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Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1427

Characterization of *Campylobacter jejuni* and *Campylobacter coli* water isolates

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ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2018

ISSN 1651-6206
ISBN 978-91-513-0232-4
urn:nbn:se:uu:diva-339839

Dissertation presented at Uppsala University to be publicly examined in Hörsalen, Klinisk Mikrobiologi, Dag Hammarskjöldsväg 17, Ing D1, Uppsala, Friday, 23 March 2018 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Mikael Skurnik (University of Helsinki, Medicum, Department of Bacteriology and Immunology).

Abstract

Nilsson, A. 2018. Characterization of *Campylobacter jejuni* and *Campylobacter coli* water isolates. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1427. 64 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0232-4.

Campylobacter jejuni and *C. coli* are together the most common cause of bacterial gastroenteritis in the European Union. *Campylobacter* can be transmitted to humans via contaminated water, but it is largely unknown how these bacteria survive in water.

The aim of this thesis was to better understand the water survival strategies and pathogenic potential of *Campylobacter* water isolates. For this purpose, *C. jejuni* and *C. coli,* originally isolated from incoming water at surface water plants, were characterized using whole genome sequencing, phenotypical assays, water survival experiments and an *in vitro* infection model.

C. jejuni water isolates included both common and uncommon sequence types for human pathogens, whereas *C. coli* isolates were assigned to clades 2 and 3, associated with environmental sources. For *C. jejuni,* comparative genomics revealed genes involved in oxidative and aerobic stress response. In *C. coli*, various carbon metabolism-related sequences were identified in clade 2 isolates and in clade 3 isolates, oxidative stress and putative virulence genes were detected. All water isolates were motile and the majority of *C. jejuni* isolates, but none of *C. coli* isolates, were able to form biofilm. *C. jejuni* survived better than *C. coli* in untreated well and lake water. Furthermore, in contrast to *C. coli,* a seasonal difference in survival was observed for *C. jejuni* with better survival in lake water collected during autumn than in spring. When tested in an *in vitro* infection model, all water isolates adhered to and induced IL-8 response in HT-29 cells indicating pathogenic potential. However, *C. coli* clade 3 isolates demonstrated a strong cytotoxic effect on human HT-29 cells leading to rapid cell death. This novel phenomenon was not observed for *C. coli* clade 2 or *C. jejuni* isolates.

This is, to the best of our knowledge, the first study on *Campylobacter* water isolates characterized using genomic, phenotypical and *in vitro* infection analyses. These findings suggest that some *Campylobacter* isolates might survive better than others in water and water survival patterns shown here help us further understand the seasonality and predominance of water-related *C. jejuni* infections.

Keywords: C. jejuni, C. coli, C.coli clade 2, C. coli clade 3, waterborne pathogens, whole genome sequencing, water survival, pathogenic potential, phenotypical identification

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ISSN 1651-6206
ISBN 978-91-513-0232-4
urn:nbn:se:uu:diva-339839 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-339839)

In wine there is wisdom
In beer there is freedom
In water there is bacteria

Unknown origin

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Nilsson A,** Johansson C, Skarp A, Kaden R, Engstrand L, Rautelin H. Genomic and phenotypic characteristics of Swedish *C. jejuni* water isolates. PloS One. 2017;12(12):e0189222.

II **Nilsson, A.,** Skarp, A., Johansson, C., Kaden, R., Engstrand, L., Rautelin, H. Characterization of Swedish *Campylobacter coli* clade 2 and 3 water isolates. MicrobiologyOpen. *In press.* DOI: 10.1002/mbo3.583

III Johansson, C., **Nilsson, A.,** Rautelin, H. *Campylobacter coli* clade 3 isolates induce rapid cell death *in vitro. Submitted*

IV **Nilsson, A.,** Johansson, C., Skarp, A., Kaden, R., Rautelin, H. Survival of *Campylobacter jejuni* and *C. coli* water isolates in lake and well water. *Submitted*

Published papers not included in this thesis

Skarp, C.P., Akinrinade, O., **Nilsson, A.J.**, Ellström, P., Myllykangas, S., Rautelin, H. Comparative genomics and genome biology of invasive *Campylobacter jejuni*. *Sci Rep.* 2015 Nov 25;5:17300

Ellström, P., Hansson, I., **Nilsson, A.**, Rautelin., Olsson Engvall, E. Lipooligosaccharide locus classes and putative virulence genes among chicken and human *Campylobacter jejuni* isolates. *BMC Microbiol.* 2016 Nov 21;16(1):116.

Contents

Abbreviations

bp	base pairs
CadF	*Campylobacter* adhesion to fibronectin
CBA	CFU-based adherence
CC	Clonal complex
CDT	Cytolethal distending toxin
CFU	Colony forming units
Cia	*Campylobacter* invasion antigen
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GBS	Guillain-Barré syndrome
IBS	Irritable bowel syndrome
Ig	Immunoglobulin
IL-8	Interleukin 8
LDH	Lactate dehydrogenase
LOS	Lipooligosaccharide
MALDI-ToF MS	Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
NGS	Next-generation sequencing
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RAST	Rapid Annotation using Subsystem Technology
ReA	Reactive arthritis
RNA	Ribonucleic acid
SGS	Second-generation sequencing
spp.	Species, plural
ST	Sequence type
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
T6SS	Type 6 Secretion System
tCDS	Translated coding sequences
TGS	Third-generation sequencing

Introduction

Historical aspects

The first known description of *Campylobacter* is by Theodor Escherisch in 1886. He observed spiral shaped bacteria in stool of infants suffering from enteritis and in colon of infants who had died of "cholera infantum", but believed that these bacteria was not the cause of disease (1). In the beginning of the 20th century, it was reported that *Vibrio*-like organisms caused abortions in sheep and cattle and also caused human infections and these bacteria were identified as belonging to *Vibrio* species (spp.) (1–5). In 1963, the new genus *Campylobacter* was proposed based on the low DNA base composition, nonfermentative metabolism and microaerobic growth and former *Vibrio* spp. were transferred to the *Campylobacter* spp. (6). The name *Campylobacter* was derived from the Greek words for curved (Campylo) rods (bacter).

In the 1970's, first filtration techniques and then selective culture media for isolating *Campylobacter* led to a renewed interest and *Campylobacter* was recognized as an important human pathogen (6,7). Since 2005 it has been the most commonly reported bacterial enteropathogen in the European Union (EU) (8).

General description

Campylobacter are Gram-negative spirally curved or corkscrew shaped rods with a width of 0.2-0.8 μm and a length of 0.5-5 μm (Figure 1) (9). The genus *Campylobacter* consists of at least 28 species (http://www.bacterio.net/campylobacter.html, last accessed 25 September 2017) and belongs to the family *Campylobacteraceae,* under the order of *Campylobacterales,* in the class of ε–proteobacteria and in the phylum of proteobacteria. Most of the *Campylobacter* spp. are motile with polar flagella at one or both ends, with exceptions of *C. gracilis (*non-motile) and *C. showae* (multiple flagella). *Campylobacter* spp. grow at temperatures between 37°C and 42°C, with *C. coli* and *C. jejuni* having an optimal growth temperature of 42°C. The majority of *Campylobacter* spp. are microaerophilic and need reduced oxygen (3-10%) and raised CO_2 (5-10%) levels (6). When *Campylobacter* are exposed to stress, such as high oxygen

levels, starvation or suboptimal temperatures, the bacteria can enter a non-culturable, but viable coccoid form (10).

In the following sections *Campylobacter* refers to *C. jejuni* and *C. coli,* unless otherwise stated.

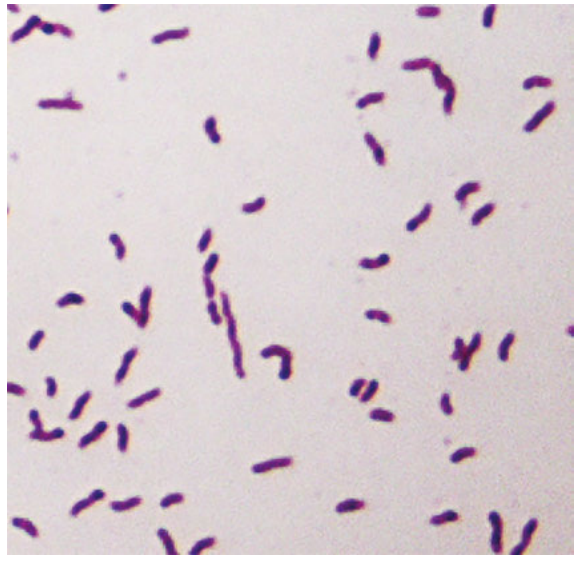

Figure 1. Gram-stained clade 3 *C. coli* showing small Gram-negative curved rods indicative of *Campylobacter* spp.

Epidemiology

Campylobacter are the most common cause of bacterial gastroenteritis in many western countries (8,11), but much less is known about the less resourced countries. Estimations of *Campylobacter* infections in the developing countries show that the incidence is higher, especially for small children, than in the developed countries (12). In general, the majority of human infections are caused by *C. jejuni* (80-90%) and *C. coli* (5-10%), which has led to studies focusing mainly on *C. jejuni* (6,13). In 2013, the number of reported confirmed human *Campylobacter* cases in the European Union (EU) was more than 200 000 (14), but true incidence predictions range from 2-20 million cases of human *Campylobacter* infections each year, making the estimated financial burden 2.4 billion euro annually in the EU alone. The large number of human infections each year makes *Campylobacter* a major threat to public health in the whole of Europe and it is mandatory to report *Campylobacter* infections in almost all EU member states (8).

In Sweden, *Campylobacter* are the most common cause of bacterial gastroenteritis and the number of reported cases has been increasing (Figure 2). Only over the last years, from 2013 to 2017, the number of reported human

Campylobacter infections has increased with around 30% (www.folkhalsomyndigheten.se, last accessed 10 January 2018; Figure 2). Until recently the majority of *Campylobacter* infections reported in Sweden were acquired while travelling abroad, but in 2015 the number of domestically acquired *Campylobacter* infections (52%) exceeded that of imported infections for the first time (https://www.folkhalsomyndigheten.se, last accessed 10 January 2018). During the last half of 2016 (July-December), the number of reported domestic human *Campylobacter* infections increased dramatically compared to the same period of the previous year, from 3464 cases in 2015 to 5693 cases in 2016 (Figure 2b, https://www.folkhalsomyndigheten.se, last accessed 10 January 2018). This increase in domestic *Campylobacter* infections lasted until the middle of 2017 (Figure 2), and in September 2017 it was reported that the number of domestic *Campylobacter* infections was back to normal. This dramatic increase in incidence was believed to be due to an increase in colonized chicken flocks in Sweden (www.folkhalsomyndigheten.se, last accessed 10 January 2018).

As in many temperate countries, a seasonal variation has been observed for *Campylobacter* infections in Sweden with a peak during the summer months (Figure 2) (15–17). This peak coincides with the period when people to a larger extent are being exposed to known risk factors for *Campylobacter* infections, such as travelling, barbeques, swimming in natural waters, drinking untreated water and animal contact (18–22). The seasonality peaks vary somewhat and in Europe the most stable ones have been observed in the Scandinavian countries and in Wales and Scotland (17).

Campylobacter are a part of the gut flora in many domestic and wild animals. Warm-blooded farm animals such as poultry, pigs, cattle and sheep are major reservoirs for *Campylobacter* and the bacteria are mainly thought to be transmitted to humans via handling and eating raw or undercooked meat, especially poultry (19,20). In 2015, in EU member states, *Campylobacter* were reported in almost one out of five broiler units (8). *C. jejuni* is the most prevalent *Campylobacter* found in most animals, with the exception for pigs for which *C. coli* is the most detected species (23). *Campylobacter* can also be passed on via unpasteurized milk (19), contact with domestic pets (18,20), untreated drinking water and swimming in natural waters (21,22). *Campylobacter* have also been found among wild birds (24,25), wild animals (26,27) and even in non-vertebrate vectors such as flies (28,29) and slugs (29).

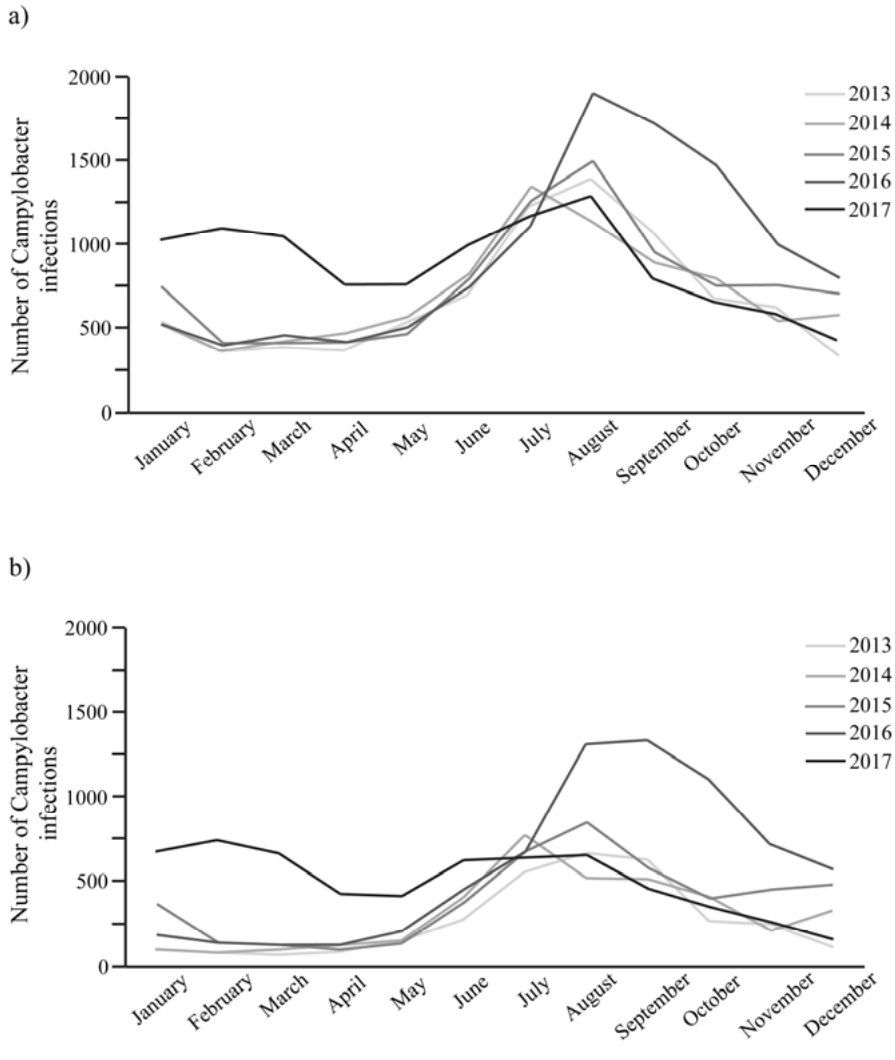

Figure 2. Total number of reported human *Campylobacter* infections in Sweden 2013-2017 showing the increased numbers as well as the seasonal peak during the late summer months. a) All reported human *Campylobacter* infections in Sweden. b) Reported domestically acquired human *Campylobacter* infections in Sweden. Based on data from https://www.folkhalsomyndigheten.se/ (last accessed 10 January 2018).

Serotyping

Before reliable genotyping methods were developed for *Campylobacter*, serotyping was commonly used to describe the *Campylobacter* population. In the early 1980's two major serotyping schemes, the Penner scheme based

on heat-stable antigens (30) and the Lior scheme based on heat-labile antigens (31), were developed. However, as serotyping methods are costly and time-consuming with a high number of untypable isolates, different genotyping methods have later replaced serotyping.

MLST and clade divison of *Campylobacter*

The multilocus sequence typing (MLST) system was originally developed for *Neisseria meningitidis* in 1998 to better describe the long term epidemiology and identify groups of strains more likely to cause disease (32). In 2001, MLST for *C. jejuni* was established and it soon became generally accepted for describing the population structure of *C. jejuni* and *C. coli* (33). *Campylobacter* MLST is based on the sequencing of the seven house-keeping genes (essential genes found in all isolates); aspartase A (*aspA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxymethyltransferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*) and adenosinetriphosphate synthase subunit α (*uncA*). Allel numbers are assigned according to the *Campylobacter* MLST reference database (http://pubmlst.org/campylobacter/) and provide a genetic profile or sequence type (ST) for each isolate (33). Related STs are grouped into clonal complexes (CCs) defined as STs that share alleles at least at four MLST loci. As MLST is a sequenced-based method, data from different laboratories and studies can easily be compared and are accessible online (http://pubmlst.org/campylobacter/).

C. jejuni show only a weakly clonal population structure and to date at least 9100 STs, divided into a multitude of CCs, are assigned in the Pub-MLST database (http://pubmlst.org/campylobacter, last accessed 31 January 2018). Some CCs are host- or niche-specific, i.e. specialists, while others are considered generalists and are capable of colonizing a large variety of hosts (34). The three most common clinically relevant CCs among *C. jejuni,* ST21CC, ST45CC and ST48CC, can be considered generalists as they have been isolated from various sources and hosts (35).

In contrast to *C. jejuni*, there are only two MLST-based CCs for *C. coli*, ST828CC and ST1150CC, the first of which includes a clear majority of genotyped *C. coli*. The majority of clinical and farm animal *C. coli* isolates belong to these two CCs, which in turn are part of one of three clades (clade 1) within *C. coli.* The remaining clades 2 and 3 of *C. coli* include mainly isolates from environmental sources (36). Clade 1 is associated with agriculture-adapted *C. coli* and up to 23% of the clade 1 gene content originate from *C. jejuni* (37).

Clinical picture

Campylobacter have a low infectious dose and as few as 500-800 colony forming units (CFU) can cause disease (38,39), but even a dose as low as 360 CFU has been estimated to be enough (40). The incubation time can vary between 18 hours up to eight days and the illness usually lasts for approximately one week (6). The symptoms of *Campylobacter* infection include watery diarrhoea, fever and abdominal cramps and in more severe cases also bloody stools and vomiting (12). *Campylobacter* can occasionally cause bacteraemia (41). Based on symptoms, *C. coli* infections cannot be distinguished from those caused by *C. jejuni*.

Important sequelae of *Campylobacter* infection include Guillain-Barré Syndrome (GBS) (42), reactive arthritis (ReA) (43) and irritable bowel syndrome (IBS) (44). GBS is estimated to develop in approximately 0.07% of *Campylobacter* cases following the infection (45). GBS is an autoimmune neurological disorder that causes paralysis and can affect respiratory and cranial nerve-innervated muscles as well (46). The symptoms are likely to be due to cross-reacting antibodies developed against the lipooligosaccharide (LOS) of *Campylobacter* and attacking human gangliosides (mainly GM1), which leads to degradation of axons. Carbohydrate mimicry between GM1 and *Campylobacter* LOS has been shown under experimental conditions (47). ReA is a postinfectious joint inflammation and has been shown to occur in 3-7% of *Campylobacter* infections (43,45). However, joint and musculoskeletal symptoms may be much more common and have been reported to occur in 39% of *Campylobacter* patients (48). IBS is estimated to follow *Campylobacter* infection in around 4% of cases. In this condition, symptoms like abdominal pain together with changed bowel habits occur without other known underlying diseases, which could cause these symptoms (45).

Laboratory diagnosis

Different types of diagnostic methods can be used for the detection of *Campylobacter* infections, such as stool cultures and PCR-based methods. For clinical diagnosis of *Campylobacter* infections, stool samples are cultivated on selective growth medium, which is useful for detecting *C. jejuni* and *C. coli* although less common species, such as *C. fetus* and *C. hyointestinalis,* can be missed (49). To identify typical catalase and oxidase positive colonies as *Campylobacter,* Gram staining is usually used and reveals a typical appearance of Gram negative curved rods, “gull wings” (Figure 1). *C. jejuni* can be distinguished from *C. coli* as showing a positive hippurate hydrolysis test, however, not all *C. jejuni* are positive for hippurate (6). Nowadays most clinical laboratories use matrix-assisted laser desorption

ionization – time of flight mass spectrometry (MALDI-TOF MS) to identify the isolates to the species level (50).

By using PCR-based methods for detecting *Campylobacter*, it is easier to detect the more uncommon species and not only the two main pathogens, *C. jejuni* and *C. coli.* However, no significant difference between the number of *Campylobacter* positive samples found was shown when a DNA-based method was compared with the conventional culturing on selective agar (49). The disadvantages with using PCR for detection include the cost, the increased workload and the lack of an isolate for further analyses, such as antimicrobial susceptibility testing (6,49). However, as intestinal infections caused by different enteropathogens cannot be distinguished from each other by symptoms only and several media are needed for culture, it has become more and more common in clinical laboratories to use multiplex PCR detecting a wide range of different enteropathogens (51).

Tests for *Campylobacter* stool antigens have also been developed and are commercially available, however, a low sensitivity often limits their clinical use (52). *Campylobacter* infection can also be identified by detecting specific antibodies of immunoglobulin (Ig) A, IgG and IgM classes in serum by e.g. enzyme-linked immunosorbent assays (ELISAs). IgA- and IgM-levels have been shown to be markers for an acute *Campylobacter* infection, as they are elevated as an early response and decrease to normal levels quite rapidly afterwards (39,53–55). The IgG-levels are also elevated in *Campylobacter* infection. However, as they can be elevated for a longer period of time than those of IgA and IgM classes, they are not so good markers of an acute infection unless paired serum samples showing significant antibody titer changes are used (54). Serological methods can be useful for revealing *Campylobacter* etiology in late sequelae.

Antimicrobial resistance

Campylobacter infections are often self-limited and antimicrobial treatment is usually not required, but drugs of choice include fluoroquinolones (e.g. ciprofloxacin) and macrolides (e.g. erythromycin) (56). Antimicrobial resistance is a growing problem in the world and *Campylobacter* are not an exception. The use of antimicrobials (fluoroquinolones) in growth promotors and as treatment for animals has been shown to be linked to the emergence of antibiotic resistance in *Campylobacter* (56,57). Fluoroquinolone resistance is nowadays very common among *Campylobacter,* and macrolides are used more often to treat *Campylobacter* infections (56).

The mechanisms behind fluoroquinolone and macrolide resistance are well known. Fluoroquinolones target the two enzymes DNA gyrase and topoisomerase IV, which are involved in the replication, transcription, recombination and repairing of DNA (56). Fluoroquinolone resistance is

mainly caused by amino acid substitutions in the quinolone resistance determining region (QRDR) of the *gyr*A gene encoding the subunits of the DNA gyrase (58). Several point mutations have been identified and the most common mutation causes the Thr86Ile substitution leading to a high-level resistance (59).

Macrolides inhibit bacterial synthesis of RNA-dependent proteins by targeting the 50S subunit (56). Macrolides bind to 23S rRNA nucleotides, which leads to changes in the ribosome and termination of elongation of the peptide chain (56). In macrolide resistant *Campylobacter* the binding sites have been modified and the antimicrobial agent is not able to bind to its target any more (56).

Fluoroquinolone and macrolide resistance can also be caused by the CmeABC multidrug efflux pump. The pump works in synergy with mutations for fluoroquinolone and macrolide resistance resulting in high-level resistance (56,60). Inactivation of the efflux pump leads to an increased susceptibility for several antibiotics, including those *Campylobacter* have intrinsic resistance against (56). By blocking the efflux pump, strains containing mutations in *gyr*A can become susceptible to fluoroquinolones (56,60).

Pathogenesis

In vitro infection models

The pathogenesis of *Campylobacter* is still largely unknown and seems to be a multifactorial process. Studies have revealed several putative factors with importance for colonization and disease. The lack of a suitable animal model has haltered the progress of studying infections *in vivo.* The disease caused by *Campylobacter* seems to vary depending on several factors such as the genetics of the bacteria (61,62), infectious dose (39), and the gut microbiota composition (63,64). Mice have been found to be suitable as colonization models, however, the colonization is sporadic, not long-lasting and disease manifestations are rare and atypical (6). The sporadic colonization and lack of typical symptoms are due to the colonization resistance against *Campylobacter* due to the commensal gut flora of mice (65). To circumvent these problems, a “humanized” mouse model with a normal immune system has been developed by removing the commensal flora with antibiotic treatments and replacing it with the human gut flora (65). This model can be used to get a better idea of the pathogenesis of *Campylobacter* and the impact of gut flora and the host immune status.

As suitable animal models have been lacking, *in vitro* infection models with different cell lines are used to study *Campylobacter* pathogenicity and assess the ability of different isolates or strains to adhere to, invade and induce a cellular response in host cells. *C. jejuni* are able to interact with a wide variety of eukaryotic cells, but even though the adherence is similar

between different cell lines other interactions such as invasion and induction of a cellular response are more dependent on the cell line. Therefore, the cell line needs to be chosen with the aim of the experiment in mind (66). Non-polarized cell lines, such as INT-407, have often been used for studying bacterial virulence even though the intestinal epithelium is polarized (67). For studies of *Campylobacter* translocation over the epithelium, cell lines that can form tight and adherent junctions connecting adjacent cells, such as HT-29, T84 and Caco-2, are required (66,67). For *C. jejuni,* mucosal colonisation has been suggested to be an important step during infection (68), but most cultured cell lines do not produce or secret mucins (66). The exception is the polarized HT-29-MTX-E12 (E12) cell line that produces a mucus layer which increases the adherence and invasion of *C. jejuni*.

To assess the adherence of *Campylobacter,* the CFU-based adherence (CBA) assay is considered to be the standard method. In short, bacteria are added to cultured cells at a defined multiplicity of infection (MOI) and non-adherent cells are removed by washing before the cells are lyzed. The lysate containing all cell associated bacteria (extracellular and intracellular) is serially diluted and plated on agar plates to enumerate the bacteria (66). As this method enumerates all cell associated bacteria and not only the adhered bacteria, the assay needs to be combined with the gentamicin assay to assess the invasion of *Campylobacter.*

Gentamicin is an aminoglycoside antibiotic substance that is unable to penetrate eukaryotic cells, but kills *Campylobacter* (67). In the gentamicin assay, the antibiotic is added to cell cultures infected with *Campylobacter* and kills all extracellular bacteria, while the intracellular bacteria are protected, and can be enumerated as described above (66).

Interleukin 8 (IL-8) is a proinflammatory cytokine produced by a number of cell types, such as macrophages and epithelial cells. IL-8 is involved in the recruitment of neutrophils and macrophages to the inflammation site and contributes to the resulting inflammation. *C. jejuni* adherence/invasion and the cytolethal distending toxin (CDT) can induce an IL-8 response in cell cultures (69,70), and IL-8 levels are commonly used to assess differences in virulence between *Campylobacter* isolates and strains *in vitro.*

Virulence factors

To colonize the human small intestine and to cause disease, *Campylobacter* need to be motile (39). The motility of *Campylobacter,* generated by the combination of the one or two polar flagella and the corkscrew shape of the bacteria, is unusual and facilitates the movement through viscous substances, such as the mucus layer (71,72). In experiments, non-motile *C. jejuni* mutants have been shown to colonize chickens at a lower level than the motile wild type strains (73). For *Campylobacter* to move in the right direction, the bacteria use chemotaxis. *C. jejuni* have been shown to display chemotactic motility towards amino acids and other components of chicken

gastrointestinal tract and of the mucus layer (74). For non-chemotactic but motile *C. jejuni* mutants, it has been shown that a much higher inoculum is needed for colonization than for the wild type (75).

When the bacteria have reached the site for colonization, they adhere to the epithelial cells. The adhesion to eukaryotic cells is mediated by several proteins, such as the *Campylobacter* adhesion to fibronectin protein (CadF) which binds specifically to fibronectin in the cell membrane (76). CadF mutants have been shown to be incapable of colonization or have a reduced colonization *in vivo* in chicken and *in vitro* in human intestinal epithelial cells (INT407) (76,77). It has also been suggested that CadF activates the GTPases Rac1 and Cdc42, which facilitates the internalization of *Campylobacter* into the host cells (78,79). For the invasion of *Campylobacter* into the target cells, a number of factors have been identified as being important, for example the *Campylobacter* invasion antigen (Cia) proteins (80). The Cia proteins have been shown to be secreted into the host cell and seem to be crucial for *Campylobacter* colonization and invasion (81). The secretion of Cia proteins into the host cells is mediated by the flagella, which is suggested to serve as a type 3 secretion system (T3SS)-like organelle in *Campylobacter* (81). The T3SS, which has not been identified in *Campylobacter,* can be found in many Gram negative bacteria and is used to deliver effector proteins to host cells and the analysis of the T3SS indicates that it has evolved from flagella (82,83).

A well-known toxin commonly produced by *Campylobacter* is the CDT, which has also been found among several other Gram negative bacteria (84). The CDT is composed of two binding proteins (CdtA and CdtC) responsible for binding of the toxin to the target cells and deliver the enzymatically active third protein of CDT, CdtB (80). CDT blocks the cells in the G2 phase and stops the cells from entering mitosis (80). For CDT to be functional all three components must be active (80).

C. jejuni cells are surrounded by a capsule consisting of polysaccharides, and the capsule is involved in the survival and the colonization of the bacteria (85). The genes coding for the biosynthesis of the capsule are located in a hypervariable region and are phase variable (86). Mutants lacking capsules show reduced resistance against human serum, less ability to colonize poultry and less invasion of INT-407 cells (80).

The regulation and transport of iron has been shown to be important for colonisation in chicken. *C. jejuni* mutants lacking iron uptake genes have been shown to have a lower ability for colonization of chickens (87,88). An increase of *Campylobacter* transcription of receptors for hemin and haemoglobin have also been observed in caecum of chicken (89).

LOS are major glycolipids on the cell surface of *Campylobacter.* Compared to the lipopolysaccharide (LPS) on many other Gram negative bacteria (including enteric bacteria), LOS lacks the O-polysaccharide chain rendering the bacteria hydrophobic and sensitive to solubilisation by bile (90). LOS displays a wide diversity and there is a large variation in the gene

content responsible for *Campylobacter* LOS biosynthesis (6). The LOS is involved in the ability of *Campylobacter* to evade the host immune system and to colonize host cells. *Campylobacter* with sialylated LOS have been suggested to have a higher potential for invasiveness (91), although conflicting results have been reported (92), and a higher serum resistance has been proposed (93).

The type IV secretion system (T4SS) is a cell-surface organelle which has been identified in *Campylobacter* (94). With the T4SS bacteria can secrete (e.g. virulence proteins into host cells) and take up (e.g. DNA) macromolecules by a cell-contact depending mechanism. Another secretion system that has been identified in *Campylobacter* is the type VI secretion system (T6SS) (95). The T6SS consists of a needle-like structure by which the bacteria injects effector proteins into adjacent cells and a platform structure that anchors the T6SS to the cell membrane of the bacteria (95). Having a T6SS may be advantageous as it may be a way for the bacteria to outcompete or to interact with other bacteria (96). The function of T6SS in *Campylobacter* is not fully understood, but has been suggested to play a part in adhesion and invasion of human epithelial cells (97).

However, the importance of the different putative virulence factors is far from clear. It was recently shown that in whole genome sequenced clinical isolates belonging to *C. jejuni* ST677CC, CDT was disrupted and the isolates lacked several virulence traits including LOS sialylation genes (62). However, isolates of ST677CC seem to be invasive as they were found among 48% of Finnish patients with *Campylobacter* bacteraemia (61). Thus, more studies on relevant clinical isolates are needed to get a clearer picture of the pathogenesis of *Campylobacter*.

Whole genome sequencing and next-generation sequencing

In 1995, the genome of *Haemophilus influenzae,* the first whole genome sequenced bacterial genome, was published (98). Sanger-sequencing had been introduced already in the 1970's (99), but only allowed for sequencing of shorter fragments. Whole genome sequencing was made possible by the combination of Sanger-sequencing with shotgun sequencing technology (first-generation sequencing) (98). For shotgun sequencing, the DNA is cut into smaller fragments and a clone library is prepared. These shorter fragments are then sequenced creating multiple reads. The reads are aligned into longer sequences and a continuous DNA strand can be created from overlapping reads (98). Shortly after 1995, several other bacterial genomes were published, including *Escherichia coli* in 1997 (100) and *C. jejuni* in 2000 (101).

A decade after the sequencing of *H. influenzae,* next-generation sequencing (NGS) made it possible to sequence multiple DNA fragments in parallel on high-throughput platforms. The introduction of NGS made whole genome sequencing more available as being both faster and cheaper (102–104). NGS can be divided into two groups; second-generation sequencing (SGS), e.g. Illumina and Ion Torrent, and third-generation sequencing (TGS), e.g. PacBio (103,104). In general, SGS produces shorter reads but with a lower error rate at a lower cost than TGS and it allows for a larger throughput of samples. However, TGS has several advantages as it can be performed in real time and the longer reads allow for easier *de novo* assembly, better sequencing of repetitive regions and easier detection of mutations (103,105).

The reads produced by NGS are assembled into contigs, i.e. longer nucleotide sequences, and the assembly can either be performed by mapping against a known genome or *de novo* without a reference genome (103,105). For the assembly of genomes either against a reference genome or *de novo*, several software programs have been developed and are optimized to handle reads from different technologies, e.g. MIRA (Mimicking Intelligent Read Assembly) (106). After assembly of the genomes, annotation can either be performed manually or automatically by using different tools and programs to gain more information of the gene content and functions. One program that can be used for automatic annotation is the RAST (Rapid Annotation using Subsystem Technology) web server. RAST uses several subsystems to predict genes in the assembled genomes and BLAST searches against reference datasets and publicly available annotated genomes to forecast the function of the predicted genes (107).

First whole genome sequenced *Campylobacter jejuni*

The first whole genome sequenced *C. jejuni* (NCTC 11168) was published in 2000 and revealed a quite small circular chromosome of approximately 1.6 Mbp with a G + C content of approximately 30.6% and 94.3% of the genome coding for proteins (101). This genome revealed hypervariable regions consisting of homopolymeric tracts and a high variation frequency due to the lack of DNA-repair genes. The majority of these hypervariable sequences were found in genes encoding proteins for biosynthesis and modification of surface structures such as LOS and flagella (101). Variation in the length of the homopolymeric tracts can result in slipped-strand mispairing by affecting the translation and lead to phase variation (108).

Campylobacter and water

Drinking untreated water from private water supplies and recreational use of water are risk factors for *Campylobacter* (21,22,109). Studies have also

shown that cattle are more likely to test positive for *Campylobacter* during the grazing periods outside when lakes have been used as the main water source (110) or if cattle get their drinking water from private supplies of untreated water (111). Waterborne outbreaks of *Campylobacter* are not uncommon and during 1998-2012, 29% of all the reported waterborne outbreaks caused by different microorganisms in the Nordic countries were caused by *Campylobacter* (112). The most common bacterial cause of waterborne outbreaks in Sweden is *Campylobacter* (113).

Groundwater for drinking water in the Nordic countries is normally not disinfected and contamination after heavy rain has been associated with waterborne *Campylobacter* outbreaks in Finland (109,112). Even though the majority of the population in Sweden are supplied with chlorinated and UV-treated surface water, many households are supplied with untreated water from private wells or untreated groundwater. In 2014, approximately 1 million people in permanent households and an additional 1 million people in their summer houses were supplied by private wells and around 400 000 persons received their drinking water from untreated groundwater (112).

Survival in water

To be able to cause sporadic infections or an outbreak via water, *Campylobacter* need to be able to survive in water. Some studies have shown that even though *Campylobacter* are considered to be fastidious and sensitive to different types of stress (114), *C. jejuni* can survive in a wide range of environments and in water at low temperatures for up to four months (115–118). How *Campylobacter* can survive in an environment such as water is not fully understood but several strategies have been suggested. *Acanthamoeba polyphaga* has been proposed to act as a reservoir for *C. jejuni* and the bacteria can evade an unfavourable environment by invading this protozoa (119). Another way for bacteria to survive in harsh conditions is by forming biofilm (120). Motility has also been shown to be of importance for the formation of biofilm. *Campylobacter* lacking flagella have been shown to be incapable of forming biofilm on a surface or unable to form cell-to-cell interactions in pellicle (121).

Several genes and stress response mechanisms have been identified for *Campylobacter*, even though many of the classic bacterial stress response mechanisms are lacking (122). In most bacteria, there are two types of superoxide dismutases (SOD), SodA and SodB, that protect against oxidative stress by catalysing the conversion of oxygen radicals to hydrogen peroxide and dioxygen (123). In *C. jejuni*, however, only SodB has been identified (123) and instead a catalase coded for by *katA* is induced by oxygen exposure and converts hydrogen peroxide to water and dioxygen (124).

The survival of *C. jejuni* in water has been shown to be affected by the nutrient concentration in the water (125). In general, *Campylobacter* are unable to utilize sugars for energy and growth and instead are reliant on

amino acids (122). A homologue of the carbon starvation protein A (CstA) in *E. coli*, Cj0917, has been shown to be upregulated in *C. jejuni* during starvation. This protein is believed to be involved in the utilisation of peptides for amino acids (122). However, a pathway for fucose uptake and metabolism in several strains and capability of glucose catabolism in certain strains of *Campylobacter* have been identified during the recent years (126).

Water and other natural environments are full of different microorganisms that can influence the survival of *Campylobacter*. One example is the proposed function of protozoa as reservoirs for *Campylobacter* mentioned earlier (119). Furthermore, by co-culturing *Campylobacter* with *Pseudomonas* spp., it has been shown that the survival at atmospheric levels of oxygen is enhanced (127). In addition to protecting themselves from harsh conditions by forming biofilm, *Pseudomonas* has been suggested to be able to create a protective environment for other bacteria as well (128). As *Campylobacter* have been suggested to be able to colonize pre-existing biofilms (129), this may be one way by which *Pseudomas* can enhance the survival of *Campylobacter*. Furthermore, it is possible that *Campylobacter* can survive by taking up resources, such as metabolites, produced by other bacteria in the surrounding environment (122).

Aims

C. jejuni and *C. coli* can be transmitted to humans via contaminated water but factors crucial for the viability of these microorganisms in water are mostly undiscovered. Water isolates of *C. jejuni* and *C. coli* have not been characterized by whole genome sequencing together with phenotypic analyses and studies on water survival have mainly been performed with reference strains of *C. jejuni* in artificially treated water samples. The general aim of this PhD thesis was to characterize *C. jejuni* and *C. coli* isolates originally cultivated from water in order to better understand their water survival strategies and their pathogenic potential. The specific aims were:

- To genetically characterize *C. jejuni* and *C. coli* water isolates using whole genome sequencing and comparative genomics to get a better understanding of the *Campylobacter* isolates that can be found in water, which genotypes can be found and differences and similarities between the isolates.

- To further phenotypically characterize the *C. jejuni* and *C. coli* water isolates using selected phenotypical analyses in order to reveal characteristics important for survival and pathogenic potential.

- To investigate the survival of *C. jejuni* and *C. coli* water isolates in untreated lake and well water. To reveal potential seasonality in water survival, by testing in lake water collected both in spring and in autumn.

- To study the pathogenic potential of *C. jejuni* and *C. coli* water isolates in an *in vitro* infection model and determine adhesion to and the cellular response in human cells. To correlate the effects on the cell to the presence and expression of selected putative virulence genes.

Materials and methods

Bacterial isolates (*I, II, III* and *IV*)

The water isolates were collected in 2000 by the Swedish National Food Agency from incoming water at surface water plants in Sweden (Table 1). The bacteria were originally isolated by filtrating water samples of 200 ml through 0.22 µm filters and incubating the filters in preheated enrichment broth (Preston broth) at 37°C for 24 h in a microaerobic atmosphere (Campygen, Oxoid, Basingstoke, UK). The broth was then cultured on CCDA plates (Blood-free campylobacter selective media) and *Campylobacter* positive colonies were collected from the plates and stored at -80°C. Seven isolates were identified as *C. jejuni* and eight as *C. coli* using MALDI-TOF Biotyping (Microflex, Bruker, Billerica, Massachusetts, US). All experiments were performed on isolates cultured directly from -80°C freezer to keep the passage number low.

In addition to the water isolates, selected reference strains and other characterized *Campylobacter* isolates were included for comparison and as positive controls in the studies (Table 2).

Genomics

Whole genome sequencing (*I* and *II*)

The isolates were cultured for 24-48h on blood agar plates before DNA extraction with MagNa Pure Compact Nucleic Acid isolation Kit I according to the manufacturer's protocol version 12. The isolates were whole genome sequenced using an Illumina HiSeq platform with a 2 x 300 paired end run. The assembly of the reads to contigs was done using the Mira plugin (version 1.0.1.) in Geneious (8.1.5.) (130). The assembled sequences were annotated using RAST (107) and the translated coding sequences (tCDS) were extracted. Orthologue clusters were determined by performing a reciprocal BLASTp query with an e-value of 1e-5 and the OrthAgogue (131) and MCL-edge tools (132). Phylogenetic analysis of the whole genome sequences of the *Campylobacter* water isolates together with *C. jejuni* and *C. coli* reference strains, and whole genome sequenced clinical *C. coli* clade 1 isolates was performed using Gegenees with a threshold of 20% (133).

Table 1. *Campylobacter* water isolates used in *papers* I-IV.

Species	Isolate	Water sample collection		Genetic description		
		Water catchment (Municipality)	Month	Sequence type (ST)	Clonal complex (CC)	Clade
C. jejuni	VA1	Öxsjön (Lerum)	March	48	48	n.a.
	VA12	Vänern (Lidköping)	April	637	1275	n.a.
	VA25	Långasjön (Karlshamn)	September	48	48	n.a.
	VA33	Motala Ström (Norrköping)	October	683	ua	n.a.
	VA48	Långasjön (Karlshamn)	November	793	ua	n.a.
	VA49	Helgasjön (Växjö)	November	8853 [a]	ua	n.a.
	VA52	Mälaren (Botkyrka)	September	48	48	n.a.
C. coli	VA6	Glan (Norrköping)	April	8850[a]	ua	2
	VA7	Glan (Norrköping)	April	ua	ua	3
	VA8	Eskilstunaån (Eskilstuna)	March	8851 [a]	ua	2
	VA15	Eskilstunaån (Eskilstuna)	April	ua	ua	3
	VA24	Mälaren (Botkyrka)	June	8852 [a]	ua	2
	VA37	Göta Älv (Trollhättan)	October	ua	ua	2
	VA38	Glan (Norrköping)	October	ua	ua	3
	VA46	Göta Älv (Trollhättan)	November	ua	ua	2

[a]New STs, ua= unassigned, n.a.= not applicable

Table 1. Additional isolates and sequences used in *papers I-IV*.

Species	Isolate	In paper	Reference
C. jejuni	NCTC 11168	I-IV	Reference strain
	81-176	I-II	Reference strain
	76577	I-II	Revez et al. 2011 (134)
C. coli	LMG 6440	II-IV	Reference strain
	76339	I-IV	Skarp-de Haan et al. 2014 (135)
	RM2228	II-III	Reference strain
	BIGS0002	III	Sheppard et al. 2013 (37)
	BIGS3	II-III	"
	BIGS0005	III	"
	BIGS0015	"	"
	BIGS0017	"	"
	BIGS0019	"	"
	BIGS0021	"	"
	A12B2	II	NCBI database
	B15B3	"	"
	F14B4C4	"	"
	F15D2	"	"
	H055260513	"	"
	H055260517	"	"
	H091320798	"	"
	H091940890	"	"
	H092560131	"	"
	H093580632	"	"
	H094560718	"	"
	H105160209	"	"
	K11	"	"
	M2D2	"	"
	M4A4	"	"
	M5D4	"	"
	C9A1	"	"
	D13A4	"	"
	H053120426	"	"
	H060740717	"	"
	H060960756	"	"
	H060960757	"	"
	H061680633	"	"
	H061980521b	"	"
	H065100506	"	"
	H065160534	"	"
	H071960681	"	"
	H072620566	"	"
	H073220518	"	"
	H073580401	"	"
	H074080509	"	"
	H074080511	"	"
	H075140555	"	"

H075200522	“	“
H081380695a	“	“
H082280513	“	“
H082280515	“	“
H082560569	“	“
H083420694	“	“
H083420701	“	“
H085160742	“	“
H085160749	“	“
H090980249	“	“
H092260569b	“	“
H093580324	“	“
H094560713	“	“
H094560720	“	“
H094860392	“	“
H102740169	“	“
H110340458	“	“
H132840800	“	“
K3D1	“	“

Gegenees was also used for a phylogenetic analysis of the whole genome sequences of the *C. coli* water isolates together with 53 additional publicly available *C. coli* clade 2 and 3 sequences derived from environmental water isolates from the NCBI database, *C. coli* clade 1 sequences from a previously published collection and sequences of six clinical *C. coli* clade 1 isolates. The results were exported as .nex-files and imported in SplitsTree4 version 4.13.1 (136) or Geneious (130) to create phylogenetic trees. Alignment and phylogenetic analyses of selected genes were performed in CLC Main Workbench (Qiagen, Hilden, Germany) using standard program settings.

For the comparative genomics analyses, the orthologue clusters were extracted to Microsoft excel where they were manually searched to find groups of orthologues shared by or unique to certain *C. jejuni* STs or *C. coli* clades. Nucleotide or predicted amino acid sequences of interest were extracted from RAST (137) and blasted against the NCBI database. BLAST searches of the sequences were also performed against all our *Campylobacter* water isolates, 53 additional *C. coli* clade 2 and 3 environmental water isolates from the NCBI database and previously published *C. coli* clade 1 isolates using Bionumerics (Bionumerics version 7.6.1 created by Applied Maths NV. Available from http://www.applied-maths.com). Alignments were performed in MAFFT version 7 (137) and in CLC Main Workbench.

For MLST, a sequence query of the genome sequences was performed against the PubMLST database (https://pubmlst.org/campylobacter/). The genome sequences that did not match any of the STs in the database were submitted to the PubMLST database for ST assignment.

Plasmids (*I* and *II*)

For the *C. jejuni* water isolates, the feature "circulize contigs with matching ends" of the Geneious *de novo* assembler version 8.1.9 (130) was used to assemble plasmids.

For the *C. coli* water isolates, the presence of plasmids was determined by extracting the plasmids using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, Massachusetts, US) and visualisation was performed with gel electrophoresis on a 0.7 % agarose gel.

PCR (*I, II* and *III*)

RNA preparation and cDNA synthesis

Bacterial RNA from overnight cultures was extracted using the ISOLATE II RNA Mini kit (Bioline Reagents Ltd, London, UK) according to the manufacturer's protocol. The RNA was treated with DNase I (Ambion by life technologies, Carlsbad, USA) both on column and on the final RNA preparation. The RNA concentration was measured using NanoDrop (NanoDrop ND-1000, Thermo Fisher Scientific) and the RNA was run on a 1% agarose gel to verify its integrity.

For cDNA synthesis, 0.5-1 µg of RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Fisher Scientific). For qPCR, 1/2000 of the total cDNA synthesis reaction volume was used.

Real time qPCR

Real time qPCRs were run in the BioRad CFX96 cycler using the DyNAmo HS SYBR green mix (Thermo Fisher Scientific) according to the manufacturer's protocol. Primers and annealing temperatures are shown in Table 3. To measure the adherence of *Campylobacter* in the *in vitro* infection model in *paper I* and *III,* previously published primers targeting the 16S rRNA gene were used (138). *C. jejuni* primers targeting *cdtA, cdtB* and *cdtC* were used to verify presence of *cdtABC* in the *C. jejuni* isolates in *paper I.* In *paper III,* the expression levels of *C. coli* virulence genes and clade 3-specific genes in overnight bacterial cultures were measured using gene-specific primers and normalized to 16S rRNA expression.

Conventional PCR

To determine the LOS classes of the *C. jejuni* water isolates in *paper I,* conventional PCR was performed using primers previously described by Parker et al. (139). The PCR was run on the Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, California, USA) using DreamTaq Green PCR

Master Mix (2x) (Thermo Fisher Scientific) according to the manufacturer's protocol.

The PCR products were visualized by gel electrophoresis on a 1% agarose gel stained with GelRed (Biotium Inc, Fremont, Californa, USA) and compared to GeneRuler Express DNA Ladder (Thermo Fisher Scientific).

Table 3. Primers used for putative virulence genes in *papers I* and *III.*

Species	Target	Sequence (5´→3´)	Ann. °C	Paper
C. jejuni	cdtA	ggcgatgctagagtttggc gaaccgctgtattgctcatagg	55	*I*
	cdtB	cgcgttgatgtaggagctaa gctcctacatctgttcctcca	55	"
	cdtC	caacaacttcagctgtgcaaa ggggtagcagctgttaaaggt	55	"
C. coli	cdtA	gacctttggagatgcaagag gctcaaattaaattcttccattacat	55	*III*
	cdtB	gtgcaaatcctatggatgttttag gagctaggtcttgatacagagcc	55	"
	cadF	gtgagaaattttatttttatggtttagc gaaagaaatttgatctcttgtttcaa	55	"
	iamA	caaaatatcatcacccgatttg ttcatattctccctcatcaaaatg	55	"
	ciaB	gatgcaatggaatttttacgaa gttttaaaatttcattttcaaaaaatctt	55	"
	pldA	cacaaagcacgaaatgcaag aaataattcatcttatatgagctgattcc	55	"
	ceuE	ctttagtttttgcatttttttgcatt ccctcatcactcatgctaatagg	55	"
	P.S.P[a]	cgatcaaggtataacagggca gctccagtagcttgcaatcc	55	"
	"	caataaaaaagttacagaatctgaaatttc cgggcttgattacaggttttac	55	"
	"	ctaaatcttacgaagctataaatcatcaca ctcgagctaattccccaagtaat	55	"
	A.H.[b]	cagccaagtttttttaggtgtagataat ctccttgccgataagatttaatct	55	"
	"	cagtggattttatagggcaaaaa gcgttatctcctaaaatttgctct	55	"

[a]Putative Serine protease, [b]Amidohydrolase

Phenotyping

All phenotypic experiments were incubated at 42ºC in a microaerobic environment and performed in duplicate with three repeats, unless otherwise stated.

Preparation of bacterial suspensions (*I-IV*)

Bacteria were grown in Brucella broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, US) for 17-18 h and collected from the cultures by centrifugation at 8000 x g for 5 mins. Bacterial pellets were resuspended to the desired concentration in phosphate-buffered saline (PBS) or RPMI 1640 without phenol red or L-glutamine (RPMI 1640; SVA, Uppsala, Sweden).

Biofilm assay (*I* and *II*)

The ability of the *Campylobacter* water isolates to form biofilm was tested as previously described by Revez *et al.* (134) with minor modifications. Bacterial colonies were harvested from a blood agar plate, suspended in PBS and added to Brucella broth in glass tubes for a final concentration of 4 x 10^6 CFU/ml and incubated at 37ºC. The broth was removed and 1% crystal violet solution was added for staining of biofilm. The isolates were identified as positive if a band was formed at the air-liquid interface. As a positive control the clinical *C. jejuni* isolate 76577, previously identified as being able to form biofilm (134), was used and broth without addition of bacteria was used as a negative control.

Motility assay (*I* and *II*)

The motility of *C. coli* water isolates was tested according to Szymanski *et al.* (71), with minor modifications. Of a bacterial suspension in PBS with the concentration of 10^8 CFU/ml, 5 µl was stabbed into a Brucella soft agar plate (0.4%) and the plates were incubated for 48 h. After the incubation, the diameters of swarming zones were measured.

Colony morphology (*II*)

During the work with the *C. coli* water isolates, clade-specific differences in the colony size and morphology were detected. To confirm these observations, the colonies of the isolates cultured on blood and CCDA agar were compared after 48 h incubation.

Use of carbon source (*II*)

The ability to use tricarballylate as an energy source was tested after the comparative genomics revealed a tricarballylate utilization system in the *C. coli* clade 2 water isolates. Bacterial suspensions were prepared in PBS from overnight cultures in Brucella broth and were added to RPMI 1640 supplemented with 20 mM tricarballylate (Sigma-Aldrich, Saint Louis, Missouri, USA) for a starting concentration of 10^6 CFU/ml. Growth was monitored over 24 h with OD-measurements at the start, after 18 h and after 24 h, respectively.

Water survival assays (*II* and *IV*)

In *paper II,* the survival of *C. coli* water isolates at 4ºC temperature was tested in autoclaved tap water in an aerobic athmosphere. Autoclaved tap water was chosen to avoid interference from native water microbiota and incubation was performed aerobically to mimic natural conditions.

For testing the water survival in *paper IV*, water was collected from the lake Erken (Uppland, Sweden) at six different time points during spring, late summer and early fall and at three different time points from a private well (Uppland, Sweden) during early summer. Before the start of the survival assay, samples of both waters were cultured on CCDA plates to ensure that the water was culture negative for *Campylobacter*. Standard methods were used to determine the chemical characteristics of the lake water samples. The pH of the well water samples was determined using pH strips (MColorpHast, pH 0-14, EMD Millipore Corp., Billerica, US).

Bacteria were added to the respective water samples for a final concentration of approximately 10^7 CFU/ml and stored at 4ºC under aerobic conditions in the dark. Samples were taken at the start of each experiments and on days 2, 4, 6 and 8 for culturable counts using a 1:10 dilution series. Two selected dilutions were plated on blood agar (*paper II*) or CCDA (*paper IV*) and incubated for 24-48 h. The *C. jejuni* reference strain NCTC 11168 and the clinical *C. coli* clade 3 strain 76339 (135) were included for comparison in all experiments and in addition, the *C. coli* clade 1 reference strain LMG 6440 was included in *paper IV*.

In vitro infection model (*I* and *III*)

The pathogenic potential of the *Campylobacter* water isolates to cause human infections was estimated with an *in vitro* infection model using human HT-29 colon cancer epithelial cells as previously described (138). The HT-29 cell line was maintained in RPMI 1640 supplemented with 10% Fetal bovine serum (Gibco by life technologies, Carlsbad, California, US), 2 mM

glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin (Swedish Veterinary Institute, Uppsala, Sweden). The cells were grown at 37ºC with 5% CO_2 and were routinely split before reaching 100% confluency. Low passage cells with a confluency of 40-60% were used for bacterial infections.

A bacterial suspension in RPMI 1640 was added to HT-29 cells grown in RPMI 1640 supplemented with 1% FBS for a MOI of 100. Before the cells and media were harvested for downstream analyses, the cells were photographed through the microscope lens.

Adhesion assay

The cells were washed with PBS four times to remove non-adhered bacteria. The cells were then lysed in 20 mM Tris (pH 7.5), 150 mM NaCl and 0.15% Triton X-100. The lysate was diluted and analysed with 16S rRNA qPCR together with the starting inoculum to determine the percentage of *Campylobacter* that had adhered to the cells.

IL-8 ELISA

IL-8 secretion is an early signal for an acute inflammatory response following a bacterial infection (69). To estimate the levels of IL-8 expressed, cell media was removed and diluted four to ten times before being analysed using the IL-8 ELISA kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). To assess the variations between experiments, a standard (included in the ELISA kit) was used. The results were expressed as fold increase over uninfected cells.

Cell viability

Released lactate dehydrogenase (LDH) to the cell culture media is an indicator for reduced cell viability. To quantify the viability of the cells, LDH activity was measured in the cell culture media at different time points after inoculation using the Pierce LDH Cytotoxicity assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The viability was expressed as relative amount of healthy cells compared to mock-infected cells, here set to 100% viability.

Statistical analyses

In *paper I-III, s*ignificance between the groups compared was determined using the Student's t-test (unpaired, two-tailed). In *paper IV,* the Mann-Whitney U test was used for determing significance between the groups. A significance level of 5 % was used in all papers.

Results and discussion

Genomic characterization of *C. jejuni* and *C. coli* water isolates (*I* and *II*)

Genetic diversity

In this thesis, seven *C. jejuni* and eight *C. coli* water isolates were whole genome sequenced. The genome sizes ranged from 1.61 to 1.91 Mbp for *C. jejuni* and from 1.66 to 1.95 Mbp for *C. coli.* A comparison of the genome sizes of the *C. coli* water isolates revealed that the clade 2 isolates, on average, had a larger genetic content than the clade 3 isolates. This was also true when genome sizes of the 53 additional clade 2 and 3 environmental water isolates from the NCBI database were included in the comparison. Three *C. jejuni* and four *C. coli* isolates were shown to include plasmids (included in the genome sizes above). A plasmid of 20801bp was identified in three *C. jejuni* isolates (VA1, VA25 and VA52) and was annotated against the plasmid sequence of *C. jejuni* F38011 after the sequence was blasted against the NCBI database. In four of the *C. coli* clade 2 isolates (VA6, VA24, VA37 and VA46) small plasmids of sizes below 20000 bp were identified by gel electrophoresis after plasmid extraction.

MLST analysis identified ST for six out of the seven *C. jejuni* isolates; ST48 (VA1, VA25 and VA52), ST637 (VA12), ST683 (VA33) and ST793 (VA48), respectively. One *C. jejuni* water isolate and all of the *C. coli* water isolates were originally unassigned to any STs and were submitted to PubMLST for ST assignment. One *C. jejuni* and three *C. coli* isolates were assigned to new STs; ST8850-53, respectively, while five *C. coli* water isolates remained unassigned to any STs. All three ST48 isolates belonged to ST48CC and the ST637 isolate to ST1275CC, whereas the remaining *C. jejuni* and all the *C. coli* isolates were unassigned to any CC (Table 1).

A phylogenetic analysis of the whole genome sequenced *Campylobacter* water isolates together with *C. jejuni* reference strains NCTC 11168 and 81-176, *C. coli* clade 1 reference strains RM2228 and LMG 6440, the clinical *C. coli* clade 3 isolate 76339 (135) and *C. coli* clade 1 from a previously published collection (37) placed the *C. jejuni* separate from the *C. coli* isolates and showed a distinct clade-division where five of the water *C. coli* belonged to clade 2 and three to clade 3 (Table 1, Figure 3). Of the *C. jejuni* water

isolates, the three ST48 isolates clustered closely together on one branch and the non-ST48 isolates placed closely together on another branch (Figure 3). Interestingly, our collection of *C. jejuni* water isolates represented both genotypes from environmental sources and those common among human isolates. *C. jejuni* ST1275 isolates have been reported to be common among wild birds and in environmental waters (35,140,141) and, according to the PubMLST database, are only rarely detected in human samples. Furthermore, *C. jejuni* ST683 and ST793 have hitherto only been found in wild birds and environmental waters. Also, as *C. coli* is concerned, the majority of isolates from environmental sources are known to belong to clades 2 and 3, as shown here for our *C. coli* water isolates, and are only rarely detected in human samples (37,135). However, ST48CC, including three ST48 water isolates of this study, is one of the most common CCs among human *C. jejuni* isolates (35,142), even though isolates from other sources are included as well. Thus, although the number of water isolates in our study was limited, the diversity of the genotypes made the collection interesting for further characterisation.

Comparative genomics

Comparative genomics was used to identify both differences and similarities between the *C. jejuni* isolates belonging to different STs and also between the *C. coli* isolates of the two clades.

In different environmental surroundings, such as water, *Campylobacter* may encounter various toxic substances, for example arsenic. For *Campylobacter* to survive in the presence of arsenic, the bacteria have developed resistence mechanisms (143). In the *C. jejuni* water isolates, genes coding for proteins involved in arsenic resistance were identified, whereof three were different arsenic effluc transporters. *ArsB* was identified in all of the *C. jejuni* water isolates and has been shown to contribute directly to arsenite resistance and indirectly to arsenate resistance in *C. jejuni* (144). The gene coding for Acr3 was found in the three ST48 water isolates and in the ST683 isolate, however, in all the three ST48 isolates *acr3* was disrupted by a premature stopcodon. Acr3, like ArsB, is suggested to contribute to *C. jejuni* arsenite resistance (143). The *arsP* gene coding for an efflux transporter for organic arsenic (145) was found in the ST1275CC isolate.

In three of the *C. jejuni* water isolates (ST1275CC, ST683 and ST793) an intact cluster coding for an anaerobic dimethyl sulfoxide reductase (DMSO-reductase) was identified. A DMSO reductase, previously described as clade 3-unique (135), was also identified in our *C. coli* clade 3 water isolates. DMSO has been suggested to act as an electron acceptor for *Campylobacter* under low-oxygen conditions (146) and may be advantageous for environmental survival. DMSO can be produced by algae (147) and exists naturally in marine environments (148).

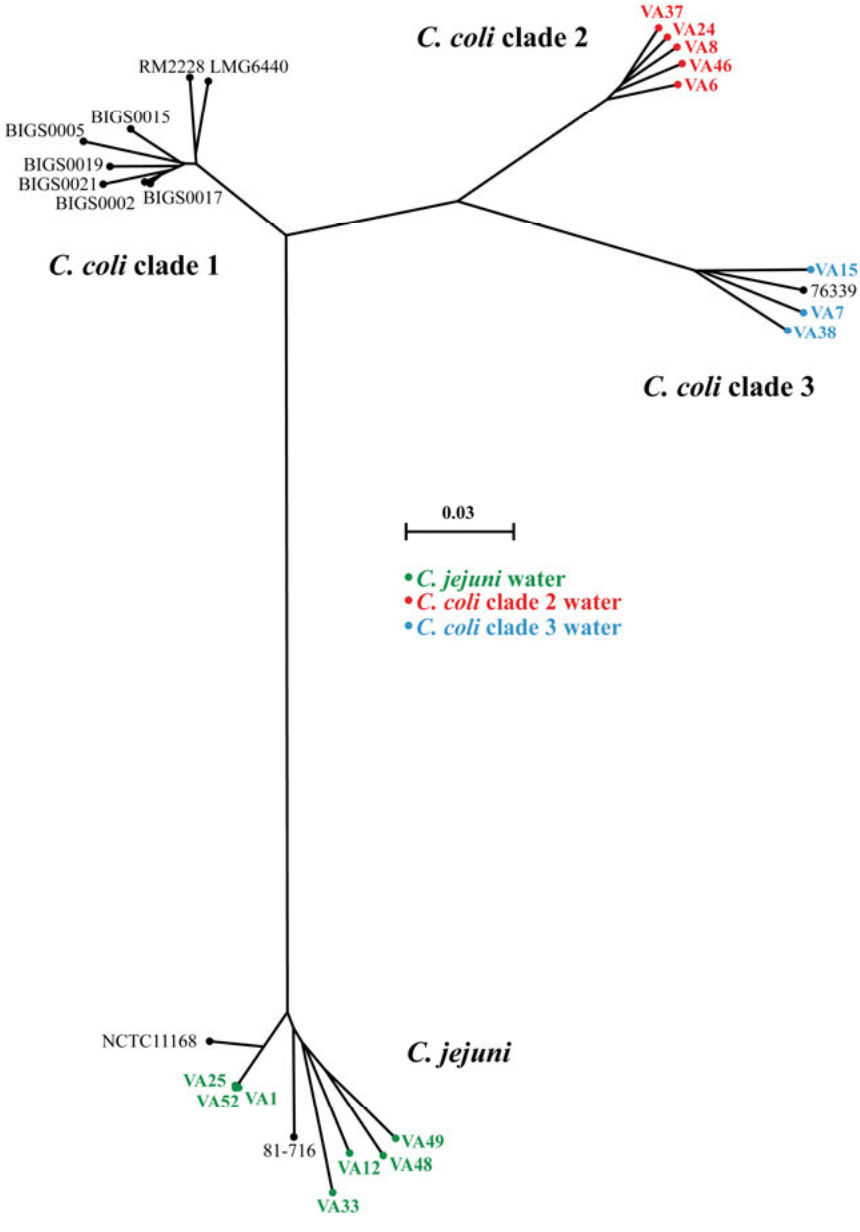

Figure 3. Neighbour-joining tree based on phylogenetic analysis of whole genome sequenced *Campylobacter* water isolates, *C. jejuni* reference strains NCTC 11168 and 81-176, *C. coli* clade 1 reference strains RM2228 and LMG 6440, the clinical *C. coli* clade 3 isolate 76339 (135) and *C. coli* clade 1 isolates from a previously published collection (37).

A Cytochrome-c (CytC) family protein that has been previously described as clade 3-specific and likely a part of the respiratory chain (135) was identified in all *C. coli* clade 3 water isolates.

An intact T6SS gene cluster was found in three *C. jejuni* isolates (ST1275CC, ST793 and ST8853) and in one of the *C. coli* clade 2 isolates (VA46). T6SS is used by bacteria to deliver effector proteins into adjacents cells (95) and has been suggested to be involved in *Campylobacter* adherence and invasion to human epithelial cells (97).

In addition, comparative genomics revealed genes involved in oxidative stress (*rrpB)*, virulence (*cdtABC*) and phenotypical features (*luxS, csrA* and *peb4*) for *C. jejuni* and genes involved in phenotypical features (*tcuABC, pseA* and *tlp8*) for *C. coli,* which are discussed further in the text below.

Phenotypic characterization of *C. jejuni* and *C. coli* water isolates (*I* and *II*)

When cultivating bacteria on agar plates, part of the identification is usually based on the varying colony appearances different bacteria can display. In this study, *C. coli* water isolates were shown to form two distinct types of colonies; small, round and even or large and blurry. The colony morphology of the *C. coli* isolates was further assessed on both blood and CCDA agar to see if the observed differences were constant or only random. The *C. coli* clade 3 isolates together with one clade 2 isolate (VA6) formed small, even and round colonies on both agar plates, while the *C. coli* isolates belonging to clade 2 formed large and blurry colonies difficult to distinguish from each other. The even and round colony forming pattern was also observed for the clinical *C. coli* 76339 of clade 3. These findings were constant for the two groups of isolates. For the *C. jejuni* water isolates we could see some variation in colony morphology, but the differences between the isolates were not constant.

As colony morphology might be associated with bacterial motility, all water isolates were tested for their motility in soft agar. All isolates tested were motile (Figure 4). For *C. jejuni*, a correlation between the size of the genome and the motility was demonstrated so that isolates with a smaller genome had a higher motility. As *C. coli* were concerned, we expected, on the basis of the large colony appearance, the *C. coli* clade 2 isolates to be more motile than the clade 3 isolates. However, the *C. coli* clade 2 isolates, with the exception of VA6 (showing small colonies), had a lower motility than the clade 3 isolates. This clade-specific difference was significant when VA6 was excluded from the analysis ($p=0.001$, Figure 4). As more genetic content was found in clade 2 than in clade 3 isolates, here too, although not so clearly as for *C. jejuni,* an association between the genome size and

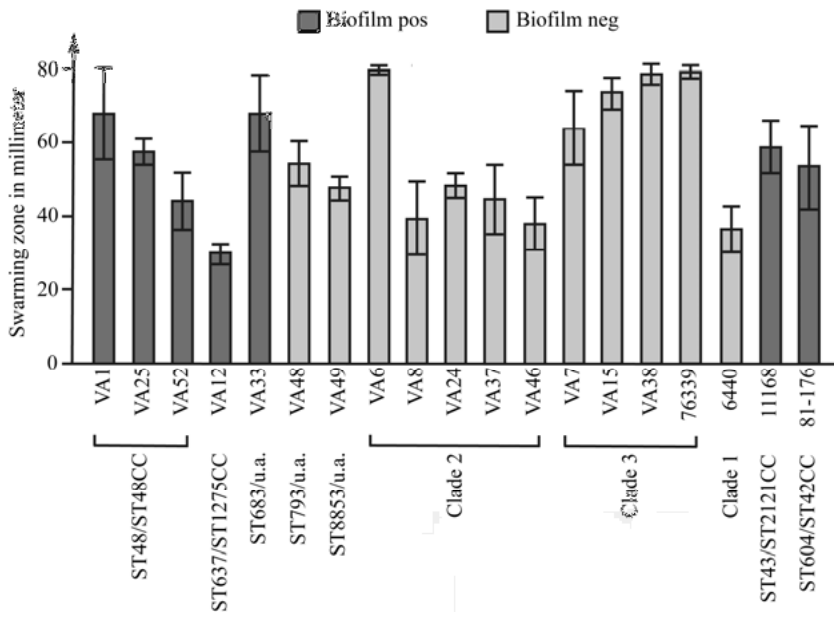

Figure 4. Motility and biofilm formation of all *Campylobacter* water isolates. Motility shown as swarming zone diameter. Mean values of 3 experiments with error bars indicating SDs are shown. Biofilm positive isolates are shown in dark grey and negative isolates in light grey. The *C. jejuni* reference strains NCTC 11168 and 81176, the *C. coli* clade 1 reference strain LMG 6440 and the clinical *C. coli* clade 3 isolate 76339 were included for comparison. ST-types, CCs and clade assignment are included where available (u.a.= unassigned).

motility could be shown. Of all the *Campylobacter* water isolates, *C. jejuni* VA12 (ST1275CC) had the lowest motility whereas *C. coli* VA6 and VA38 (clade 3) had the highest motility.

To further study motility, a BLASTn search for major motility genes, such as *flaA, flaB, motA, motB, flh, flg* and *fli*, was performed and revealed that these genes were present in the genomes. The nucleotide sequences of the motility genes in the *C. jejuni* water isolates were more closely analyzed and compared to the corresponding sequences in *C. jejuni* reference strain 11168 and revealed deletions and insertions in some of the genes. However, these nucleotide variations only led to a disrupted ORF in few of the cases. Further genetic analyses verified the presence of the gene *pseA* in the *C. coli* clade 3 isolates and in all *C. jejuni* isolates, but not in any of the clade 2 isolates. In addition, we performed a BLAST search for the *pseA* gene sequence in the 53 additional *C. coli* clade 2 and 3 sequences from environmental water isolates and of previously published *C. coli* clade 1 genomes (37) and showed this particular gene to be present in *C. coli* clade 1 and most of the clade 3 genomes, but not in any of the clade 2 genomes. *PseA* is involved in the glycosylation of the flagella and even though the absence of this gene in

C. jejuni has not been shown to result in motility loss (149), it may still have an effect on the motility of *C. coli.*

Furthermore, it has previously been reported that *C. jejuni* CJ1110c (*tlp8*) mutants have an increased swarming ability (150). This is in contrast with the present results on the motility of our *C. coli* water isolates, as the clade 2 isolates displaying the lowest motility were all lacking CJ1110c while this particular gene was present in the isolates belonging to clade 3 and showing higher motility. Also, a BLAST search for the nucleotide sequence of Cj1110c was performed in previously published *C. coli* clade 1 (37) and in the 53 publicly available environmental clade 2 and 3 genomes from the NCBI database together with the genomes of the *C. jejuni* water isolates. This revealed that CJ1110c was present in all of the *C. jejuni* isolates and clade 3 genomes, but not in any of the clade 1 or 2 genomes analyzed.

As motility has been shown to be an important prerequisite for biofilm formation (121), the ability to form biofilm was tested in all isolates. Although differences in the ability to form biofilm have previously been seen among *Campylobacter*, with *C. coli* in general showing less formation of biofilm as compared to *C. jejuni* (134,151–153), the results were striking in the present study. Of the *C. jejuni,* five out of seven isolates were able to form biofilm whereas none of the *C. coli* isolates were positive for biofilm formation (Figure 4). Motility and biofilm formation did not show any association as *C. jejuni* with both low and high motility were able to form biofilm whereas not even the *C. coli* isolates with highest motility, clade 3 isolates and clade 2 isolate VA6, could form biofilm. The presence of *csrA* and *peb4*, associated with biofilm formation in *C. jejuni* (154,155)*,* was confirmed in all of the *C. jejuni* water isolates with only minor variations in the nucleotide and predicted amino acid sequences between the isolates. The sequences of *luxS* gene involved in quorum sensing and suggested to be important for biofilm formation (156) were found in four out of the five biofilm positive but in none of the biofilm negative *C. jejuni* water isolates. However, the *luxS* gene sequence was also found in all of the *C. coli* water isolates of which none was able to form biofilm.

As a putative tricarballylate utilization locus consisting of three genes was identified in all the *C. coli* clade 2 isolates, we wanted to test the functionality of this particular locus and all the *C. coli* water isolates were cultured in minimal media with and without supplement of tricarballylate. As expected, all clade 2 isolates grew better in the supplemented than in the unsupplemented media. Although all the clade 3 isolates lacked the genes coding for the tricarballylate locus, one of the isolates (VA38), showed better growth in the presence of tricarbyllate. Tricarballylate is formed by rumen microorganisms as a fermentation product of trans-aconitate (157) and the ability to utilize tricarballylate as an energy source may be advantageous for bacteria colonizing ruminants but this remains to be verified in further experiments. A BLAST search was also performed in the 53 additional clade

2 and 3 environmental water genome sequences from the NCBI database and revealed that the tricarballylate locus only could be identified in the clade 2 sequences.

Survival of *Campylobacter* in water (*II* and *IV*)

Survival of *C. coli* in autoclaved tap water (*II*)

As, to the best of our knowledge, there were no studies on the water survival of *C. coli* water isolates or *C. coli* isolates of clades 2 and 3, we tested the water survival of our *C. coli* water isolates. For this, autoclaved tap water was chosen in order to have controlled conditions and to avoid the possible influence of aquatic microbiota on survival.

The water survival was measured over eight days and compared between the isolates of the two clades. On day 2, clade 3 isolates showed a better survival but otherwise there were hardly any clade-specific differences. Furthermore, when the results were expressed as the rates of culturability loss, no significant differences were seen between clade 2 and clade 3 isolates (Table 4). Of the individual isolates tested, VA6 (clade 2) had the highest rate of culturability loss and the lowest water survival of all the isolates, whereas the *C. jejuni* reference strain NCTC 11168 survived better than any of the *C. coli* isolates tested, which is in accordance with previous reports on *C. jejuni* showing better water survival than *C. coli* (125,158). Interestingly, the clinical *C. coli* clade 3 isolate 76339 included for comparison had a higher rate of culturability loss than the clade 3 water isolates.

Survival of *C.jejuni* and *C. coli* in lake and well water (*IV*)

To better mimic real environmental conditions, further water survival experiments were performed in untreated and unfiltered lake and well water samples and both *C. jejuni* and *C. coli* water isolates were included. Also, to study the effect of seasonality, we included lake water samples collected both during spring and autumn.

The survival in all three water samples was in general better for *C. jejuni* than for *C. coli* and significant differences in the rate of culurability loss were seen both in the autumn lake water and in the well water ($p<0.01$ and $p<0.05$, respectively; Table 4). For *C. coli* the survival was quite the same in all the three water samples, while for *C. jejuni* the survival varied more and the best survival, with the lowest rate of culturability loss, was demonstrated in the autumn lake water samples (Table 4). In Sweden, both domestically acquired sporadic infections and waterborne outbreaks of *Campylobacter* show a seasonal variation over the year with a clear increase during late summer/early autumn and with a predominance of *C. jejuni*

(www.folkhalsomyndigheten.se and www.livsmedelsverket.se, last accessed 26 September 2017). Although differences in seasonality were reflected in the water survival of the *C. jejuni* isolates, this could not be demonstrated for the *C. coli* isolates. In addition, interestingly, when looking at the original collection dates for the water isolates included in the study, we noticed that while half of the *C. coli* isolates had been collected during spring and half during autumn, the majority of the *C. jejuni* isolates had been collected during autumn (five out of seven). It needs to be verified with a higher number of isolates if *C. coli*, as compared to *C. jejuni*, show less seasonality.

Table 4. Rate of culturability loss of *C. jejuni* and *C. coli* water isolates and reference strains in the four types of water. The rate is expressed as Log_{10} CFU ml^{-1} bacteria that dies per day and is the absolute value of the slope of the linear best fit of respective survival curve. Standard deviation calculated from the individual isolates in each groups is included. [a]*C. coli* water isolates only, n=8.

	n	Autoclaved tap water	Spring lake water	Autumn lake water	Well water
C. jejuni	8	n.d.	0.31 ± 0.11	0.16 ± 0.03	0.23 ± 0.07
C. coli	10	0.30 ± 0.19[a]	0.28 ± 0.07	0.30 ± 0.04	0.32 ± 0.09
C. jejuni ST48	3	n.d.	0.26 ± 0.08	0.14 ± 0.03	0.17 ± 0.02
C. jejuni non-ST48	4	n.d.	0.38 ± 0.09	0.18 ± 0.03	0.28 ± 0.07
C. coli clade 2	5	0.34 ± 0.22	0.28 ± 0.09	0.32 ± 0.03	0.37 ± 0.05
C. coli clade 3	3	0.29 ± 0.09	0.30 ± 0.07	0.32 ± 0.03	0.23 ± 0.03

n.d. not determined

Among the *C. jejuni* water isolates, no significant differences in survival were demonstrated between ST48 and non-ST48 (ST637, ST683, ST793 and ST8853) groups, even though ST48 isolates in general had the lowest rates of culturability loss in all three water samples in our study. In addition, these two *C. jejuni* groups showed the same kind of seasonal variation with a lower rate of culturability loss in the autumn than in the spring lake water (Table 4).

When the water survival was compared between *C. coli* clade 2 and 3 isolates, a better survival was shown for clade 3 isolates in well water ($p<0.05$); which also better supported the survival of clade 3 isolates than autumn lake water (Table 4).

Cj1110c (*tlp8*), detected in the clade 3 (but not in the clade 2) *C. coli* isolates and in the *C. jejuni* water isolates, has been suggested to mediate

movement away from environment with high oxygen content to more microaerobic environment (150). Whether this would mean that the *C. jejuni* and the *C. coli* clade 3 water isolates are better to avoid high oxygen tension and therefore survive better than *C. coli* clade 2 isolates in water, needs to be further studied. Furthermore, one plausible explanation for the generally better water survival of *C. jejuni* than that of *C. coli* could be the absence of the MarR-type transcriptional regulator rrpB (regulator of response to peroxide) (159,160) in all but one *C. jejuni* water isolates (VA1275CC). RrpB has been associated with livestock-associated CCs and rrpB positive *C. jejuni* isolates have been shown to be more sensitive to aerobic and peroxide stress than isolates lacking rrpB (160).

Pathogenic potential of *Campylobacter* water isolates (*I* and *III*)

To assess the pathogenic potential of the *Campylobacter* water isolates, an *in vitro* infection model with human HT-29 colon carcinoma cells for measuring the bacterial adherence and the induced IL-8 response was set up. Genetic analyses were also performed on selected putative virulence genes.

To our surprise, the *C. coli* clade 3 water isolates together with the clinical clade 3 isolate 76339 had a rapid cytotoxic effect on the cells. This phenomenon could not be shown with any of the *C. coli* clade 2 or the *C. jejuni* water isolates. Already 1-3 hours after inoculation with the clade 3 isolates, the cells showed clear signs of necrosis and after 3 hours, viability less than 10 % was demonstrated by LDH activity. The most rapid induction of necrosis in the cells was obtained with the clinical isolate 76339 and the water isolate VA7.

All the *Campylobacter* water isolates were able to adhere to HT-29 cells (Figure 5), but the adherence levels 1 h after inoculation of bacteria varied. Compared to the *C. jejuni* reference strain NCTC 11168, the *C. jejuni* water isolates adhered at a lower level to the HT-29 cells. The three ST48 isolates showed both the highest and lowest adherence levels of *C. jejuni* water isolates (data not shown). For *C. coli* water isolates, the adherence was significantly higher than for the *C. jejuni* water isolates ($p=0.040$, Figure 5). Clade 3 isolates showed a significantly higher adhesion than clade 2 isolates shortly after infection (30 min, data not shown), but at 1 h post infection there was no significant difference between the two groups of isolates (data not shown). Interestingly, the clinical *C. coli* clade 3 isolate 76339 had the lowest adhesion of the clade 3 isolates. However, this may be due to the strong cytotoxic effect leading to a possibly lower number of harvested HT-29 cells for the adhesion assay. Instead of the standard CBA method with agar plating (66), we chose to use 16S rRNA qPCR to assess the level of adherence,

as the cell lysis buffer inhibited the growth of the *Campylobacter* isolates on agar plates.

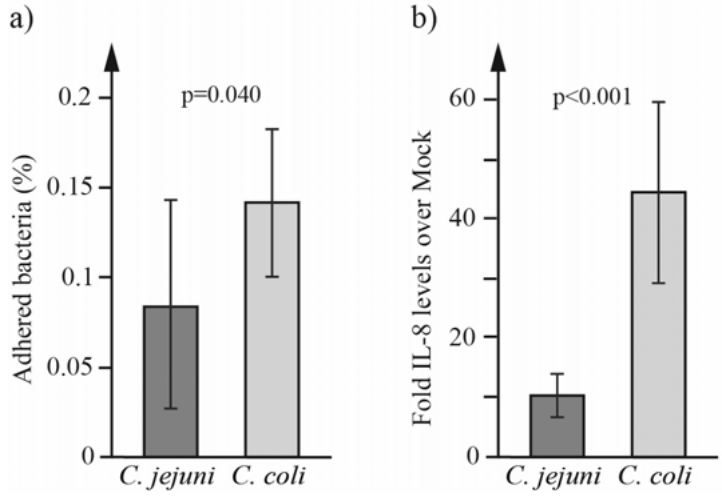

Figure 5. Adherence and IL-8 induction of *Campylobacter* water isolates. a) The adherence of *Campylobacter* water isolates to HT-29 cells 1 h post infection shown as percentage of the starting inoculum. b) The induction of IL-8 at 4 h post infection shown as fold increase over uninfected cells. Mean values of isolates of each species with error bars indicating SDs are shown.

In addition to the adherence levels, also the IL-8 responses were higher for the *C. coli* than for the *C. jejuni* water isolates ($p<0.001$) 4 hours after inoculation. On the individual level, the IL-8 responses varied between the isolates among *C. jejuni* and among *C. coli* water isolates, and there was no significant correlation between adherence and IL-8 response when comparing the individual isolates. We included the *C. jejuni* reference strain NCTC 11168 and the clinical *C. coli* clade 3 isolate 76339 for comparison and could see that the IL-8 response was higher for NCTC 11168 than for any other isolate while the IL-8 response induced by 76339 was the lowest of all the isolates. The low value for 76339 may, as suggested for the adhesion, be caused by the strong cytotoxic effect on the cells.

As variations were seen for both the adherence and the induced IL-8 response among the water isolates, a BLAST search for the presence of some selected putative virulence genes (*ciaB, pldA, cadF* and *ceuE)* was performed. These four genes were identified in the genomes of all *C. jejuni* and all *C. coli* water isolates. For *C. coli,* the genomes were also blasted for the presence of the putative virulence factor invasion-associated marker (IamA) and was identified in all clade 2 and clade 3 isolates. For *C. jejuni* only minor differences in the predicted amino acid sequences were identified but for

C. coli alignment and phylogenetic analyses revealed highly conserved clade-specific differences in the predicted protein sequences (Figure 6). For three of the genes; *ciaB, cadF* and *iamA,* the protein sequence variations were highly clade 3-specific as the protein sequence differed from the clade 1 and 2 sequences (Figure 6). Furthermore, we also identified genes coding for CDT in all *C. coli* water isolates, in all three ST48 water isolates and in the *C. jejuni* water isolate VA33 (ST683). While the other putative virulence genes had clade-specific sequence variations, the *cdtABC* genes were more conserved with a high level of identity between the predicted amino acid sequences of *C. coli* clades 2 and 3 predicted amino acid sequences (Figure 6). CDT has been shown to induce an IL-8 response in host cells, but when the bacteria were able to adhere to the host cells CDT did not have any additional effect on the IL-8 response (70). As all the *C. jejuni* water isolates were able to adhere to the cells, this may explain why no difference could be see in the induced IL-8 response whether or not genes coding for the CDT were present.

Campylobacter with sialylated LOS have been suggested to be more invasive than isolates with nonsialylated LOS and also to have a higher serum resistance (91,93). Four of the *C. jejuni* water isolates were identified as belonging to the potentially sialylated LOS locus classes B2 (all three ST48 isolates) and C (ST1275). Of the remaining three isolates, one was identified as belonging to either of the nonsialylated LOS locus classes F, J or S (ST683), while the other two remained untypeable for LOS locus class.

To better understand the cytotoxic effect of *C. coli* clade 3 isolates, the expression of selected putative virulence factors and clade 3-unique hypothetical proteins was analyzed in overnight cultures. Although the cytotoxic effect would have suggested a higher expression of the putative virulence genes in the clade 3 isolates, our results showed the opposite. For *cdtA, cdtB, ciaB* and *pldA,* no significant differences could be seen, but for *cadF, iamA* and *ceuE* the expression was significantly lower for clade 3 than clade 2 isolates. However, as discussed above, the predicted amino acid sequences contained clade-specific differences that may affect the function of the protein.

In addition to the putative virulence factors present in all isolates, we also took a closer look at two clade 3-specific genes identified by comparative genomics to see if they could be linked to the cytotoxic effect. Many of the clade 3-specific genes seemed to code for hydrolytic proteins involved in degradation of cellular components. One of the proteins we found interesting was a putative secreted serine protease. This serine protease was also identified in previously published *C. coli* clade 3 sequences (37,135) and in the highly virulent *C. jejuni* strain 81-176 (161). The RNA expression of this serine protease was analyzed here to verify its presence and similar levels were seen in all the clade 3 isolates of the present study. In *E. coli,* the Pet toxin, which contains a serine protease motif, has been shown to have a cyto-

toxic effect on Hep-2 and HT-29 cells (162), similar to those observed in our *in vitro* infection model. The effect of the Pet toxin is suggested to be due to the serine protease motif as the effect could be inhibited by serine protease inhibitors and was absent in Pet mutants (162). We also confirmed the expression of an amidohydrolase specific for clade 3 isolates, but with unknown function. However, further studies are needed to determine whether any of these hydrolytic proteins are involved in the cytotoxicity of the clade 3 isolates.

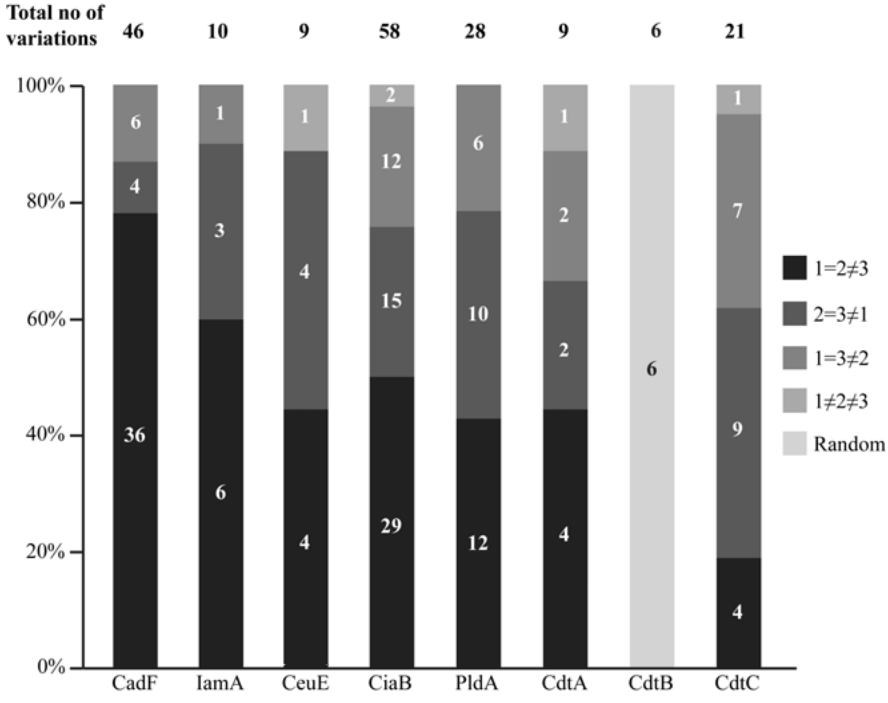

Figure 6. Variations in the predicted protein sequences of putative virulence factors in *C. coli.* Here, the amino acid variations are presented as shared between two clades and unique for one clade, not shared by any clades or random.

Conclusions

C. jejuni and *C. coli* isolates originally derived from water were characterized with whole genome sequencing, comparative genomics and selected phenotypical analyses. In addition, the survival of the isolates was tested in water samples and their pathogenic potential was analyzed using an *in vitro* infection model.

- *C. jejuni* water isolates included both common (ST48CC) and uncommon (ST1275CC, ST683, ST793 and ST8853) human pathogens whereas *C. coli* water isolates were assigned to the clades 2 and 3 known mainly to include isolates from environmental sources.
  Further genomic characterization of *C. jejuni* isolates revealed potential for better survival under oxidative and aerobic stress. For *C. coli,* clade-specific differences were demonstrated. *C. coli* clade 2 isolates with larger genomes possessed a variety of carbon metabolism-related coding sequences, such as a tricarbyllate utilization locus, while clade 3 isolates showed genes involved in oxidative stress, like Tlp8, a Cytochrome-c family protein and a DMSO reductase system.

- In the phenotypic analyses, all the isolates were motile, however, *C. coli* clade 3 isolates showed higher motility compared to clade 2 isolates. In addition, although most of the *C. jejuni* isolates formed biofilm, none of the *C. coli* water isolates were able to establish biofilm formation.

- *C. jejuni* survived better than *C. coli* in well and autumn lake water samples. In contrast to *C. coli,* seasonal differences were evident for *C. jejuni* as a better survival was demonstrated in the autumn lake water samples as compared to the spring lake water samples. These results are in accordance with the predominance and seasonality of *C. jejuni* in *Campylobacter* waterborne infections.

- All the *Campylobacter* water isolates tested were able to adhere to and induce an IL-8 response in a human colon cancer cell line and thus, showed pathogenic potential. Furthermore, all *C. coli* clade 3 isolates, but none of the clade 2 or *C. jejuni* isolates, displayed a strong cytotoxic effect with cell necrosis rapidly induced after inoculation of the bacteria. The exact mechanism for the rapid cell death remains to be revealed but

some putative virulence genes showed lower expression in clade 3 than in clade 2 isolates.

The genomic and phenotypic analyses together suggest that certain *Campylobacter* isolates could have potential to survive better in the environment; *C. jejuni* and *C. coli* clade 3 isolates might benefit from their potential to handle oxidative stress, for instance. Furthermore, the water survival patterns described here increase our understanding on the seasonality and predominance of *C. jejuni* in water-related infections. The role of the novel cytotoxic effect of *C. coli* clade 3 isolates in human infections remains to be shown.

Future plans

In this PhD project, a collection of *Campylobacter* isolates was characterized and studied with focus on water survival strategies. Even though the number of isolates available was limited, the collection not only included isolates of STs common in human infections, but also isolates with new STs and STs or *C. coli* clades common among environmental isolates. The majority of studies on *C. coli* have been performed with isolates belonging to clade 1, but here we had the opportunity to take a closer look at clade 2 and 3 isolates.

- We are interested in the role of other bacterial species in the water survival of *Campylobacter*. Practically nothing is known about the impact of the water microbiota on the survival of *Campylobacter*. Our group has previously shown that the composition of the human faecal microbiota is connected to the susceptibility of *Campylobacter* infection. We plan to analyze by NGS the abundance and diversity of bacterial communities in the water samples from the present study, with the hope to identify certain species that might explain the seasonality and predominance of *C. jejuni.* These findings could then be further validated by co-culturing of *Campylobacter* and selected bacterial species to see the impact on survival.

- In this study, we could see that all the isolates tested had a pathogenic potential after they hade been cultivated in nutrient-rich media at optimal temperature and oxygen levels. We would like to further test the ability of the isolates to adhere to and induce a cell response in the *in vitro* model after incubation in water at a low temperature in an aerobic environment. This could answer the question whether *Campylobacter* are still pathogenic after prolonged sustenance in environmental waters.

- One striking finding in this study was the demonstrated cytotoxic effect of the *C. coli* clade 3 isolates. Although we found some clade 3-unique genes, the mechanism of the cytoxicity remains unknown. It would be interesting to study the role of the unique genes in mutational analyses and the impact of the many hydrolytic proteins of clade 3 with the use of chemical inhibitors. Identified effector proteins could subsequently be recombinantly expressed, purified and added to cell cultures to try to mimic the necrotic effects seen with clade 3 isolates.

Sammanfattning på svenska

Campylobacter är den vanligaste orsaken till bakteriell gastroenterit i Sverige och i hela EU, och majoriteten av fallen orsakas av *C. jejuni* och *C. coli*. Campylobacter är också den vanligaste bakterien bakom vattenburna utbrott i Sverige och i Norden orsakas nästan en tredjedel av alla vattenburna utbrott av campylobacter. Tidigare har de flesta i Sverige som drabbats av campylobacterinfektioner smittats då de vistats utomlands, men under senare år har detta förändrats och 2015 var första året då fler än 50% blev smittade i Sverige.

Campylobacterinfektioner ger symptom som vattnig diarré, feber och magkramper och i de svårare fallen även blodig avföring och kräkningar. I vissa fall kan campylobacterinfektioner också ge upphov till följdsjukdomar som till exempel reaktiv artrit, irritabel tarm (eng. irritable bowel syndrom, IBS) och den autoimmuna neurologiska sjukdomen Guillain-Barrés syndrom.

Den vanligaste orsaken till campylobacterinfektioner är att man har fått i sig rå eller dåligt tillagad kyckling som är kontaminerad med bakterien, men man kan även bli smittad via opastöriserad mjölk, obehandlat dricksvatten eller genom att bada i naturliga vattendrag där bakterien finns.

I många länder med tempererat klimat kan man se en säsongsvariation av campylobacterinfektioner och i Sverige ser man en ökning av rapporterade campylobacterfall under sensommaren. Den här ökningen sammanfaller med den tid då svenskar utsätter sig för flera av de kända riskfaktorerna; resor, grillad mat, kontakt med djur och obehandlat dricksvatten från privata brunnar.

Den här avhandlingen har fokuserat på att studera campylobacter som isolerats från vatten. Detta för att bättre förstå hur en bakterie som anses vara känslig och krävande kan överleva i vatten och även studera dessa isolats potential som patogener.

De campylobacterisolat som har studerats i denna avhandling kommer från vattenprover som tagits innan vattnet gått in i ytvattenverk i Sverige. Isolaten karaktäriserades med helgenomsekvensanalyser i kombination med fenotypiska tester, vattenöverlevnadsexperiment och en *in vitro* infektionsmodell.

Artbestämning visade att av de 15 isolaten tillhörde sju *C. jejuni* och åtta *C. coli. C. jejuni* isolaten genotypades med multilokus sekvenstypningssystemet (MLST) för campylobacter och det visade sig att bland våra isolat

fanns både vanliga och ovanliga humanpatogener enligt de sekvenstyper (ST) som var representerade. En fylogenetisk analys av *C. coli* isolaten visade att de tillhörde två olika grupper, clade 2 och 3 (clade är en grupp av evolutionärt besläktade organismer med ett gemensamt ursprung). Majoriteten av alla *C. coli* isolat som ursprungligen kommer från olika miljökällor tillhör clade 2 och 3, medan isolat från kliniska prover och från djur på bondgårdar oftast tillhör clade 1. Jämförande genomisk analys av *C. jejuni* isolaten påvisade gener som är involverade i hantering av oxidativ och aerob stress. Samma analys visade att de *C. coli* isolaten som tillhörde clade 2 innehöll flera gener involverade i kolvätemetabolism, medan clade 3 isolaten hade gener för hantering av oxidativ stress och förmodade virulensgener.

Alla vattenisolaten var motila och majoriteten av *C. jejuni* isolaten, men inte något av *C. coli* isolaten, hade förmåga att bilda biofilm.

Isolatens förmåga att överleva i vatten testades i obehandlat vatten från en privat brunn och från en sjö. Dessa tester visade att *C. jejuni* överlevde generellt bättre än *C. coli* i de olika vattentyperna. Överlevnaden testades dessutom i sjövattenprover från både vår och höst för att se om det fanns någon skillnad i överlevnad mellan de två årstiderna. För *C. jejuni* kunde vi se en markant bättre överlevnad i höstsjövattnet än i det från våren, vilket stämmer överens med den säsongsvariation som kan ses för campylobacterinfektioner i Sverige.

Vattenisolatens förmåga att binda till och inducera ett cellsvar (IL-8) i en human koloncancer cellinje (HT-29) testades i en *in vitro* infektionsmodell. IL-8 tillhör en grupp proteiner som kan utsöndras vid en infektion och bidra till inflammation. Genom att mäta mängden IL-8 som utsöndras kan man uppskatta campylobacterisolatens patogenicitet. Alla isolaten kunde binda till HT-29 cellerna och inducera ett IL-8 svar, vilket tyder på att alla isolaten hade patogen potential. *C. coli* clade 3 isolaten hade dessutom en snabb toxisk effekt på cellerna och orsakade nekrotisk celldöd. Den här toxiska effekten kunde endast ses bland clade 3 isolaten och inte bland varken clade 2 eller *C. jejuni* isolaten. Vad som orsakar den här effekten är forfarande oklart, men clade 3 isolaten hade flera specifika gener som inte hittades bland clade 2 isolaten och som skulle kunna bidra till deras toxicitet.

Sammanfattningsvis visar denna studie att vissa campylobacterisolat överlever bättre än andra i vatten och variationer i överlevnad kunde ses för *C. jejuni* som reflekterar den säsongsvariation av campylobacterinfektioner som rapporterats.

Acknowledgements

There are many people I would like to thank for helping me finish this thesis. I would like to especially express my gratitude to following people:

My supervisor **Hilpi Rautelin,** for giving me the opportunity to do this PhD. Thank you for all your support and the trust you have shown me over the years. Also, thank you for movie nights and excellent dinners with great company.

My co-supervisor and former roommate **Astrid Skarp,** thank you for all your advice, support and discussions. My second co-supervisor **Cecilia Johansson**, I am so happy you joined the group. Thank you for all your support both in the lab and with tying everything together in the end.

My co-authors: **René Kaden**, my work-neighbour, for answering my many questions and for good discussions, both scientific and non-scientific. **Lars Engstrand** for all your input and advice. **Stefan Bertilsson** for your many ideas, enthusiasm and advice.

I would also like to thank the Swedish Research Council FORMAS for financial support.

To all the people at Klinisk mikrobiologi who has taught me about microbiology and answered my questions over the years, especially **Mehri Moghadam** and **Christina Öhrmalm**.

To **Björn Herrmann, Anders Bergqvist, Jonas Blomberg, Johan Lennerstrand, Åsa Melhus, Thomas Tängdén** and **Kåre Bondesson** for keeping up the good work with Clinical microbiology and Antibiotic research, making it an inspiring research environment.

To all the present co-workers and fikaroom friends: **Ayda Shams**, **Christer Malmberg**, **Priya Devi**, **Pernilla Lagerbäck**, **Pikkei Wistrand-Yuen**, **Anna Olsson**, **Sofia Rydahl, Carolina Rönnberg, Muhammad Rizwan, Bengt Rönnberg** and **Markus Hong.** And to all former co-workers: **Lotta**, **Stina**, **Markus**, **Ylva**, **Rachel**, **Linus**, **Maria**, **Akofa**, **Linn**, **Patricia**, **Matti**, **Karolina**, **Amal**, **Magnus**, **Resha, Guma**, **Anna**, **Anna, Christian, Ronnie** and **Toffe**. **Patrik Ellström** who once upon a time introduced me to the world of *Campylobacter* and got me started in the lab. I also want to thank **Jonas Blomberg** and **Shaman Muradrasoli** for taking me in as a master student. A special thanks to **Katarina Wallmenius** and **Jenny Isaksson**, without you two it wouldn't have been the same!

My friends out in the “real world”! Thank you for always being there. **Lina,** it is always nice to see you and I will miss you here in Uppsala. **Ebba,** even though we don’t see or talk to each other nearly as often as we should, it always seems like yesterday when we do. **Alexandra**, what would I do without you? Thank you for being you and for keeping me sane all these years.

And last but not least: My family, I love you all! **Mamma** and **pappa,** thank you for all your support and encouragement throughout my life. My brother **Henrik** and his wife **Cecilia,** for all your support and curious questions about what I’m really doing (No Henrik, you will not find any ebola in this thesis). To my brother **Per,** you once talked to me about the wonderful world of mathematics and that led me to choose biomedicine instead. I also want to thank my wonderful grandparents, who always had time for me and never said no to show me how to do something. I miss you and I wish you could have been here.

Anna Nilsson
Uppsala, February 2018

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