylated carbohydrates closely resemble some of the human gangliosides (Gilbert et al., 2002). Some *C. jejuni* serotypes have been associated with the development of GBS, particularly Penner serotype O:19 (Allos et al., 1998). Sialylated LOS is more prevalent than nonsialylated LOS in GBS-associated *C. jejuni* strains (Koga et al., 2006), but not all *C. jejuni* isolated from patients who developed GBS have sialylated LOS (Godschalk et al., 2007). Certainly, many other bacterial and host factors might affect the pathogenesis of GBS, as molecular mimicry at present insufficiently explains why certain patients develop GBS.

2.7.5 Other sequelae

Antecedent *Campylobacter* infection is considered a risk factor for reactive arthritis (Hannu et al., 2002; Locht & Krogfelt, 2002). The prevalence of reactive arthritis among patients examined within a median of 11 weeks after the onset of *Campylobacter* infection was 7% (Hannu et al., 2002). However, self-reported musculoskeletal symptoms are more prevalent, and studies have shown that between 16% and 39% of patients, after *Campylobacter* infection, have reported joint symptoms which could indicate arthritis or tendinitis (Bremell et al., 1991; Locht & Krogfelt, 2002; Schönberg-Norio et al., 2010). In one study, a longer duration of diarrhea was significantly associated with later arthralgia (Locht & Krogfelt, 2002). Antimicrobial treatment does not prevent the development of reactive joint symptoms (Locht & Krogfelt, 2002; Schönberg-Norio et al., 2010).

An association is known between bacterial enteritis and the development of irritable bowel syndrome (IBS), and in studies where *Campylobacter* infection has been prevalent, the incidence of IBS as a post-infectious complication is estimated to be between 4 and 13% (Rodríguez et al., 1999; Spiller, 2007).

2.7.6 Treatment

It is generally accepted that *Campylobacter* enteritis is a self-limited disease which resolves spontaneously in the majority of infected patients, and that antimicrobial treatment should be reserved primarily for immunocompromised individuals and patients with severe symptoms (Blaser, 1997; Rautelin & Hänninen, 2000; Butzler, 2004). For a long period of time, fluoroquinolones were considered the appropriate treatment for campylobacteriosis, but due to the increasing fluoroquinolone resistance which has developed rapidly in many parts of the

world, these antimicrobials are no longer considered to be optimal empirical treatment of *Campylobacter* infections (Allos, 2001). Traditionally, erythromycin has been regarded as a primary choice of treatment for campylobacteriosis, and has been presented as the most favorable option for treating uncomplicated intestinal illness (McNulty, 1987). Although some studies have suggested that antibiotic treatment shortens the duration of illness (Salazar-Lindo et al., 1986; Kuschner et al., 1995), double-blind placebo-controlled trials have shown that differences in the duration of symptoms are minimal between the patients treated with erythromycin and those who receive placebo (Anders et al., 1982; Mandal et al., 1984). In addition, a recent meta-analysis showed that the beneficial effect of antibiotics is limited, possibly only shortening the duration of gastrointestinal symptoms by little more than a day (Ternhag et al., 2007). In a recent Finnish study, appropriate antimicrobial therapy, based on the MIC values of the isolates, shortened the duration of illness only when initiated within two days after onset of symptoms (Schönberg-Norio et al., 2010). However, several studies have clearly shown that treatment with erythromycin effectively eradicates *Campylobacter* from patients' stools (Anders et al., 1982; Mandal et al., 1984; Williams et al., 1989).

When considering antimicrobial treatment for *Campylobacter* bacteremia, antimicrobial susceptibility of the blood culture isolate is of even greater importance and should naturally be taken into consideration when the therapy is planned. The beneficial effect of appropriate antimicrobial treatment on the outcome of *Campylobacter* bacteremia has been either evident, particularly for species other than *C. fetus* (Pacanowski et al., 2008), or non-existent (Fernández-Cruz et al., 2010).

While not regarded as primary antimicrobial agents for campylobacteriosis treatment, carbapenems may be an alternative treatment for severe *Campylobacter* infection, based on susceptibility-testing results (Pacanowski et al., 2008; Fernández-Cruz et al., 2010). Even multi-drug-resistant *Campylobacter* isolates are still susceptible to carbapenems, at least *in vitro* (Lehtopolku et al., 2010). In Finland, *C. jejuni* and *C. coli* have been highly susceptible to aminoglycosides (Rautelin et al., 1991; Lehtopolku et al., 2010). Further, based on the susceptibility patterns of the isolates tested, clindamycin (Wagner et al., 2003), tetracyclines (Wagner et al., 2003), and tigecycline (Lehtopolku et al., 2010) are possible alternatives for antimicrobial therapy of *Campylobacter* infection.

2.7.7 Immunological aspects

Several immunological factors affect both the susceptibility of the human host to *Campylobacter* infection, as well as its outcome and course.

Innate immunity to *Campylobacter* infection can be divided into the defense mechanisms of the gastrointestinal tract and those defenses present in the intestinal submucosa and systemic circulation (Iovine, 2008). The gastrointestinal tract is an important first-line defense barrier, and important innate immune defenses include salivary nitrite, gastric acid, the microbiome of the large intestine, mucins, defensins, Toll-like receptors (TLRs), and, to a certain degree, bile (Iovine, 2008).

Interestingly, bile has triggered CiaB expression in *C. jejuni in vitro* (Rivera-Amill et al., 2001). On the other hand, *Campylobacter* is generally bile-resistant, and cholecystitis sometimes occurs as a complication during or after campylobacteriosis.

Lipopolysaccharides are known ligands to TLR2 and TLR4 on the surface of dendritic cells (DCs) (Moll, 2003). *C. jejuni* activates DCs through TLR4 signalling (Rathinam et al., 2009), and sialylation of LOS may enhance this activation (Kujif et al., 2010), and possibly even lead to the development of anti-ganglioside antibodies (Kujif et al., 2010), which are associated with the neurological complication GBS (Yuki et al., 2004).

The inflammatory response leading to neutrophil and macrophage recruitment is the basis for the systemic defensive mechanisms of the innate immunity mechanism in the human host. Proinflammatory responses of cytokines and chemokines as well as phagocyte recruitment and the activation of complement are essential innate immunity defense mechanisms (Janssen et al., 2008). Very few studies show any association between *Campylobacter* bacteremia and sensitivity to normal human serum (NHS); *C. fetus* is essentially serum-resistant, while the serum susceptibility of *C. coli* and *C. jejuni* varies considerably (Blaser et al., 1985; 1986). The complement-mediated killing of serum-susceptible isolates is important for restricting the access of pathogens as well as of commensal organisms to the bloodstream; however, *C. jejuni* blood isolates are not always particularly serum-resistant (Blaser et al., 1985). Sialylated LOS may be important for the development of serum resistance in *C. jejuni* (Guerry et al., 2000), while another group speculates that LOS plays a role in resisting some defensins and proteins, and that the capsule would instead be associated with serum resistance (Keo et al., 2011).

In addition to the diagnostic usefulness of understanding the activation of humoral immune responses during and following *Campylobacter* infection, the role of the adaptive immune system in disease severity and the development of cross-reactive immunity at an individual level has been studied (Janssen et al., 2008). Vast epitopic variation in immunogenic *Campylobacter* surface components such as flagellin, the major outer membrane protein (MOMP), capsule structures, and LOS, is mostly attributed to the fact that these antigenic regions are particularly poorly conserved (Havelaar et al., 2009). Due to the antigenic diversity, it is therefore probable that although the presence of *Campylobacter*-specific antibodies indicates exposure to infection, the possible protection provided by these immunoglobulins is mostly limited to homologous *Campylobacter* strains (Havelaar et al., 2009). Hence, it seems that repeated exposure to heterologous strains may be a requirement for development of cross-reactive immunity.

When considering the immunological importance of various immunoglobulins, it is worth noting that empirical evidence for at least two major classes of immunoglobulins, IgA and IgG, exists. The protective effect of IgA, and of secretory IgA (sIgA) in particular, has been shown (Mégraud et al., 1990). Furthermore, children with elevated IgG levels in developing countries are less likely to develop the more severe symptoms like bloody diarrhea (Blaser et al., 1985; Blaser et al., 1986; Janssen et al., 2008).

In accordance with differences between the characteristics of *C. jejuni* infection in different parts of the world (Table 2), findings reflecting the development of partial immunity against this heterogenous species have been presented in a study by Miller et al., in which common serotypes were over-represented among patients under age 40, while uncommon serotypes were over-represented among the older (Miller et al., 2005).

2.7.8 General health burden and cost

Among estimates of the costs and health care burden due to campylobacteriosis, in Sweden (population approximately 8 million) the total cost, with both direct healthcare costs and indirect, patient-, and loss-of-earning-related costs included, came to as much as 179 to 352 million Swedish crowns (data from Government Offices of Sweden). This estimate is based on a prevalence multiplier of 9.02, and the direct healthcare costs accounted for approximately 15% of total cost. The cost of acute campylobacteriosis to patients and the health services in the UK, excluding the costs of post-infectious complications, was estimated to be around 20 million UK pounds (personal communication, Tam CC, 2012). An earlier estimate from the UK attributed roughly 10% of the total cost of intestinal infectious diseases (743 million UK pounds) to *Campylobacter* infection, indicating that the most significant proportion of the socio-economic impact of bacterial enteropathogens was due to campylobacteriosis (Roberts et al., 2003).

3. Aims of the study

Bacterial traits which could lead to a more severe outcome of *Campylobacter* infection are not well known. Although experimental studies have proposed a number of possible virulence factors as important for colonizing and invading the human host, the need is evident for studies with non-selected clinical materials with patient data to study these findings' relevance. The aim of this PhD thesis was to clarify whether factors, involving patients' and bacterial characteristics, are important for development of a more severe *Campylobacter* infection. The specific aims of the study were:

1. To study whether ciprofloxacin-resistant *C. jejuni* or *C. coli* isolates cause more severe disease.
2. To screen for putative virulence factors of *C. jejuni* isolates and to study their possible association with disease outcome, or with severity of symptoms.
3. To characterize *C. jejuni* and *C. coli* bacteremia patients as well as the course and outcome of the disease.
4. To study the bacterial characteristics of *C. jejuni* blood culture isolates, and more specifically;

whether certain MLST CCs are associated with bacteremia,

whether serum resistance is needed for *C. jejuni* to cause bacteremia,

whether sialylated LOS is prevalent among *C. jejuni* bacteremia isolates,

and whether any of these bacterial characteristics are associated with *C. jejuni* bacteremia in certain patient subgroups, for example, those with severe underlying diseases.

4. Materials and methods

4.1 Patient data

4.1.1 *Campylobacter* enteritis patients (I, II)

Patients from the Helsinki-Uusimaa region for whom *Campylobacter*-positive stool samples were detected at Helsinki University Central Hospital Laboratory (HUSLAB) from July 1 through December 31, 2006 were asked to participate in the study. Questionnaires, along with information about the study, went to the physicians who had referred the patients to laboratory tests, and the physicians forwarded the questionnaires to the patients, who completed a questionnaire and returned it with their written consent. For children, parents or guardians filled in the questionnaire, and gave their informed consent.

As 206 patients in Studies I and II returned the questionnaire, the response rate was 57% (Figure 3). Further, 14 patients were excluded because the isolates were lacking or because they had a co-infection. A total of 192 patients with *C. jejuni*/*C. coli* enteritis were included in Study I, and 166 patients with *C. jejuni* enteritis in Study II.

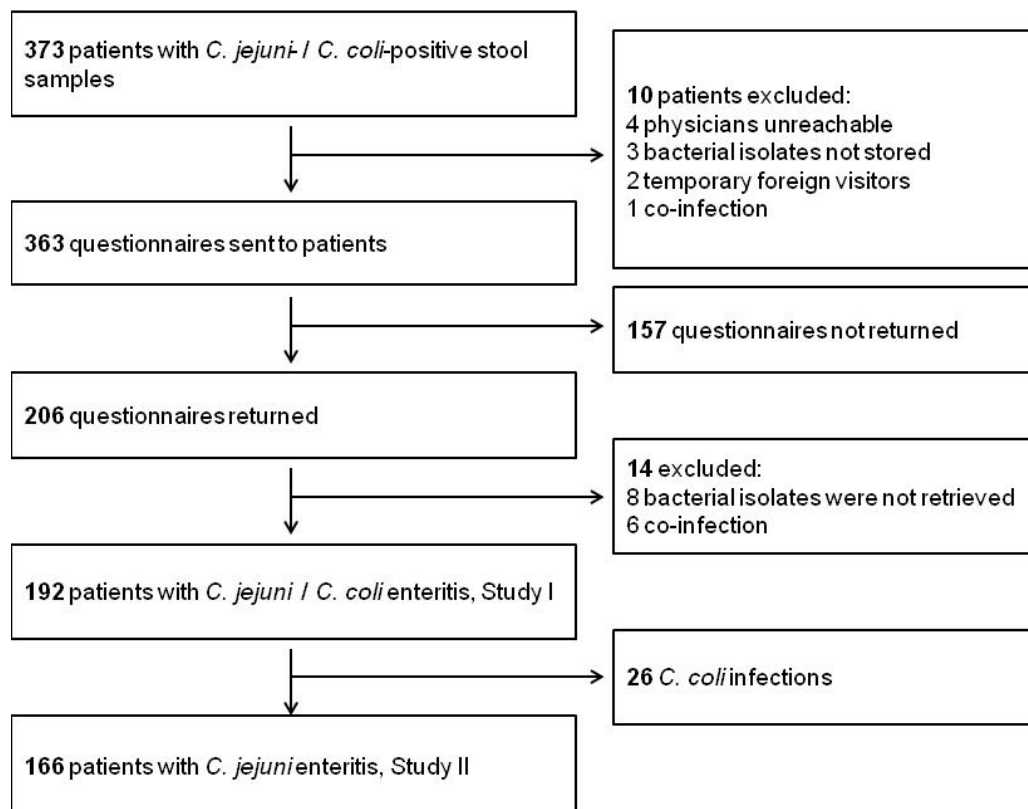


Figure 3 Flow chart of patients included in *Campylobacter* enteritis Studies I and II.

Questionnaires

The questionnaires contained detailed questions about occurrence, timing, and time-period of symptoms, background information such as underlying diseases or medical conditions, foreign travel, and medication. In addition, they asked for data on treatment and post-infectious complications, with emphasis on timing and length of antimicrobial therapy.

Occurrence of the following symptoms was specifically inquired; diarrhea, watery stools, bloody stools, slimy stools, headache, abdominal pain, fever, chills, cramps, nausea, vomiting, myalgia, and weight-loss. The patients filled in the length of time with diarrhea, headache, abdominal pain, fever, nausea, vomiting, myalgia, and other self-reported symptoms, and whether they had prodromal symptoms, and their timing.

Travel information covered any foreign or domestic travel within two weeks prior to onset of symptoms. The patient's own opinion regarding the source of infection, as well as suspicion of horizontal disease transmission, was included. The patients reported the length and timing of any hospital treatment for *Campylobacter* infection.

The patients stated whether they had used any medication in the month prior to onset of illness, and described their use of antibiotics, cortisone, and antacids or any antibiotic treatment for *Campylobacter* infection, the names of the drugs, and length and timing of antimicrobial therapy.

As to underlying diseases, these specifically were diabetes, gastric, intestinal, cardiovascular, and rheumatic diseases, as well as diseases increasing susceptibility to infections, and any episodes of *Campylobacter* infection.

Occurrence of post-infectious symptoms was included, such as arthralgia, tendinitis, eye symptoms indicating conjunctivitis/iritis, and skin blisters, nodules and rashes, angina pectoris, arrhythmias, prolonged intestinal symptoms, as well as neurological symptoms. The patients stated whether they had been treated at a hospital or had received antimicrobial therapy for post-infectious symptoms.

Finally came total length of illness and the patients' own descriptions of their convalescence.

4.1.2 *Campylobacter* bacteremia patients (III, IV)

The *Campylobacter* bacteremia study was a nationwide retrospective study, essentially covering all *C. jejuni* and *C. coli* bacteremia cases diagnosed in Finland within the 10-year period 1998-2007. The patients were identified from the National Infectious Diseases Register (NIDR), as well as from the blood culture isolates received from microbiological laboratories throughout the country. In total, 119 *Campylobacter* bacteremia cases were reported to the NIDR in 1998-2007, and 95 bacterial isolates were obtained from microbiological laboratories. However, as comparison of patient information and characteristics of *Campylobacter* bacteremia isolates was of special interest, only those cases

for which all information and bacterial isolates were available were included. A flow chart for the selection and exclusion of patients in Studies III and IV is in Figure 4.

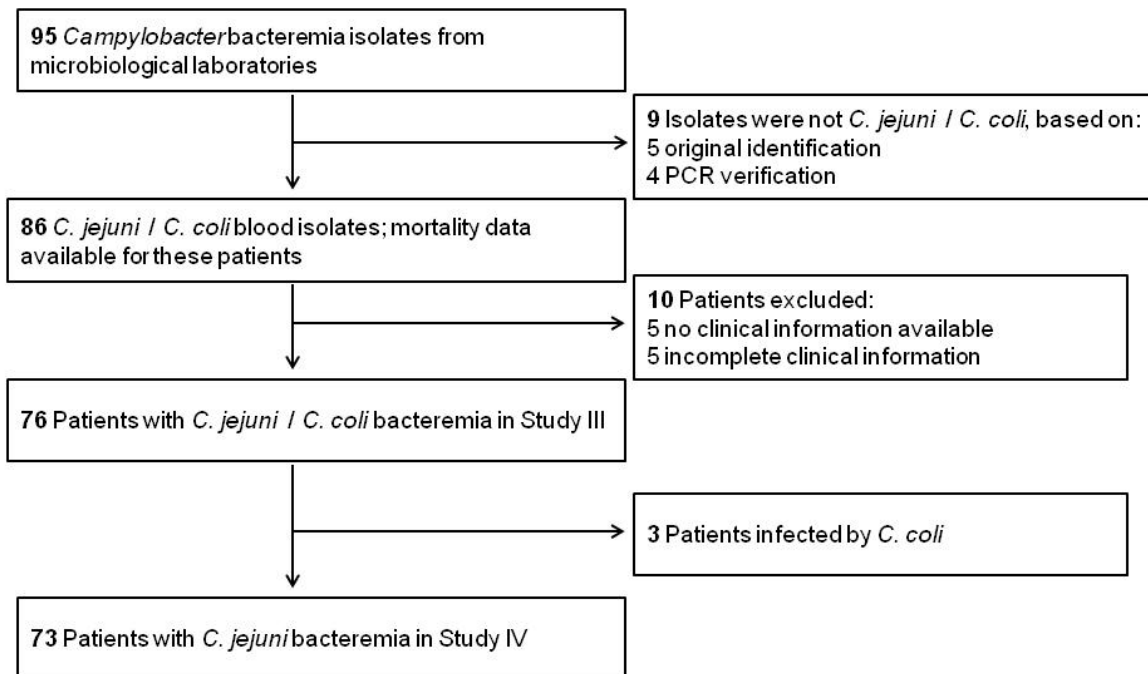


Figure 4 Flow chart of patients included in the *Campylobacter* bacteremia Studies III and IV.

Patient information

The hospital treatment records of patients are routinely stored in hospital archives. From such archives throughout Finland, detailed information was reviewed regarding the course of disease, symptoms, and treatment. The time-range for follow-up of patient data was from admission until one year after the *Campylobacter*-positive blood culture. Information regarding background, symptoms, laboratory results, course of disease, treatment, outcome, and follow-up, when available, was documented for all patients.

Relevant background information included documented antibiotic use before the bacteremic episode, foreign travel within two weeks before the onset of symptoms, initial symptoms, and referral diagnoses.

Relevant information regarding all available patient symptoms from hospital treatment records was systematically documented as follows: occurrence and length of diarrhea and fever, and occurrence of bloody stools, slimy stools, watery stools, abdominal cramps, abdominal pain, vomiting, myalgia, and arthralgia. Fever was defined as an axillary or rectal temperature of $>37.9^{\circ}\text{C}$ documented at least once during the hospital stay; the highest

measured temperature was also noted, as were blood pressure levels (at admission, and lowest), and tachycardia at admission.

All relevant laboratory test results were documented, and levels at admission to hospital and discharge from hospital, as well as lowest and highest levels. Laboratory test results included hemoglobin, leukocyte, and platelet count, alkaline phosphatase (ALP), amylase, bilirubin, *Campylobacter* antibodies, C-reactive protein (CRP), calcium, creatinine, potassium, sodium, and transaminases ALAT and ASAT, human leukocyte antigen B27 (HLA-27), as well as stool cultures and urine findings.

Detailed hospital treatment information specifically involved length of hospital treatment, need for intensive care unit (ICU) treatment, length and timing of antimicrobial therapies, and radiological examinations such as computer tomography (CT) scan, echocardiography, magnetic resonance (MR) imaging, and thorax radiography.

Data regarding underlying diseases were analyzed and scored according to the Charlson index (Table 5) (Charlson et al., 1987). The Charlson index score is cumulative: a Charlson index score ≥ 1 indicates that the underlying disease is significant, and ≥ 2 that the underlying disease is severe.

Table 5. Scoring of underlying diseases according to the Charlson weighted index of comorbidity (adapted data from Charlson et al., 1987).

Condition	Weight
Cerebrovascular disease	1
Chronic pulmonary disease	1
Congestive heart failure	1
Connective tissue disease	1
Dementia	1
Diabetes	1
Mild liver disease	1
Myocardial infarction	1
Peripheral vascular disease	1
Ulcer disease	1
Any tumor	2
Hemiplegia	2
Leukemia	2
Lymphoma	2
Moderate or severe renal disease	2
Moderate or severe liver disease	3
Metastatic cancer	6
AIDS	6

Information regarding antimicrobial treatment after hospitalization, post-infectious complications, and possible relapse or re-admission to hospital was noted, and records of hospital visits were reviewed. Inflammatory, neurological, or rheumatic symptoms, indicating arthritis, iritis, IBS, paralysis, or tendinitis, as well as possible surgical procedures, were noted from the next year following the bacteremic episode. Duration of sick-leave was documented. Any results from colonoscopy or gastroscopy were included. Mortality data

came from Statistics Finland, from the treatment period for *Campylobacter* bacteremia and the following year.

4.2 Bacterial isolates

4.2.1 Species identification

Determination of *Campylobacter* enteritis isolates to species level was based on a hippurate hydrolysis test; positive isolates were classified as *C. jejuni* and negative isolates as *C. coli* (I, II). Furthermore, characteristic PFGE patterns for a subgroup of the isolates confirmed typing to species level. In the *Campylobacter* bacteremia study, isolates were genotyped by species-specific PCR analyses for *C. jejuni* and *C. coli* (III). For the initial analyses, primers were designed to target the *mapA* gene in the *C. jejuni* genome and the *ceuE* gene in the *C. coli* genome, with findings verified by additional analyses targeting hippuricase in *C. jejuni* and aspartokinase in *C. coli* (III). The reference strains *C. jejuni* NCTC 11168, *C. jejuni* 811176, and *C. coli* LMG6440 served as positive controls; negative controls included one with no added template DNA for all bacteremia isolates, as well as the *C. coli* reference strain for the *C. jejuni* isolates and the *C. jejuni* reference strains for the *C. coli* isolates (III).

4.2.2 Genotyping

To determine whether the *C. jejuni* enteritis isolates were essentially clonal or diverse, all domestic isolates and all those isolates presumably of foreign origin from July 2006 were analyzed by PFGE (II). The *Campylobacter* bacteremia isolates were genotyped by MLST (IV).

The PFGE was performed with DNA from the *C. jejuni* isolates that were harvested from Brucella blood agar after two days' growth. Shortly, after preparation of the DNA and digestion with *KpnI*, DNA plugs were loaded into sample wells of the agarose gel, and separated by electrophoresis with ramped pulses (Hänninen et al., 1998). Differences in at least one band indicated that the PFGE profiles differed.

MLST was performed according to techniques described in detail (Dingle et al., 2001; Kärenlampi et al., 2007; de Haan et al., 2010). In short, DNA was isolated with the Wizard genomic DNA purification kit (Promega, Germany), to be used for PCR. PCR conditions and annealing temperature were chosen as earlier described (Korczak et al., 2009; de Haan et al., 2010), and the PCR products were purified on Multiscreen PCR plates (Millipore, Billerica, MA, USA). Sequencing was performed by the BigDye terminator version 3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) at the Institute of Biotechnology, Helsinki, and the assembly and editing of the sequence data were performed with BioNumerics version 5.1 software (Applied Maths, Kortrijk, Belgium).

4.2.3 Antimicrobial susceptibility

Antimicrobial susceptibility of stool culture isolates was first tested for ciprofloxacin (I) and then also for erythromycin (II) and doxycycline (II). In addition to these three antimicrobial agents, blood culture isolates were also evaluated for susceptibility to gentamicin, meropenem, clindamycin, and metronidazole (III). The MIC values of ciprofloxacin (Bayer Health Care AG, Leverkusen, Germany), erythromycin (Amdipharm Ltd, Dublin, Ireland), doxycycline (Orion Pharma, Espoo, Finland), clindamycin (Sigma-Aldrich, St. Louis, MO, USA), metronidazole (B. Braun Melsungen AG, Melsungen, Germany), gentamicin (Sigma-Aldrich) and meropenem (Sandoz AG, Basel, Switzerland) were determined by an agar dilution method according to CLSI recommendations (CLSI, 2005). Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates supplemented with defibrinated sheep blood (5%) and containing antimicrobial agent were prepared within 24 hours of use. The concentrations of the antimicrobials were as follows: ciprofloxacin 0.008–128 mg/L, erythromycin 0.125–1024 mg/L, doxycycline 0.032–128 mg/L, gentamicin 0.016–32 mg/L, metronidazole 0.064–256 mg/L, clindamycin 0.016–32 mg/L, and meropenem 0.001–32 mg/L. Direct colony suspension was prepared from overnight growth on blood agar plates by suspending the culture in sterile distilled water in order to obtain a turbidity of a 0.5 McFarland standard.

Approximately 10^4 colony-forming units (CFU) were applied per spot with a multipoint inoculator. Quality-control strains included *C. jejuni* American Type Culture Collection (ATCC) 33560, *Staphylococcus aureus* ATCC 29213 (for clindamycin), and *Helicobacter pylori* ATCC 43504 (for metronidazole). The inoculated agar plates were incubated in a microaerobic atmosphere (BBL CampyPak Plus, Becton Dickinson CO., Sparks, MD, USA) at 36°C for 48 h. The MIC was the lowest concentration of the antimicrobial agent completely inhibiting visible bacterial growth. Antimicrobial susceptibility of the isolates for ciprofloxacin, doxycycline, and erythromycin were interpreted according to the CLSI guidelines, whereas for clindamycin, gentamicin, and meropenem, breakpoints came from the National Antimicrobial Resistance Monitoring System (NARMS), and for metronidazole breakpoints suggested by Hariharan et al. were used (Table 6).

Table 6. Breakpoints for ciprofloxacin, clindamycin, doxycycline, erythromycin, gentamicin, meropenem, and metronidazole.

Antimicrobial agent	Susceptible	Intermediate	Resistant	Reference
ciprofloxacin	≤1 mg/L	2 mg/L	≥4 mg/L	CLSI
clindamycin	≤2 mg/L	4 mg/L	≥8 mg/L	NARMS
doxycycline	≤2 mg/L	4 mg/L	≥8 mg/L	CLSI
erythromycin	≤8 mg/L	16 mg/L	≥32 mg/L	CLSI
gentamicin	≤2 mg/L	4 mg/L	≥8 mg/L	NARMS
meropenem	≤4 mg/L	8 mg/L	≥16 mg/L	NARMS
metronidazole	≤4 mg/L	8 mg/L	≥16 mg/L	Hariharan et al., 2009

4.2.4 Virulence factors

C. jejuni enteritis isolates found to be *ggt*-positive by comparative genomics were further tested for production of GGT by a qualitative detection method (Shibayama et al., 2003). To study the presence of other putative virulence factors, DNA was extracted from the *C. jejuni* enteritis isolates and tested for the presence of the genes *ceuE*, *cgtB*, *ciaB*, *cj0486*, *pldA*, *virB11*, and *wlaN*, as well as the *cdtABC* operon, with primers selected for detecting each of these genes (Table 7a).

To describe the virulence factor profiles of the *C. jejuni* bacteremia isolates, we measured qualitative production of GGT as described for *Helicobacter pylori* (Shibayama et al., 2003), and presence of the genes *ceuE*, *ciaB*, *cj0486*, and *virB11*, by the same method as described above for the enteritis isolates. For screening for LOS locus classes, the presence of open reading frame (ORF) orf12 (*waaV*) served as verification for successful DNA extraction from all isolates. A summary of the primers used for the detection of the open reading frames orf7ab (sialyltransferase *cstII*), orf6ab1 (galactosyltransferase *cgtB-1*), orf6ab2 (galactosyltransferase *cgtB-2*), and orf5bII (*cgtA2*) used for identification of sialylated LOS locus classes A and B, detection of orf6c (galactosyltransferase Cj1139c) and orf7c (sialyltransferase *cstIII*) in strains of sialylated LOS locus class C, and detection of orf26e and orf27e for identification of nonsialylated LOS locus classes E, H, O, and P, as well as orf12 are summarized in Table 7b.

Table 7a. Primers in Studies II and IV.

Virulence factor gene	Primer 5'-3'	Primer sequences
<i>cdtABC</i>	1	CTTTATGCATGTTCTTCTAAATTT
	2	GTAAAGGTGGGGTTATAATCATT
<i>ceuE</i>	1	GATAAAGTCGTTGGCGTTCC
	2	GCGAGATTGGAGGACCAAAGG
<i>ciaB</i>	1	CAGAAGGAGAAATTTGTGAGC
	2	ATATCCCATTCTAATGCCACC
<i>cj0486</i>	1	GATAGAGCATTAAATTGGGATG
	2	CCTATAAAGCCATACCAAGCC
<i>cgtB</i>	1	TTAAGAGCAAGATATGAAGGTG
	2	GCACATAGAGAACGCTACAA
<i>pldA</i>	1	AAGCTTATGCGTTTTT
	2	TATAAGGCTTTCTCCA
<i>virB11</i>	1	TCTTGTGAGTTGCCTTACCCCTTTT
	2	CCTGCGTGTCTGTGTTATTTACCC
<i>wlaN</i>	1	TTAAGAGCAAGATATGAAGGTG
	2	TGCTGGGTATACAAAGGTTGTG

Table 7b. Primers for LOS classification.

ORF (gene)	LOS locus class	Primer 5'-3'	Primer sequences
12 (<i>waaV</i>)	all	1 2	GCCACAACCTTTCTATCATAATCCCGC CGCCGTAACCTCAAACGCTCATCTATT
6ab1 (<i>cgtB-1</i>)	A1*, B1*	1 2	CAAGGGCAATAGAAAGCTGTATCA ACAAGCACTTCATTCTTAGTATTACAAAT
6ab2 (<i>cgtB-2</i>)	A2*, B2*	1 2	TCATCTTGCCAACTTATAATGTGGA TCTAGCGATATTAACCAACAGCCT
7ab (<i>cstII</i>)	A1*, A2*, B1*, B2*	1 2	ACTACACTTTAAAACATTTAATCCAAAATCA CCATAAGCCTCACTAGAAGGTATGAGTATA
5bII (<i>cgtA2</i>)	B1*, B2*	1 2	CTGTGATGATGGGAGTGAAGAGC GGTAATCGTTTCGGCGGTATT
6c (Cj1139c)	C*	1 2	GTAGTAGATGATTGTGGTAATGATAAA ATAGAATTGCTATTTACATGCTGG
7c (<i>cstIII</i>)	C*	1 2	TTGAAGATAGATATTTTTGTGGGTAAA CTTTAAGTAGTGTTTTATGTCACTTGG
26e	E, O	1 2	ATATTGCCGTTAATTCATTACAGTT TTTGAGCGATAATTTTAAATCCATC
27e	E,H, O, P	1 2	GTAGATGATTGTTCAAATGATAATAGCACA GTTTTAGATTCTAAGGCCATTATTCC

*Sialylated LOS locus classes.

Primers for *orf18df* (5'-GCAGCAAGAAATAATGGTGTAAAC-3', and 5'-AAATAATCATCCAAACATTCCTGAA-3') show bands for the nonsialylated LOS locus classes D, F, I, J, K, N, S, or Q and were used to attribute isolates non-typable using the primers above.

4.2.5 Serum resistance (IV)

A serum-sensitivity assay of the *C. jejuni* bacteremia isolates was performed as described (Blaser et al., 1985; Guerry et al., 2000). The same pool of normal human serum (NHS) from the blood samples of ten healthy blood donors served for all experiments. Heat-inactivated NHS (HINHS) was prepared by placing the test tubes in a waterbath of 56°C for 45 min. Bacterial cells from the 73 *C. jejuni* isolates were cultured in Brucella broth (Becton Dickinson) overnight, optic density at 405 nm was measured, and then bacterial density was adjusted to 5×10^4 CFU/mL by serial dilution. Next, 100 µl of the diluted bacterial suspension was added to 350 µl of PBS and 50 µl of NHS or HINHS, followed by incubation for 120 min at 37°C, after which 100 µl aliquots were plated out on blood agar plates (Columbia agar II (Neogen, Lansing, MI, USA) containing 8% vol/vol of defibrinized horse blood), and the plates were incubated under microaerobic conditions for 24 h at 42°C. The

number of colonies recovered from the NHS plates, divided by the number recovered from HINHS plates, and multiplied by 100, gave the percentage (%) of bacterial survival. The final analysis used mean values of two or three separate experiments. *C. jejuni* NCTC 11168 and a *C. fetus* blood isolate received while collecting the *C. jejuni* and *C. coli* blood isolates, served as control organisms. The *C. fetus* isolate was incubated at 37°C.

4.3 Statistical analyses

The statistical analyses were performed with Graphpad Prism version 4.03 (Graphpad Software, San Diego, CA, USA), SPSS version 15.0 (SPSS, Chicago, IL, USA), and PASW 18 (SPSS Inc). Fisher's exact test and the χ^2 -test and Mantel-Haenszel test were used for comparison of categorical variables. The Mann-Whitney test was used for comparison of continuous variables. Multivariate analyses were performed with stepwise binary logistic regression models. All tests were two-sided, and a p-value <0.05 was considered statistically significant.

4.4 Ethical considerations

The studies were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (Studies I and II). Written informed consent came from all patients– or their parents or guardians– included in Studies I and II. Studies III and IV were approved by the Finnish Ministry of Social Affairs and Health.

5. Results

5.1 Patient data

5.1.1 Overview of patient characteristics

Included in the studies were 192 patients with *C. jejuni* or *C. coli* enteritis (I), 166 *C. jejuni* patients of whom were also included in Study II, and 76 patients with *C. jejuni* or *C. coli* bacteremia (III), 73 *C. jejuni* patients of whom were also included in Study IV.

A summary of patient characteristics, including a comparison between patients with either *C. jejuni*/ *C. coli* enteritis or bacteremia, is presented in Table 8.

Table 8. Background, travel, and treatment data for patients in Studies I and III.

Patient characteristics	Enteritis study, 2006 (I) N=192	Bacteremia study, 1998-2007 (III) N=76
Age, years, median	39	46
Gender, proportion of male patients	42%	74%
Travel-associated infection (%)	148/192 (77)	16/76 (21)
Travel destination:		
Baltic states/ Poland	11	2
Bulgaria	21	
Central Europe/ UK/ Ireland	20	3
Cyprus/ Greece/ Italy	12	
India/ Nepal	14	
Northern Africa/ Middle East	8	1
Spain/ Portugal	25	
Sub-Saharan Africa	3	
Sweden/ Denmark	2	2
Thailand/ Cambodia/ Malesia	14	3
Turkey	11	2
Other	5	1
Unknown	3	2
Underlying disease, any (%)	43/187 (23)	48/76 (63)
alcohol abuse/ liver disease	0	6
chronic pulmonary disease	0	5
diabetes	3	4
diverticulosis/ celiac disease	4	2
dyspepsia/ gastritis/ GER/ peptic ulcer	12	5
malignant disease	2	4
HIV-infection	2	1
hypertension	10	14
rheumatoid arthritis/ fibromyalgia	5	1
Hospitalized for ≥ 2 days (%)	31/190 (16)	67/75 (89)
Received antimicrobial treatment (%)	140/191 (73)	73/76 (96)

GER= gastroesophageal reflux

The median age of the enteritis patients in Study I did not differ between the 26 patients infected by *C. coli* (38 years), and the 166 patients infected by *C. jejuni* (39 years), and the proportion of presumably imported infections was also similar between these two patient groups (85% and 76%).

Younger age-groups were slightly under-represented in Study I (Figure 5).

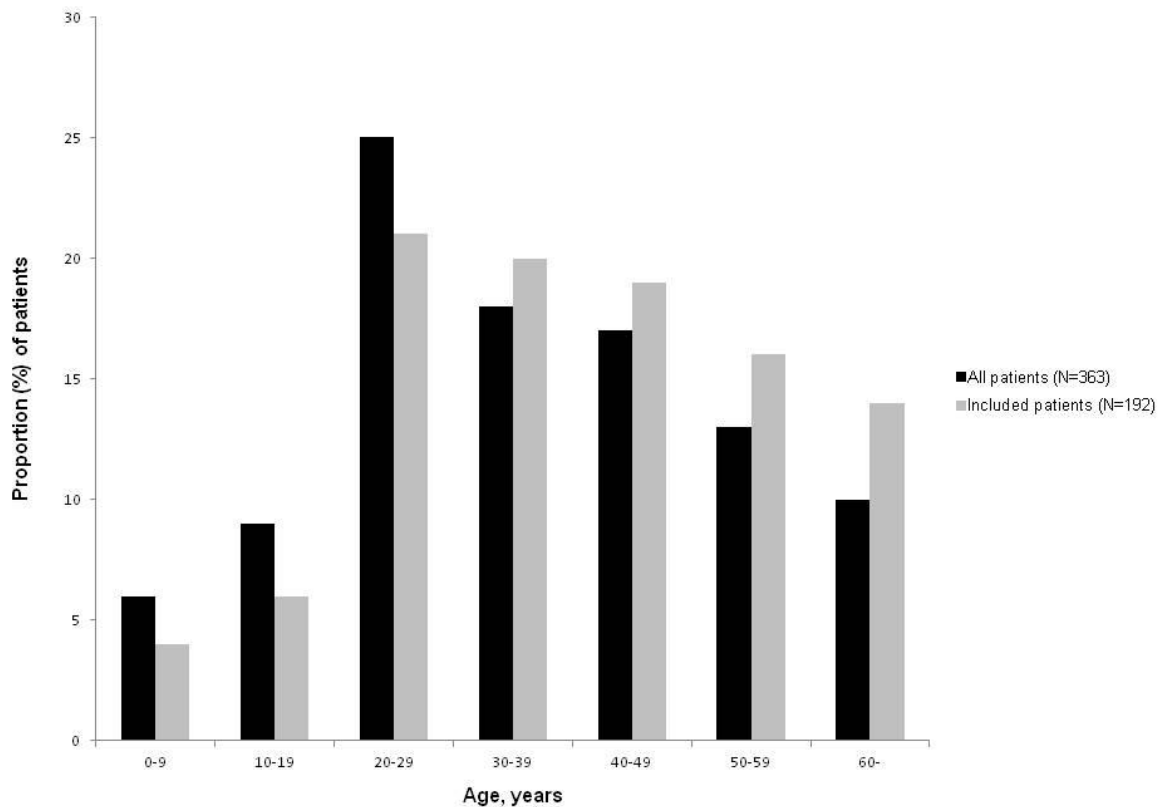


Figure 5 Age distribution of patients in Study I, for all *Campylobacter*-positive patients during the 6-month study period, and those included in the study.

A patient was classified as having an underlying disease if any medical condition or disease was either self-reported by the patient (I), or had been noted in the hospital records (III). In the bacteremia study, significant underlying diseases were further identified by classification of the illnesses according to the Charlson index score (Table 5), and the patients were divided into three groups; Charlson index scores 0, 1, and ≥ 2 . Although the majority of the bacteremic patients had some underlying disease, as shown in Table 8, the proportion of patients who had significant underlying diseases was clearly lower; only 30% were grouped with Charlson index score ≥ 1 .

Enteritis patients had underlying diseases less frequently than did bacteremic patients (Table 8), and although the data were self-reported by the patients, the more severe diseases seemed less frequent among these patients as well, compared to the bacteremic patients in Study III. Further, alcohol-related diseases and chronic pulmonary diseases seemed either under-represented or under-reported among the patients with enteritis, or over-represented among the bacteremia patients (Table 8).

The median age of the patients with *C. jejuni*/*C. coli* bacteremia was 46 years, and the median age of patients with Charlson index scores 0, 1, and ≥ 2 was 41, 54, and 57 years. Patients without significant underlying diseases were younger than those with a Charlson index score of either 1 ($p=0.002$), or ≥ 2 ($p=0.004$).

5.1.2 Foreign travel before onset of illness

An infection was regarded as imported if the patient had traveled abroad within two weeks prior to the onset of symptoms (I, III). The majority of the patients in the *Campylobacter* enteritis study (I) had presumably been infected abroad (Table 8). When comparing characteristics of domestically infected patients and those patients with presumably imported infection in Study I, we found that the age distribution differed, but no significant differences in the distribution of any underlying diseases or gender were detectable (Table 9).

Table 9. Distribution of age, any underlying diseases, and gender among 44 patients with domestic and 148 patients with imported *C. jejuni/ C. coli* enteritis (I).

Patient characteristic	Domestic infection	Imported infection	p-value
Age, years, median	46	37	0.03*
Any underlying disease, no. of patients (%)	13 (30%)	30 (21%)	0.2
Male gender, no. of patients (%)	21 (48%)	60 (41%)	0.4

In contrast to the finding that foreign travel was common among the patients with *C. jejuni* or *C. coli* enteritis, only 21% of the *C. jejuni* or *C. coli* bacteremia patients were known to have traveled abroad before onset of illness (Table 8). Further, some of the most common travel destinations among the patients with enteritis, for example Bulgaria, Spain, and India, were not included for patients with bacteremia.

5.1.3 Clinical signs and symptoms

Information regarding clinical symptoms and the course of disease came from either questionnaires filled in personally by the patients with *C. jejuni* or *C. coli* enteritis (I), or collected from the medical records at the hospitals where the *C. jejuni* or *C. coli* bacteremia episodes were diagnosed (III). The prevalences are summarized in Table 10.

Table 10. Prevalence of specific clinical signs and symptoms, either reported directly by patients with *C. jejuni* or *C. coli* enteritis, or noted in the medical records of patients with *C. jejuni* or *C. coli* bacteremia.

Clinical symptom or sign	<i>C. jejuni</i> / <i>C. coli</i> enteritis patients		<i>C. jejuni</i> / <i>C. coli</i> bacteremia patients	
	Reported no. of patients (%)	Answer rate (%)	Reported no. of patients (%)	Mention rate (%)
Abdominal cramps			15/31 (48)	41
Abdominal pain	158/182 (87)	95	35/54 (65)	71
Diarrhea	191/192 (99)	100	60/64 (94)	84
duration ≤ 5 days	74/192 (39)		15/64 (23)	
duration >5 days	112/192 (58)		22/64 (34)	
duration ≥ 10 days	47/192 (24)			
duration uncertain	5/192 (3)		23/64 (36)	
Bloody stools	24/133 (18)	69	7/24 (29)	32
Fever	160/183 (87)	95	64/75 (85)	99
Myalgia	73/174 (42)	91	11/15 (73)	20
Vomiting	48/179 (27)	93	13/43 (30)	57

Information on the occurrence and length of specific clinical symptoms seemed to be more detailed in the questionnaires returned by the patients with enteritis (I) than in the data from the medical records of the bacteremia episodes (III), as shown in Table 10.

Because of the low mention rate of many symptoms in the medical records, the actual proportion of all patients with bacteremia with any specific symptom is lower than the estimated prevalence, calculated for those patients for whom the presence or absence of the symptom was clearly stated in the medical records. For example, 79% (60/76) of the bacteremia patients had diarrhea, compared to 94% of those for whom this information was available, and similarly 9% (7/76) had bloody stools, compared to 29% of those clearly reported (Table 10).

When the patients with enteritis were divided into three major age-groups (< 30 years, 30-59 years, 60-89 years), to exclude age as a confounding factor, two evident age-related associations emerged. First, among those aged 0 to 29, bloody stools were associated with diarrhea lasting ≥ 10 days (p=0.02). Second, among the patients aged 30 to 59, bloody stools were associated with domestic infection (p=0.01). None of the patients aged 60 to 89 reported bloody stools.

In addition to the apparent differences in symptoms between patients with *C. jejuni* or *C. coli* enteritis and bacteremia, some variance appeared in the symptoms and findings between patients of different Charlson index scores in Study III. Patients with significant underlying diseases had less frequently fevers (>37.9°C), and generally lower levels of initial hemoglobin and C-reactive protein (CRP) levels, than did patients without significant underlying diseases (Table 11).

Table 11. The most common symptoms and findings in patients with *C. jejuni* or *C. coli* bacteremia, among patients with Charlson index scores 0, and ≥ 1 .

Symptom/finding	Charlson index score 0 (N=53)	Charlson index score ≥ 1 (N=23)	p-value
Diarrhea, patients	43	17	0.5
Fever, patients	48	16	0.02*
Hemoglobin, initial, median, g/L	144	128	0.01*
Leukocyte count, initial, median, cells x 10 ⁹	9.3	8.0	0.2
CRP, initial, median, mg/L	131	93	0.03*
CRP, peak, median, mg/L	152	151	0.5

Note. CRP= C-reactive protein.

5.1.4 Hospital treatment

Referral to diagnostic stool culture tests and initiation of treatment of a patient with *C. jejuni* or *C. coli* enteritis was usually by a physician at a health care center, whereas all blood cultures were made at a hospital. Consequently, the majority of patients with *C. jejuni* or *C. coli* bacteremia were primarily examined at a hospital emergency clinic, but relatively fewer patients with *C. jejuni* or *C. coli* enteritis were referred to a hospital. For analyses of factors which could lead to need for hospital treatment, patients treated at a hospital for at least two days were considered as hospitalized patients (I). The proportion of patients treated at a hospital for ≥ 2 days was 16% among the patients with *C. jejuni* or *C. coli* enteritis, and 89% among the patients with *C. jejuni* or *C. coli* bacteremia (Table 8).

5.1.5 Antimicrobial treatment

Information regarding the antimicrobial treatment came from questionnaires sent to the enteritis patients, and data on whether antimicrobials had been administered, which antimicrobial drugs were used, and the timing of antimicrobial treatment came from hospital records of patients treated for bacteremia. The majority of patients received antimicrobial treatment, 73% of the patients with *C. jejuni* or *C. coli* enteritis (I), and 96% of the patients with *C. jejuni* or *C. coli* bacteremia (III) had received at least one antimicrobial agent (Table 8). The proportion of patients receiving antimicrobial therapy with agents from more than one group of antimicrobials was 19% among the enteritis patients, and 71% among those with bacteremia. The distributions of different antimicrobial agents used in the treatment of the patients included in Studies I and III are shown in Table 12.

Table 12. Antimicrobial agents in treatment of patients in Studies I and III.

Antimicrobial agent	Enteritis study (I) N=192	Bacteremia study (III) N=76	
		At hospital	After hospitalization
Fluoroquinolone	68	44	46
Ciprofloxacin	65	38	43
Levofloxacin		5	2
Norfloxacin	3		
Ofloxacin		1	1
Macrolide	76	15	13
Azithromycin	5	3	0
Clarithromycin	0	2	1
Erythromycin	33	5	5
Roxithromycin	38	5	7
Aminoglycoside	1	6	0
Amoxicillin-clavulanic acid	0	0	1
Carbapenem	0	1	0
Cephalosporin	8	38	7
Clindamycin	0	3	0
Doxycycline	3	1	1
Metronidazole	11	19	9
Penicillin	0	1	0
Vancomycin	0	1	0

5.1.6 Outcome of illness

Campylobacter enteritis patients (I)

The proportion of enteritis patients reporting post-infectious musculoskeletal symptoms was approximately 24%, but because these data came from the patients' questionnaires, no other information regarding follow-up or outcome was available.

Bloody stools, diarrhea lasting ≥ 10 days, and hospitalization were chosen as indicators for more severe disease, and respectively 18%, 24%, and 16% of the patients had reported these in the questionnaires (Tables 8 & 10).

No known fatalities occurred among patients in Study I.

Campylobacter bacteremia patients (III)

Outcome of *Campylobacter* bacteremia was measured by length of hospital treatment, by 30-day and one-year mortality records, and by occurrence of severe post-infectious complications documented in the hospital treatment records.

Length of hospitalization was compared for patients with differing severity of underlying diseases. Patients without significant underlying diseases (Charlson index score 0) had a median duration of hospitalization of 3.5 days; the Mann-Whitney test revealed a significant difference in median duration of hospitalization of the patients with a Charlson index score of 1 (6 days; $p=0.04$), as well as for those patients whose Charlson index score was ≥ 2 (7.5 days, $p=0.002$).

Data regarding length of hospitalization for bacteremia patients, who had received either appropriate empirical antimicrobial treatment or inappropriate, delayed, or no antimicrobial treatment, are shown in Table 13.

Table 13. Median duration of hospitalization of 75 patients with *C. jejuni* / *C. coli* bacteremia, by appropriateness of antimicrobial therapy.

	All patients (N=75) ^a	Appropriate antimicrobial therapy			Inappropriate or no antimicrobial therapy (N=25)	p
		All (N=50)	Empirical (N=30)	Delayed (N=20)		
Hospitalization (median, days)	4	5	4	6	3	
Comparison of patients receiving:						
Appropriate vs. inappropriate or no antimicrobial therapy		5			3	0.01*
Appropriate empirical vs. delayed appropriate antimicrobial therapy			4	6		0.03*
Appropriate empirical vs. delayed appropriate, inappropriate, or no antimicrobial therapy (N=45, median duration 4 days)			4		4	NS

^aFor one patient this information was unavailable.

Two deaths occurred within 30 days after the *Campylobacter*-positive blood culture, and thus the mortality attributable to bacteremia was 3%. In addition, 6 patients died within one year

of the bacteremic episode, but these deaths were either attributed to severe underlying disease, old age, or trauma.

For the patients with bacteremia, clinical follow-up data were reviewed from the hospital archives of the same hospital where the blood culture had been performed; the time period for follow-up was one year, starting from the date of the blood culture. Although some of the patients had documented later visits to the hospital, hospital records revealed only a limited amount of information regarding the outcome of infection and possible complications.

One patient was diagnosed with Guillain-Barré syndrome, and after hospital treatment was referred to a neurological rehabilitation clinic.

Another patient was diagnosed with *C. jejuni* bacteremia and concurrent cervical spondylodiscitis. Microbiological verification from the drainage aspirate was lacking, but the patient was already receiving appropriate macrolide therapy at the time of the surgical procedure.

Appendectomy was performed on two patients while they were hospitalized, and two other patients were cholecystectomized during the one-year follow-up. Further, two patients were diagnosed with an inflammatory bowel disease within the follow-up period, as colonoscopy and biopsies revealed suspected findings of Crohn's disease in one patient, and lymphocytic colitis in another.

5.2 Bacterial isolates

5.2.1 Genotype distribution

PFGE analysis of fecal isolates (II)

All domestic fecal *Campylobacter* isolates from July through December 2006 were analyzed with PFGE, revealing a high diversity of PFGE profiles, as 33 different PFGE types were detected among the 40 domestic *C. jejuni* isolates included in Study II. Three isolates had an identical PFGE, and five other PFGE types had two isolates each.

Grouping domestic isolates further into crude clusters with $\geq 70\%$ similarity, revealed two larger crude clusters including 7 isolates each.

Of the imported 35 *Campylobacter* isolates from July 2006, all *C. jejuni* isolates were of different PFGE types.

MLST analysis of the blood isolates (IV)

All *C. jejuni* and *C. coli* blood isolates were typed with MLST; the 73 *C. jejuni* isolates were of 11 different clonal complexes (CC), and all three *C. coli* isolates were of the ST-828 CC (Table 14).

Table 14. MLST clonal complexes of 73 *C. jejuni* and three *C. coli* blood isolates.

CC	N	Species	Sequence types (N)
ST-677	35	<i>C. jejuni</i>	677 (27), 794 (8)
ST-45	12	<i>C. jejuni</i>	11 (4), 45 (3), 137 (2), 230 (2), 5201 (1)
ST-21	10	<i>C. jejuni</i>	50 (5), 883 (2), 1948 (1), 5670 (1), uncertain* (1)
ST-48	2	<i>C. jejuni</i>	38 (1), 48 (1)
ST-464	2	<i>C. jejuni</i>	464 (1), 3140 (1)
ST-52	1	<i>C. jejuni</i>	52 (1)
ST-354	1	<i>C. jejuni</i>	3155 (1)
ST-443	1	<i>C. jejuni</i>	new ST (1)
ST-460	1	<i>C. jejuni</i>	606 (1)
ST-508	1	<i>C. jejuni</i>	508 (1)
ST-1332	1	<i>C. jejuni</i>	1332 (1)
ST-828	3	<i>C. coli</i>	825 (1), 872 (1), new ST (1)
Unassigned	5	<i>C. jejuni</i>	468 (1), 1080 (1), 1972 (1), 5673 (1), 5674 (1)
Mixed	1	<i>C. jejuni</i>	

*Sequence data obtained for 5 loci.

There was an evident over-representation of ST-677 CC isolates among the *C. jejuni* blood isolates; the comparatively common ST-45 CC and ST-21 CC were also found more frequently than were other CCs, and of all *C. jejuni* blood isolates, 78% were of one of these three MLST CCs (Table 14).

Although the majority (71%) of the bacteremia isolates were isolated from blood cultures taken during the months of May through April, the seasonal peak was attributed to some MLST CCs more closely than to others; 100% of the isolates of the ST-677 CC, 67% of the isolates of the ST-45 CC, and 40% of the isolates of the ST-21 CC were diagnosed during the summer seasonal peak, May through August (Figure 6).

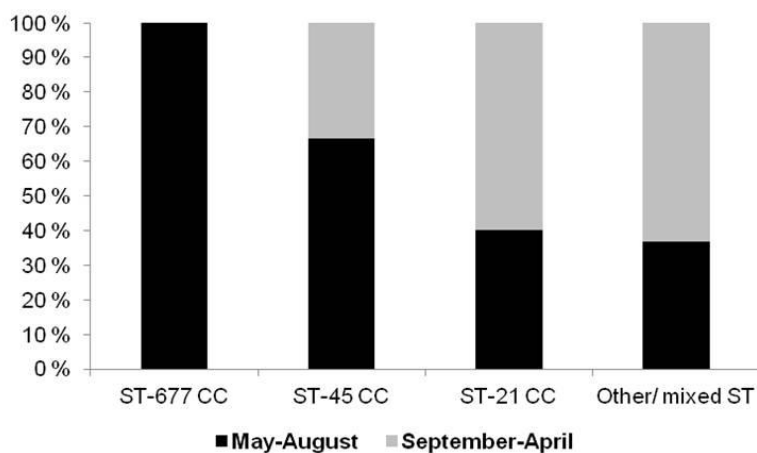


Figure 6 Seasonal distribution of bacteremia-associated *C. jejuni* MLST CCs.

5.2.2 Antimicrobial susceptibility

Minimal Inhibitory Concentration (MIC)

All isolates were tested with an agar dilution method to determine the MIC values for the antimicrobial agents most commonly used as treatment for *Campylobacter* infection. We tested by an agar-dilution method the susceptibilities of the bacterial isolates to ciprofloxacin (I,III); doxycycline, and erythromycin (II,III); as well as to clindamycin, gentamicin, meropenem, and metronidazole (III) (Table 15).

Table 15. Number of isolates resistant to different antimicrobial agents, among enteritis isolates, and in Study III.

Antimicrobial agent	No. of isolates resistant (%)	
	Enteritis isolates N=192	Bacteremia study (III) N=76
ciprofloxacin	97 (51%)	5 (7%)
clindamycin	n.a.	0
doxycycline	73 (38%)	3 (4%)
erythromycin	8 (4%)	0
gentamicin	n.a.	0
meropenem	n.a.	0
metronidazole	n.a.	12 (16%)

A clear difference emerged in resistance patterns between the enteritis and the bacteremia isolates. In both groups of isolates, antimicrobial resistance to ciprofloxacin, doxycycline, and erythromycin was detected almost exclusively in imported isolates. Of the isolates which were resistant to ciprofloxacin, all except one were of foreign origin (Figure 7).

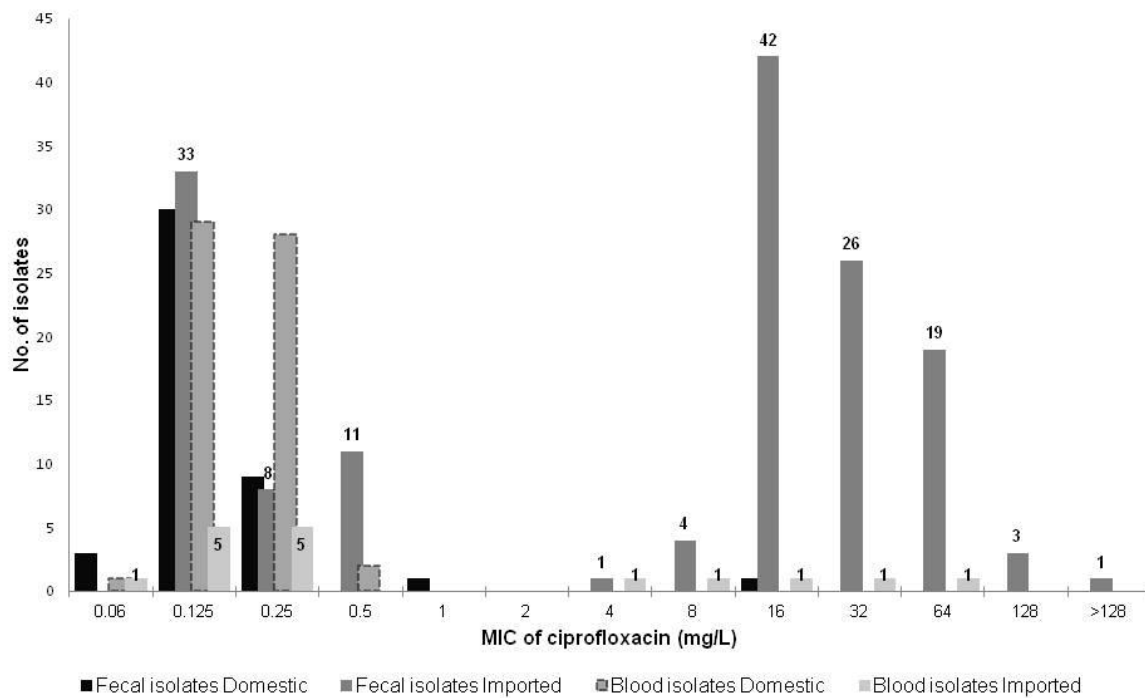


Figure 7 Distribution of ciprofloxacin MICs for 192 *C. jejuni* / *C. coli* fecal isolates and 76 *C. jejuni* / *C. coli* blood isolates. Data labels are shown for the imported isolates (N=164).

Effect of ciprofloxacin resistance on disease outcome (I)

There was a significant difference in distribution of ciprofloxacin MICs between the patients who reported bloody stools (67% of their isolates were highly susceptible, MIC 0.06-0.25 mg/L, to ciprofloxacin), and those patients who reported noticing no bloody stools ($p=0.04$); a significant difference in the distribution of MICs was also evident between patients hospitalized ≥ 2 days (65% of their isolates were susceptible to ciprofloxacin), and those who had not been hospitalized ≥ 2 days ($p=0.01$). In the light of these results, a more severe outcome of infection seemed to be associated with a lack of ciprofloxacin resistance in the *Campylobacter* enteritis strains.

5.2.3 Putative virulence factors (II, IV)

The isolates were analyzed for the presence of the putative virulence factor genes *ceuE*, involved in iron uptake (II, IV), *cgtB*, involved in sialylation of LOS (II), *ciaB*, encoding the *Campylobacter* invasion antigen (II, IV), *cj0486*, encoding a putative fucose permease (II, IV), *pldA*, encoding phospholipase A (II), *virB11* of the pVir plasmid (II, IV), and *wlaN*, involved in sialylation of LOS (II), and the operon *cdtABC*, encoding the cytolethal distending toxin (II), as well as for the production of GGT (II, IV) (Table 16).

Table 16. Prevalence of the putative virulence factor genes *ceuE*, *cgtB*, *ciaB*, *cj0486*, *pldA*, *virB11*, and *wlaN*, as well as the operon *cdtABC* among *C. jejuni* isolates in Studies II and IV.

Study	<i>cdtABC</i>	<i>ceuE</i>	<i>cgtB</i>	<i>ciaB</i>	<i>cj0486</i>	<i>pldA</i>	<i>virB11</i>	<i>wlaN</i>
II	131 (79%)	142 (86%)	31 (19%)	164 (99%)	81 (49%)	101 (61%)	4 (2%)	39 (23%)
IV	n.a.*	71 (97%)	n.a.*	73 (100%)	17 (23%)	n.a.*	2 (3%)	n.a.*

*n.a.=not available.

GGT production was detected in 25/166 (15%) of the *C. jejuni* enteritis isolates, and in 13/73 (18%) of the *C. jejuni* blood isolates. It should be noted that the proportion of *cj0486*-positive isolates was significantly higher among the *C. jejuni* enteritis patients than among the *C. jejuni* bacteremia patients (Table 16; $p=0.0002$).

Associations between putative virulence factors and disease among C. jejuni enteritis patients (II)

Univariate analyses revealed that bloody stools reported by the patients were a clinical sign associated with the presence of gene *cgtB* ($p=0.03$), and the production of GGT ($p=0.03$). The absence of the gene *ceuE* was associated with hospitalization ($p=0.03$). Isolates shown to produce GGT were associated with ciprofloxacin susceptibility ($p=0.001$), and doxycycline susceptibility ($p=0.003$), while isolates positive for either *ceuE* or *cj0486* were associated with resistance to ciprofloxacin ($p<0.001$, $p=0.005$, respectively), and resistance to doxycycline ($p<0.0001$, $p=0.002$). Those isolates which were positive for either *ceuE* or *cj0486* were associated with imported infection ($p<0.0001$), while isolates which produced GGT were associated with domestic infection ($p<0.0001$).

All results could not be analyzed by multivariate analysis, due to missing data. When those factors possible to test with a multivariate analysis model underwent testing, the only associations remaining significant were those with production of GGT, and the presence of genes *ceuE* or *cj0486*, compared to the origin of infection. Isolates which produced GGT were significantly associated with domestically acquired infection (OR 8.7, $p<0.0001$), and isolates positive for *ceuE* or *cj0486* were significantly associated with infection of presumably foreign origin (OR 6.7, $p<0.0001$, respectively).

Associations between virulence factors and the *C. jejuni* MLST CCs of the bacteremia isolates (IV)

Certain putative virulence factors were associated with certain MLST CCs. Isolates of the ST-45 CC were mostly GGT positive (83%), compared to only three other GGT-positive strains ($p < 0.0001$). The fucose permease gene *cj0486* was associated with ST-21 CC isolates (100% of ST-21 CC *cj0486* positive, vs. 14 % of all other isolates; $p < 0.0001$). All isolates were *ciaB* positive, all isolates except two were *virB11* negative, and all isolates except two *ceuE* positive (Table 16). All the 35 ST-677 CC isolates were *cj0486* negative and GGT negative. The most important putative virulence factor associations are summarized in Figure 8.

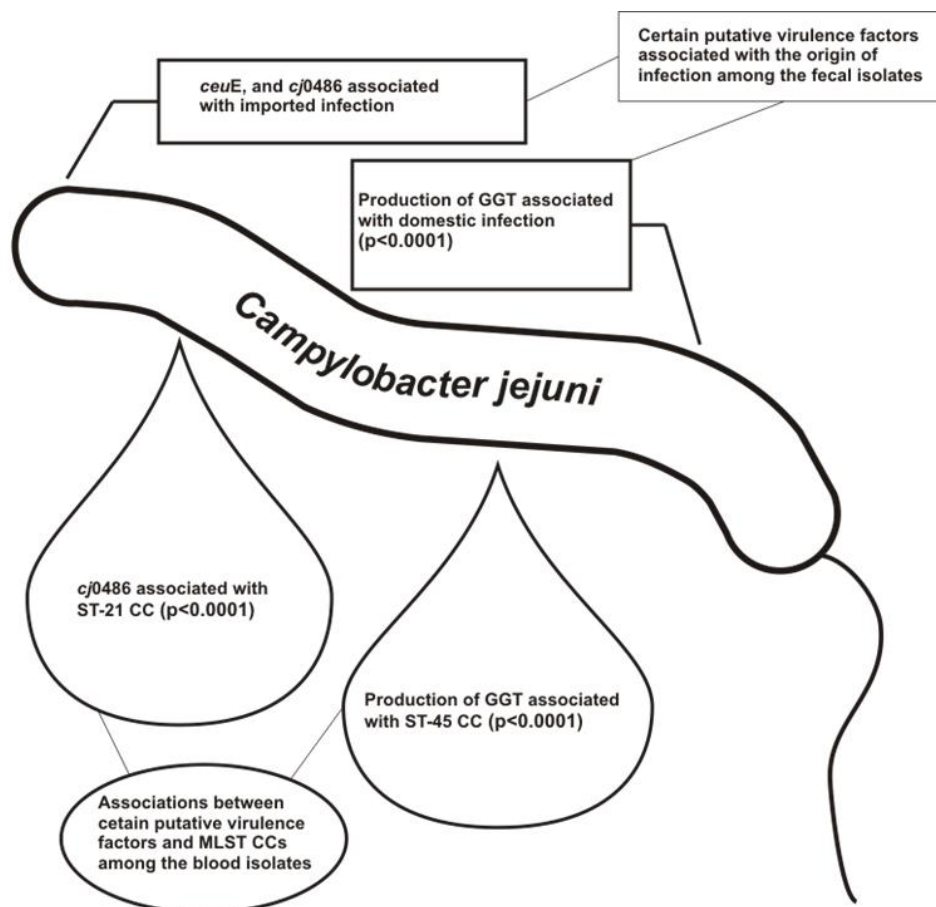


Figure 8 Summary of the associations of some putative virulence factors with origin of infection among *C. jejuni* fecal isolates (II), and with certain MLST CCs among the *C. jejuni* blood isolates (IV).

Sialylated LOS (IV)

Only 17 (23%) of the 73 *C. jejuni* blood culture isolates in Study IV had a gene profile indicating that these isolates had sialylated LOS in their outer membranes. In total, four isolates had LOS locus class A1, five isolates had LOS locus class B2, and eight isolates had LOS locus class C. *C. jejuni* blood isolates with sialylated LOS were associated with the

presence of gene *cj0486* ($p < 0.0001$), and those patients infected by a *C. jejuni* isolate with sialylated LOS had significant underlying diseases (Charlson index score ≥ 1) more often than the other patients ($p = 0.02$). However, no difference existed in the duration of hospitalization among those patients infected by a *C. jejuni* isolate with either sialylated or nonsialylated LOS.

All isolates of the ST-677 CC, as well as the ST-45 CC, belonged to one of the nonsialylated LOS locus classes E, H, O or P. CCs associated with sialylated LOS were ST-21 CC (LOS locus classes A1 and C), ST-48 CC (LOS locus class B2), and ST-464 CC (LOS locus class B2). All *C. jejuni* isolates with sialylated LOS locus class C were of the ST-21 CC.

5.2.4 Serum resistance (IV)

Between *C. jejuni* isolates of different MLST complexes susceptibility to NHS varied significantly; isolates of the ST-677 CC were significantly less susceptible to NHS than were all other isolates ($p < 0.0001$), whereas the isolates of the ST-45 CC were significantly more susceptible to NHS than were all other isolates ($p < 0.0001$).

No significant difference emerged in susceptibility to NHS of the *C. jejuni* blood isolates with nonsialylated LOS (27% bacterial survival, median), compared to that of isolates with sialylated LOS (15% bacterial survival, median) ($p = 0.28$), and the serum susceptibility of *C. jejuni* isolated from the blood of patients with or without significant underlying diseases did not differ statistically (patients with Charlson index score 0 compared to all others, $p = 0.7$) (Figure 9).

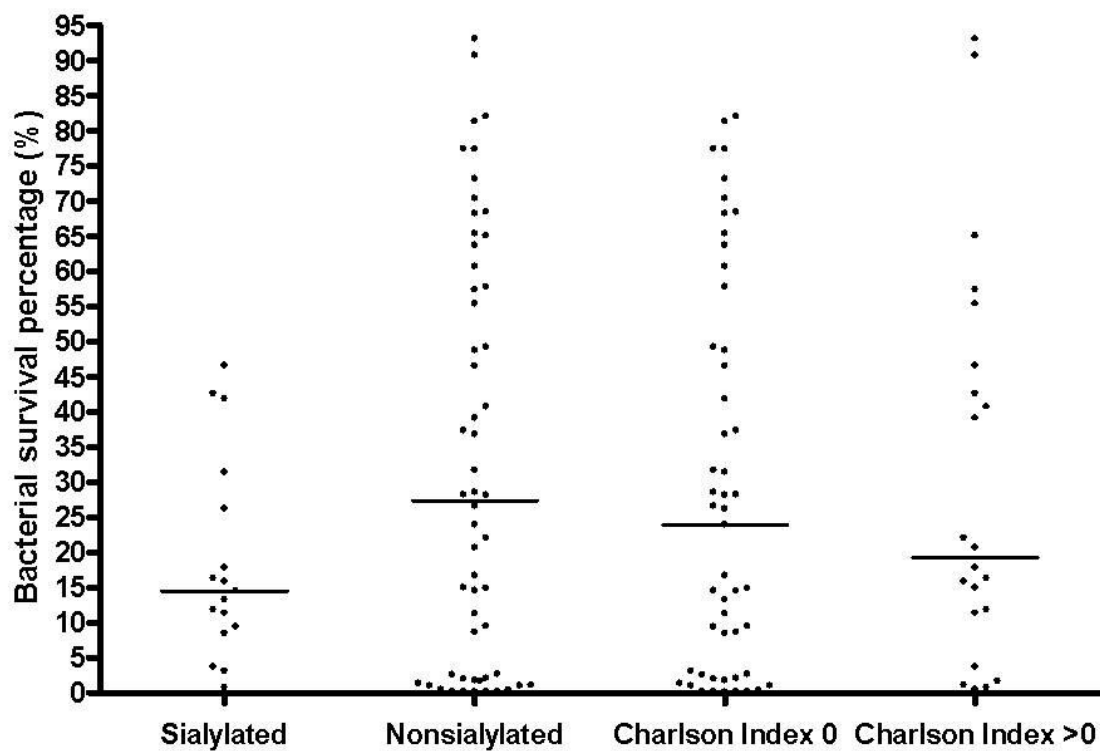


Figure 9 Scatter plot of the values and medians of bacterial survival (%), showing the serum susceptibility pattern of *C. jejuni* blood isolates with sialylated or nonsialylated LOS, as well as for the same isolates according to the Charlson index score of the host.

6. Discussion

6.1 Effect of ciprofloxacin resistance on outcome of *C. jejuni* or *C. coli* infection (I, III)

No consensus exists in the literature regarding the effect of antimicrobial resistance of *C. jejuni* on the severity of symptoms or the disease outcome. Fluoroquinolone resistance may impair the fitness of *C. jejuni* and *C. coli* (Zeitouni & Kempf, 2011), but the opposite effect was earlier noted for *C. jejuni* (Luo et al., 2005). Some studies have proposed a link between ciprofloxacin resistance and more severe infections (Engberg et al., 2004; Nelson et al., 2004; Helms et al., 2005), while others have shown no difference in the length or severity of symptoms (The Campylobacter Sentinel Surveillance Scheme Collaborators, 2002; Unicomb et al., 2006). Further, a re-analysis of some of these conflicting data is in line with those studies which found no association between ciprofloxacin resistance and severe infection (Wassenaar et al., 2007). Our results support those of the re-analysis, as we found no evidence that ciprofloxacin-resistant isolates would have caused a more severe disease than did the isolates susceptible to ciprofloxacin. Conversely, the trend was toward those isolates highly susceptible to ciprofloxacin causing more severe disease, characterized by more frequently reported bloody stools, as well as need for hospital treatment. Moreover, the clear majority of blood culture isolates, which per definition caused a more severe disease, were susceptible to ciprofloxacin. If ciprofloxacin resistance were of importance for the disease outcome, then one would expect *C. jejuni* and *C. coli* bacteremia isolates resistant to ciprofloxacin to be more prevalent in general.

The main weaknesses in our study were that the response rate was quite low, that the younger age groups were somewhat under-represented, and that not all patients had answered all questions clearly enough to be included in the analyses. However, more than half of all patients who had a *Campylobacter*-positive stool sample within the HUSLAB area during the six-month study period were included in the study, and all age groups were fairly well represented among our patients. A general problem with questionnaire-based studies is that patients may have difficulties remembering specific details such as the exact duration of a symptom or facts concerning the medication.

The fact that the association between domestically acquired infection and bloody stools was detected only among patients aged 30 to 59 is of specific interest. This particular age group was well-represented and included the majority of the patients in Study I. Among the middle-aged, the association between bloody stools and domestically acquired *Campylobacter* infection was valid, and had the answer rate been higher generally, this association may have been more evident in some other age-groups as well. The younger patients typically had bloody stools in combination with a longer duration of diarrhea, and this might be a finding which reflects the absence of immunity among patients in developed countries (Blaser, 1997). Thus, it seems logical that this association was found among the younger patients. Intriguingly, none of the patients aged 60 to 89 reported bloody stools.

These results suggest a link may exist between the absence of ciprofloxacin resistance to the severity of symptoms experienced by patients with *C. jejuni* or *C. coli* enteritis, or domestic isolates in Finland may harbor some specific virulence traits which lead to more severe infection. On the other hand, it is possible that because the risk for contracting gastrointestinal pathogens while traveling abroad is higher than otherwise, patients may become infected by less virulent isolates abroad more easily, whereas partial immunity to those isolates encountered domestically may actually lead to more frequent detection of particularly virulent domestic isolates. Further, in theory, patients with imported infection may have felt more concerned about their infection and thus may have contacted their health care units more easily than those patients who had not traveled abroad prior to onset of illness. This may have led to an over-representation of patients with imported infection in Study I. If so, ciprofloxacin-resistant isolates from patients with less severe symptoms may be comparatively more common, especially among the younger patients; this may have affected the analyses.

6.2 Virulence factor profiles of *C. jejuni* isolates (II, IV)

Although several virulence factors have been proposed to be of importance for motility, adhesion, colonization, invasion, iron uptake, metabolism, and survival of *C. jejuni* in the human host, none of those studied have been clearly linked to a more severe outcome of human disease. For example, both the pVir plasmid (Bacon et al., 2000), and CiaB (Konkel et al., 1999), have been proposed to somehow affect the invasiveness of *C. jejuni*. On the other hand, pVir has been very uncommon in clinical isolates (Talukder et al., 2008), and while *ciaB* is commonly detected in clinical isolates (Datta et al., 2003; Talukder et al., 2008), it has even been negatively associated with invasiveness *in vitro* (Fearnley et al., 2008). Cj0486 has been suggested to be associated with hyperinvasiveness *in vitro* (Fearnley et al., 2008), but information on the prevalence of *cj0486* in clinical isolates is scarce. GGT has so far been shown to affect the colonization potential of *C. jejuni* only in animal models (Hofreuter et al., 2006; Barnes et al., 2007).

The prevalence of the putative virulence factor genes *ceuE*, *cgtB*, *ciaB*, *cj0486*, *pldA*, *virB11*, and *wlaN*, and the gene cluster *cdtABC*, as well as the production of GGT were studied for a well-characterized patient material. In general, the virulence factor profiles of *C. jejuni* isolates were quite similar to those described in the literature (Datta et al., 2003; Talukder et al., 2008). Most evidently, *virB11* was very rare, and *ciaB* very common among the isolates. However, our major findings were that the domestic and presumably imported *C. jejuni* fecal isolates differed from each other not only regarding the susceptibility to antimicrobial agents, but also when comparing putative virulence factor profiles. The imported isolates were significantly more likely to harbor the genes *cj0486* and *ceuE*, as compared to the domestic isolates, which were associated with the ability to produce GGT. This suggests significant geographical variation in bacterial characteristics among the *C. jejuni* isolates which cause enteric disease in humans.

We noted some associations between certain virulence factors and clinical characteristics. Both production of GGT and the presence of the gene *cgtB*, encoding a galactosyltransferase which is present in some isolates with sialylated LOS, were associated in the univariate analyses with bloody stools. These findings could not, however, be verified in a multivariate model, due to the relatively large amount of missing data regarding this particular clinical finding. Another association which could not be confirmed in a multivariate analysis was whether those isolates which lacked the *ceuE* gene were to some degree associated with hospitalization lasting at least two days.

The putative virulence factor profiles of the *C. jejuni* blood isolates were essentially similar to those of the *C. jejuni* enteritis isolates, with the exception of the fucose permease gene *cj0486*, which was significantly more prevalent among the enteritis isolates. A possible explanation for this finding may be that foreign travel was comparatively uncommon among the bacteremia patients, and because *cj0486* was associated in Study II with imported isolates the proportion of *cj0486*-positive isolates was lower among the bacteremia patients due to the differences in travel history between the enteritis and the bacteremia patients. However, no association appeared between foreign travel and *cj0486* among the bacteremia patients. A more logical explanation would be that because the isolates of the ST-677 CC, all of which were *cj0486* negative, were so clearly over-represented among the bacteremia isolates, the proportion of *cj0486* obviously also differed from that of the enteritis isolates.

6.3 Patient characteristics and treatment aspects of *Campylobacter* bacteremia patients (III)

In studying the outcome and severity of an infection, bacteremia is an important complication to consider. Regarding *Campylobacter* bacteremia, only a few studies have presented epidemiological data from a wider geographical region (Skirrow et al., 1993; Nielsen et al., 2010a) or sufficiently detailed clinical information enabling analyses of the impact of antimicrobial therapy (Pigrau et al., 1997; Tee & Mijch, 1998; Pacanowski et al., 2008; Fernández-Cruz et al., 2010). Furthermore, the species of the isolates included have not been typed by molecular methods. A need for studies which have combined these data is therefore evident.

The basis for the current study was a unique clinical series of patients with *Campylobacter* bacteremia from a 10-year period in Finland. Most importantly, the characteristics of the human hosts and of the corresponding bacterial isolates could be compared through linkage of the information from hospital records to the *C. jejuni* and *C. coli* isolates. Further, all isolates were verified to species level by PCR, which provided sufficient reliability for analyses of the bacterial isolates.

Surprisingly, the majority of the patients had no significant underlying diseases, and all age-groups were represented, although the median age was lower than expected, 46 years. These findings differed much from several earlier studies, both with regard to the proportion of underlying diseases (Pigrau et al., 1997; Pacanowski et al., 2008; Nielsen et al., 2010a;

Fernández-Cruz et al., 2010), as well as to patient age (Pacanowski et al., 2008; Nielsen et al., 2010a; Fernández-Cruz et al., 2010). However, the results of the largest epidemiological *Campylobacter* bacteremia study to date (Skirrow et al., 1993) are in line with ours.

Unexpectedly, we found no evident effect of appropriate empirical antimicrobial treatment, on either the duration of hospitalization, or on the mortality attributable to the infection. This finding contrasts with the results of a French study which found the absence of treatment with appropriate antimicrobials to be associated with death (Pacanowski et al., 2008), but in line with a Spanish study finding no such significant association (Fernández-Cruz et al., 2010).

The low mortality rate in this study (only two patients died within 30 days after the blood culture) was in line with that of a recent Danish study (Nielsen et al., 2010a). Two other of our patients developed severe complications, one patient being diagnosed with GBS, and another on MRI with cervical spondylodiscitis. These patients were not known to have any severe underlying medical conditions, and it would be tempting to suggest that some characteristics of the bacterial isolates enabled the development of these complications. We further noted that comparatively few of our patients with bacteremia had traveled abroad, compared to those infected by *Campylobacter* in Finland in general (I), and the majority of the bacterial isolates were susceptible to all antimicrobial agents we tested. These results actually support findings in Study II, that the domestic infections in Finland may in fact have some characteristics or traits leading in the human host to a more severe disease.

6.4 Clonal distribution, LOS locus classification, and serum susceptibility among *C. jejuni* bacteremia isolates (IV)

C. jejuni is known to be weakly clonal and highly diverse, as also indicated by the fact that over 5000 registered STs have been submitted to the PubMLST database (<http://pubmlst.org/campylobacter>). Isolates of the ST-21 CC and the ST-45 CC are generally common (Dingle et al., 2001; Lévesque et al., 2008), but ST-677 CC has been uncommon among human *C. jejuni* isolates. In the light of these data, it was surprising to find that among the *C. jejuni* blood isolates the ST-677 CC was so clearly over-represented. Nevertheless, in Finnish studies, covering essentially the same time period as ours (Kärenlampi et al., 2007; deHaan et al., 2010), isolates of the ST-677 CC have been relatively prevalent (Kärenlampi et al., 2007), although the proportion of these isolates among human *C. jejuni* infections has been diminishing (deHaan et al., 2010).

Intriguingly, though, the ST-677 CC isolates also differed from the other *C. jejuni* isolates in many other aspects. All the isolates of the ST-677 CC had nonsialylated LOS as well as very similar profiles of putative virulence factors, and most importantly of all *C. jejuni* blood isolates the ST-677 CC isolates were significantly more serum resistant. *C. jejuni* blood isolates are not always serum resistant (Blaser et al., 1985). However, *C. jejuni* and *C. coli* isolates from systemic isolation sites, indicating those that spread hematogenously, have been serum resistant (Blaser et al., 1986). It can thus be argued that serum resistance may actually be necessary for development of extraintestinal *Campylobacter* infection foci due to

hematogenous spread, but not specifically for the development of *C. jejuni* bacteremia. In the latter study by Blaser et al, the majority of patients were either <1 year or ≥ 60 years old, and half had some kind of medical condition probably predisposing the patient to extraintestinal infection. It should therefore be pointed out that the majority of patients in the current study were neither from the ends of the age spectrum, nor did they commonly have severe underlying diseases. Further, it is important to note that although serum resistance was not an absolute prerequisite for the *C. jejuni* isolates to spread into the bloodstream, the isolates of the ST-677 CC may have predominated due to the fact that these particular isolates were more serum resistant than the isolates of other MLST complexes.

The role of differing outer membrane and capsule structures with regard to the serum resistance of *C. jejuni* remains unexplained. An earlier experimental study proposed that a *C. jejuni* mutant with a nonsialylated LOS becomes more susceptible to human serum than does the wild type, indicating that sialylated LOS may play a role in the development of serum resistance (Guerry et al., 2000). However, another group recently suggested that capsule expression is more likely to be of importance for complement resistance than is LOS (Keo et al., 2011). Only 23% of our *C. jejuni* blood isolates had a sialylated LOS locus class, and those isolates were not more serum resistant, than were all other isolates. Hence, no evident link between sialylated LOS and serum resistance in *C. jejuni* blood isolates emerged in our study, and sialylated LOS seems not to be necessary for the development of *C. jejuni* bacteremia. Interestingly, isolates with a sialylated LOS locus class were significantly more often from patients with a Charlson index score ≥ 1 . This finding further puts into question the role of sialylated LOS in *C. jejuni* bacteremia of patients without significant underlying diseases.

In conclusion, it is strongly emphasized that in order to understand the real significance of certain bacterial characteristics for the outcome of *Campylobacter* infection, experimental *in vitro* models must be complemented with studies on clinical, non-selected, series.

7. Conclusions

1. Ciprofloxacin resistance was not associated with a more severe course of *Campylobacter* infection; rather, isolates highly susceptible to ciprofloxacin seemed to cause more severe infection, characterized by bloody stools and need for hospitalization (I). Further, the majority of *Campylobacter* bacteremia isolates were susceptible to all antimicrobial agents tested, including ciprofloxacin (III).
2. None of the putative virulence factor genes *ceuE*, *cgtB*, *ciaB*, *cj0486*, *pldA*, *virB11*, or *wlaN*, nor the operon *cdtABC*, nor production of GGT, were significantly, or independently associated with more severe symptoms among the *C. jejuni* enteritis patients. However, multivariate analyses revealed that *ceuE* and *cj0486* were significantly associated with *C. jejuni* isolates of foreign origin, and production of GGT was associated with isolates of domestic origin (II). The *C. jejuni* bacteremia isolates essentially showed a similar proportion as being positive for the genes *ceuE*, *ciaB*, *virB11*, as well as the expression of GGT, but the fucose permease gene *cj0486* was comparatively less prevalent among the bacteremia-associated isolates than among the enteritis-associated ones.
3. The patients with *C. jejuni* or *C. coli* bacteremia were moderately young, and the majority had no significant underlying diseases. Appropriate antimicrobial treatment did not seem to affect the outcome of *C. jejuni* or *C. coli* bacteremia to any greater extent, neither with regard to <30 d mortality, nor when the duration of hospitalization was compared to that of those who received inappropriate, delayed appropriate, or no antimicrobial therapy (III).
4. Among the *C. jejuni* bacteremia isolates a striking over-representation of isolates of the otherwise quite uncommon ST-677 CC occurred. Although serum resistance was not needed for *C. jejuni* to cause a bacteremic infection, the isolates of the ST-677 CC were actually significantly more serum resistant than others. Only 23% of blood culture isolates had sialylated LOS, but sialylated LOS, and particularly LOS locus class C, was associated with patients' having significant underlying diseases. These results indicate that certain genotypes of *C. jejuni* may lead to bacteremia more easily than others (IV).

8. References

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9. Acknowledgements

This study was carried out at the Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki during 2006 to 2011. Funding, gratefully acknowledged, was provided by the Academy of Finland, ELVIRA project.

I am deeply grateful to my supervisor Professor Hilpi Rautelin for always having the time and will to aid, support, and guide me through one phase of this project after another. Her enthusiasm and motivating attitude are traits that I admire, and I appreciate having had the chance to work in her research group.

I thank Professor Seppo Meri, the former Head of Haartman Institute, for the opportunity to work in these exemplary scientific surroundings.

I warmly thank the reviewers of this thesis: Docent Heikki Kauma and Docent Risto Vuento for their expert comments and rapid responses.

I am sincerely grateful to Professor Marja-Liisa Hänninen for her help and instructive comments, to Professor Seppo Sarna for his aid with statistics, to Docent Anneli Lauhio for sharing her expertise in infectious diseases, to Patrik Ellström for his tutoring and friendship, to Astrid de Haan for performing the MLST and keeping me on track, to Heidi Hyytiäinen for cooperation and the PFGE analyses, and to Anna Nilsson and Marko Haverinen for their excellent laboratory assistance. Further, Patrik and Anna are especially acknowledged for their enormous efforts in performing PCR and serum sensitivity assays.

I thank Professor Mikael Skurnik for his friendly advice as well as for the calm and friendly atmosphere at the Department.

I thank Professor Petri Ruutu and Mikko Virtanen at the National Institute for Health and Welfare for smooth cooperation, the personnel in all the microbiological laboratories across the country for sending us the bacteremia isolates, and all the nice and helpful people working in the hospital archives for their efficient and precise work.

I warmly thank my other scientist friends for their kind support: Daniela Schönberg-Norio and Emeritus Associate Professor Timo Kosunen for their practical advice and encouragement, and my friends and colleagues Daniel Gordin, Joakim Janér, and Erik Litonius for all the scientifically relevant discussions over lunches at Unicafé Meilahti.

Many thanks to all of my friends for all the good times; let's keep them rolling! Specific acknowledgements to my "personal trainers" Björn von Alftan and Fredrik Zitting for getting me off my couch now and then, and to Kim Forsberg for his help in moving it to where it now stands, as well as for trying to teach me how computers work.

I am grateful to my father-in-law Jukka and mother-in-law Tarja for all their practical advice, aid, and care.

I thank all in my family for their continuous love and support, and I warmly acknowledge my sisters Nicolina and Nina for all the action and connection throughout the years, my father Thomas for showing trust in my decisions, my grandfather Yrjö for sharing his wisdom and for his genuine interest, and my mother Marina for always believing in me.

I am not able to express enough affection and appreciation to our beloved children Anton and Livia - you fill my days with joy, and hearing your laughter erases all mundane worries. Also, cheers to all your grandparents for looking after you when needed; special thanks to Tarja for the numerous home-cooked, and child-friendly, meals brought along!

Finally, my deepest loving thanks to Maija, who stands beside me, always encourages me, and helps me to grasp happiness. I owe a considerable part of the fulfillment of this work to you, my love.

Espoo, March 2012

Benjamin Feodoroff