Population genetics and molecular epidemiology of *Campylobacter jejuni*

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Academic Dissertation

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2. Abstract

Campylobacteriosis is the leading cause of bacterial gastroenteritis worldwide. The number one cause of human Campylobacter infection, Campylobacter jejuni, has a broad host range, from penguins to cattle, but the most acknowledged source of campylobacteriosis has been the consumption or handling of broiler meat. However, since the European Food Safety Authority Panel on Biological Hazards estimated that consumption of broiler meat explains only 20-30% of the European infections, attempts to identify additional sources of C. jejuni have been made.

In these studies, we exploit the genetic material of this interesting bacterium to evaluate the potential usefulness of three virulence and metabolic markers in source attribution, and to investigate the short- and long-term epidemiology of C. jejuni in birds and humans. The possible host adaption of three metabolic markers, gamma-glutamyl transpeptidase, and the genes of secretory L-asparaginase and fucose permease, was investigated by examining their distribution among isolates collected from a variety of reservoirs and human patients, and by assessing their association with C. jejuni lineages as expressed by multilocus sequence typing. An association between gamma-glutamyl transpeptidase and the secretory L-asparaginase gene was found, while the presence of gamma-glutamyl transpeptidase and fucose permease gene was mutually exclusive. This suggested a possible gene loss of gamma-glutamyl transpeptidase and, to a lesser extent, secretory L-asparaginase during the C. jejuni evolution. Furthermore, the distribution of fucose permease gene seen among different C. jejuni lineages could be explained by a horizontal transfer event of this gene to the ancestor of sequence type ST-21 complex, with subsequent vertical transmission throughout this clonal complex. The presence and absence of these three traits were linked to multilocus sequence types, i.e. lineages, and no evidence for host association independently of population structure was found. Therefore, these metabolic markers are unsuitable as the sole subtyping scheme in source attribution.

The character, dynamics, and epidemiology of the C. jejuni population in wild and domesticated birds were investigated by multilocus sequence typing and, partly, whole-genome sequencing of C. jejuni collected from barnacle geese and chickens over two and five years, respectively. In line with other studies, the C. jejuni population in barnacle geese was possibly host-adapted, as sequence types ST-702 and ST-1034 complexes were overrepresented in the collection. Furthermore, we proved that the C. jejuni in this wild bird species generally differed from the C. jejuni population found in agricultural animals, with a special focus on chickens, and those C. jejuni infecting humans. Therefore, barnacle geese are most probably not a major source for human campylobacteriosis.

In a longitudinal study of C. jejuni in Finnish chickens over a nine-year period, we characterized nearly 90% of all C. jejuni-positive flocks by multilocus sequence typing and linked this information to farm and collection time-points. Multivariate logistic regression was used to assess the effect of site, year, and season of collection on the presence of the three most common clonal complexes. During these years we found a highly skewed C. jejuni population on Finnish chicken farms, dominated by sequence type 45 complex and sequence type 45. There was a mild effect of site of collection on sequence type 21 and sequence type 45 complexes, and in 2004 and 2012 more sequence type 45 complex relative to sequence type 21 complex was collected. Season did not effect the occurrence of any clonal complex. The third most common clonal complex, sequence type 677 complex, was equally distributed among years, sites, and seasons. Furthermore, the
chicken farms were only sporadically and infrequently colonized by *C. jejuni*, as is typical when no persistent colonization source is present on the farms.

Whole-genome sequencing was used to reinvestigate a waterborne *C. jejuni* outbreak, revealing that pulsed-field gel electrophoresis overestimated the clonal relationship between some of the apparent outbreak-related strains. Through the reanalysis, it became clear that the outbreak was caused by at least two different strains, or an unrelated, sporadic human case was mistakenly classified as part of the outbreak. Furthermore, the study provided knowledge on how to utilize whole-genome data in future real-time investigations of *Campylobacter* outbreaks.

This thesis utilized an array of subtyping methods on both long- and short-time scales. The results highlight that more traditional methods, such as pulsed-field gel electrophoresis, still have their use in the current genomic era, and stress the usefulness of multilocus sequence typing as a preliminary screening tool to determine the population structure in the *C. jejuni* collection being investigated. However, we are convinced that whole-genome sequencing will be the subtyping scheme of choice in the near future, as it provides the full resistome, toxicone and virulome concurrently with the genotyping depth of need in a single operation.
3. Acknowledgments

This work was carried out at the Department of Food Hygiene and Environmental Health, University of Helsinki, from 2011 to 2015, and was part of the Center of Excellence in Microbial Food Safety Research (CoE-MiFoSa) and the projects EMIDA Era-Net entitled “Biology and Control of Campylobacter in the Chicken Supply Chain (CamChain)” and “Kampylobakteeritriskit Elintarvikeketjussa ja Ympäristössä” (in collaboration with Evira). Financial support was received from the Academy of Finland (grant no. 141140), the Finnish Ministry of Agriculture and Forestry (grant nos. 2054/312/2011 and 776/312/2012), and the Walter Ehrström Foundation (grant no. 4703496). Funding received from the Finnish Veterinary Foundation, the Doctoral School in Environmental, Food, and Biological Sciences, and the Chancellor’s Grant of Helsinki University is also gratefully acknowledged.

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4. List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

Study I:

Association of *Campylobacter jejuni* metabolic traits with multilocus sequence types.
*de Haan and Llarena contributed equally to this work

Study II:

Characterization of the *Campylobacter jejuni* population in the barnacle geese reservoir.

Study III

Genome analysis of *Campylobacter jejuni* strains isolated from a waterborne outbreak.

Study IV

Predominant *Campylobacter jejuni* sequence types persist in Finnish chicken production.

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5. Abbreviations

AFLP: amplified fragment length polymorphism fingerprinting
ansB(s): secretory L-asparaginase
ART: Artemis comparative tool
BAPS: Bayesian analysis of population structure
BIOHAZ: Panel on biological hazards
BRIG: Blast ring image generator
CC: clonal complex
CFUs: colony forming units
CLSI: clinical and laboratory standards institute
CNP: cluster of nucleotide polymorphism
DALY: disability-adjusted life-years
ECOFF: epidemiological cut-off value
EFSA: European food safety authority
EMBL: European Bioinformatics Institute
EU: European Union
EUCAST: European committee on antimicrobial susceptibility testing
Evira: Finnish Food Safety Authority
FST: the F-statistic, a fixation index in the subpopulation relative to the total population
fucP: fucose permease
GBS: Guillain-Barré syndrome
GGT: gamma-glutamyl transpeptidase
HGT: horizontal gene transfer
K: Number of subpopulations in Bayesian clustering methods
LOS: lipooligosaccharide locus
MALDI-TOF MS: matrix-assisted laser desorption ionization-time of flight mass spectrometry
mCCDA: Campylobacter blood-free selective agar
MIC: minimum inhibitory concentration
MLEE: multilocus enzyme electrophoresis typing
MLST: multilocus sequence typing
MRSA: methicillin-resistant Staphylococcus aureus
MS: member states
NBA: Nutrient agar supplemented with defibrinated horse blood
NGS: next-generation sequencing
OR: odds ratio
PAF: population attributable factor
PAR: population attributable risk
PCR: polymerase chain reaction
PFGE: pulsed-field gel electrophoresis
PSI: proportional similarity
QRDR: quinolone resistance-determining region
SDI: Simpson diversity index
SID: Shannon index of diversity
SNP: single nucleotide polymorphism
ST: sequence type
SVRs: sequence variable regions
TCA: citric acid cycle
UA: unassigned to clonal complex
WGS: whole-genome sequencing
6. Review of the literature

6.1 The organism

6.1.1 History
The first documentation of *Campylobacter* was in 1886, when Theodor Escherich noted the presence of a spiral bacterium in stool from children deceased of what he called “cholera infantum”.¹ In 1938, Levy *et al.* resolved a gastroenteritis outbreak in a state prison, and although the etiologic agent was believed to be the bovine pathogen *Vibrio jejuni*,² these are the most probable second human campylobacteriosis cases recorded.

King proposed the term “related vibrios” for the thermophilic bacteria isolated from patients suffering from diarrhea.³ Due to the lack of selective isolation methods, these “related vibrios” were only isolated from blood and were thereafter infrequently reported as a causative agent of human septicemia and abortion between the 1940s and 1970s.⁴⁵ In 1963 and 1973, these organisms were renamed *Campylobacter* by Sebald and Véron due to differences in the DNA base composition, growth requirements, and metabolism between vibrios and *Campylobacter*.⁶⁷ The development of the filtration technique⁸ and later the selective Skirrow-media⁹¹⁰ enabled *Campylobacter* isolation from stool, a crucial step in the reevaluation of the *Campylobacter* epidemiology. Only in the 1980s, nearly 100 years after its first discovery, was *C. jejuni* acknowledged as one of the world’s most frequent causative agents of human gastroenteritis.

Contrary to this, the organisms now known as *Campylobacter* have been recognized as causative agents of animal disease since 1909. McFadyean and Stockman attributed abortion in sheep to a vibrio-like bacterium,¹¹ while Smith isolated what he suspected to be the same organism from bovine abortions in 1919¹² and named it *V. fetus*.¹³ Furthermore, *V. jejuni (C. jejuni)* and *V. coli (C. coli)* were reported to cause winter dysentery in calves and swine dysentery in 1931 and 1944, respectively.¹⁴¹⁵

6.1.2 Taxonomy
The genus *Campylobacter* constitutes together with the genera *Arcobacter* and *Sulfurospirillum* a family of Gram-negative, generally microaerophilic organisms with a low G+C content called *Campylobacteraceae*.¹⁶ Currently, 26 species and 12 subspecies are classified in the genus *Campylobacter*, of which eight species and one subspecies were described after 2009 ([http://www.bacterio.net/campylobacter.html](http://www.bacterio.net/campylobacter.html), accessed 02.04.2015). The majority of *Campylobacter* are human and animal commensals in reproductive organs, intestinal tracts and oral cavities, but several species are opportunistic or primary zoonotic pathogens. *C. jejuni* and *C. coli* are the two most clinically important species seen from a human perspective.¹⁷

There are two subspecies of *C. jejuni, C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*, and although the pathogenic role of *C. jejuni* subsp. *doylei* is unclear, it has been isolated from infants with bacteremia.¹⁸ The two subspecies differ biochemically and can be separated by polymerase chain reactions (PCR) based on the *nap* locus.¹⁹ *C. jejuni* subsp. *jejuni* will hereafter be referred to as *C. jejuni*.

6.1.3 Microbiology
*C. jejuni* is a slender, spirally curved, non-spore forming, Gram-negative rod, with a width of 0.2 to 0.8 µm and a length of 0.5 to 5µm. In older cultures, *C. jejuni* may form coccoid or spherical
shapes. *C. jejuni* is motile due to the presence of a single unsheathed polar flagellum and has microaerobic and thermophilic growth requirements. *C. jejuni* cannot, in the majority of cases, utilize carbohydrates for energy, but rather uses amino acids and tricarboxylic acid cycle (TCA) intermediates. C. jejuni requires a temperature of at least 30°C to grow, and as one of four thermophilic campylobacters it will also grow at 42°C. It survives well at 4°C in a moist, sun-sheltered environment, but is killed by temperatures reached in pasteurization, cooking, and frying.

*C. jejuni* can be cultivated from feces on antimicrobial-containing selective media (blood-based or charcoal blood-free) in a microaerobic atmosphere at an elevated temperature (42°C). The minimum standard for identifying *Campylobacter* spp. includes colony morphology, Gram’s stain, motility, and an oxidase test. To separate *C. jejuni* from *C. coli*, a hippurate-hydrolysis test has traditionally been used, although there are several hippurate-negative *C. jejuni*. Due to the biochemical inertness of *Campylobacter*, molecular methods such as genus-specific PCR or sequencing of the 16s rRNA gene have been applied to differentiate between different *Campylobacter*-species. However, the sequence variability between the 16s rRNA gene in *C. jejuni* and *C. coli* is too small to differentiate between the two, so such alternative methods as species-specific PCR, targeting the hipO-gene, or groEL- and rpoB-gene sequencing have been developed. In addition, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and genotyping methods, such as amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST), can be used to determine *Campylobacter* species in addition to molecular typing.

### 6.1.4 Genome and population structure of *C. jejuni*

In 2000, Parkhill et al. sequenced the 1.64Mbp chromosome of the reference strain NCTC 11168, and soon genome sequences of three other reference strains (81-176, 81116, RM1221) were available. A major part of the overall sequence has been conserved in these strains, but their genomes do differ at several points. Today, 142 *C. jejuni* genomes, 32 of which are complete, have been sequenced and deposited in Genbank (http://www.ncbi.nlm.nih.gov/pubmed, accessed 29.7.2015), and over 5000 draft genomes are available from the PubMLST database (http://pubmlst.org/).

The average *C. jejuni* genome size is fairly small, between 1.6 and 1.8 Mbp, and has a relatively low G+C content (=32%). As most bacteria, the species *C. jejuni* can be described by a pan-genome consisting of a core genome shared by all isolates, plus an accessory genome composed of partially shared and strain-specific genes. According to Lefébure et al., the core- and pan-genome of the species *C. jejuni* are finite, while Meric et al. reported the pan-genome of *C. jejuni* to be open, i.e. infinite. Only fragmentary knowledge of the nature, size, and content of the genome of *C. jejuni* is available, and several aspects are debated and ambiguous. For instance, the size-estimates of the core genome lies between 866 and 1350 genes, while the pan-genomes assessments range from 2500 to 4000 genes. The genes of the core genome are predicted to be involved in vital functions such as energy metabolism, cell division, protein and peptide secretion, and synthesis of macromolecules including DNA, RNA, and proteins. However, many core genes remain uncharacterized. Extensive research efforts have been directed to describing the accessory genes since the organism’s variability in virulence, pathogenicity, and host-specificity is believed to reside in this part of the genome. The accessory genome includes for instance plasmids, integrated elements, hypervariable regions, and single or paired variable genes. For example, the 81-176 and RM1221 carry one to three of the plasmids, pVir, pTet, and pCC31, while four integrated elements, CJIE1-CJIE4, possibly of prophage or plasmid origin, have been characterized in
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In addition, a fifth integrated element was recently described in sequence type (ST)-677 clonal complex (CC) (Zhang, personal communication). Three of these integrated elements carry genes for production of extracellular DNAases.\(^{46,47}\) The variable regions of the accessory genome, referred to as hypervariable plasticity regions (PR1-PR7),\(^{41}\) hypervariable regions (HR01-HR16),\(^{48}\) and/or regions of divergence (PH01-PH15),\(^{49}\) are involved in cell surface structures, restriction-modification systems, and metabolism.\(^{41,48,50}\) As for single or paired variable genes, Morley *et al.* found 10 loci unique to ST-403 CC, which possibly reflects niche differentiation of this lineage.\(^{51}\)

Most *C. jejuni* strains are naturally competent for DNA uptake,\(^{52}\) and recombination is extensive\(^{53}\) and the main driver of diversity.\(^{43}\) This ability has resulted in a partial clonal population structure,\(^{54}\) in which the boundaries between the different clusters of related genotypes are blurred.\(^{40,55}\) Even so, recombination in *C. jejuni* has not been great enough to wipe out all signals of clonal structure, and MLST does provide information on the genealogical relationship between strains and does correlate with phenotypic traits in certain cases.\(^{56}\) For instance, MLST studies have revealed the existence of both generalist lineages containing isolates from multiple sources (like ST-21 CC and ST-45 CC)\(^{57,58}\) and possibly host-adapted specialist lineages such as ST-61 CC in cattle and ST-257 CC in poultry.\(^{58}\) In addition, there is evidence of the existence of subclusters of host specialists within generalist lineages, maybe due to ongoing adaption to that species.\(^{53,59}\)

### 6.1.5 Metabolism

To be successful, *C. jejuni* needs to replicate in a wide variety of hosts, from birds to humans, and keep viable in a range of different environments such as food and water. Therefore, *C. jejuni* is metabolically flexible, possibly due to its large pan-genome. For acquisition of energy, *C. jejuni* is dependent on the citric acid (TCA) cycle and feeds the cycle through different intermediates mainly by degradation of amino acids. Indeed, due to lack of carbohydrate transporters and key enzymes in the glycolytic pathway,\(^{34}\) *C. jejuni* is asaccharolytic, meaning that the organism is unable to utilize common carbohydrates to create energy.\(^{60}\) Therefore, the primary nutrient source for *C. jejuni* is amino acids, and preferentially L-aspartate, L-serine, L-asparagine, and L-glutamate are taken up and catalyzed in this order.\(^{60}\) *C. jejuni* also uses L-proline, but only after exhausting the above amino acids. In addition, *C. jejuni* metabolizes the fatty acids acetate\(^{61}\) and lactate,\(^{62}\) but only when all other nutrients are finished.

Iron is absolutely necessary for the growth of *C. jejuni*\(^{63}\) and acts together with biological donor-ligands like oxygen, nitrogen, and sulfur in DNA synthesis and electron transfer. The iron-sulfur complex is a co-factor in several enzymes, catabolizing key metabolic pathways, and the oxygen-sensitivity of this molecule is thought to be the reason why *C. jejuni* is microaerophilic.\(^{64}\) Iron exists as ferrous ion bound to organic acids, amino acids, and prosthetic groups, like heme, and host-derived iron-binding proteins in the intestine, and *C. jejuni* acquires iron by uptake of these complexes through various transporters. Since *C. jejuni* lacks enterobactin, a siderophore scavenging iron from host proteins, *C. jejuni* utilizes enterobactin originally produced by other bacteria\(^{60}\) to acquire ferrous-enterobactin complexes from the intestinal lumen. In addition to iron, *C. jejuni* uses molybdate and tungsten as co-factor in various metabolic reactions, which is uncommon compared with other bacteria.\(^{60}\)

Some strains carry metabolic features hypothesized to enhance colonization and survival potential.\(^{65-67}\) Gamma-glutamyl transpeptidase (GGT), secretory L-asparaginase (*ansB*(s)), and L-fucose are among these and are carried in the accessory genome of *C. jejuni*.\(^{50,51}\)
GGT hydrolyzes glutathione and glutamine to glutamate in the periplasm,\(^\text{68}\) which is subsequently transported to the cytoplasm and converted to the TCA cycle intermediate fumarate. According to Barnes et al. and Hofreuter et al., GGT is necessary for the persistent colonization of chickens\(^\text{65}\) and immunodeficient mice.\(^\text{36}\) However, colonization was only impaired in some strains, suggesting that alternative pathways of metabolism exist for \textit{C. jejuni} isolates lacking GGT. The cytoplasmic enzyme L-asparaginase deaminates asparagine to the essential amino acid aspartate, and almost all \textit{C. jejuni} isolates carry the coding \textit{ansB} gene. A secretory signal, present in for instance \textit{C. jejuni} strain 81-176, makes utilization of asparagine in the periplasm possible. Hofreuter et al. showed that \textit{C. jejuni} carrying this secretory signal \textit{ansB(s)} had an enhanced colonization potential in mice livers.\(^\text{68}\) Even though \textit{C. jejuni} is generally an asaccharolytic organism, an alternative carbohydrate pathway has been discovered in certain strains, namely the L-fucose pathway. This pathway includes the production of L-fucose permease (fucP) transporter coded by the \textit{Cj0486/fucP} gene.\(^\text{67,69}\) It is hypothesized that the presence of this transporter gives the bacteria the ability to utilize the carbohydrate fucose as energy-source in nutrient-poor environments, which could give a metabolic advantage over \textit{fucP}-negative \textit{C. jejuni} strains in certain hosts.\(^\text{69}\)

### 6.1.6 Antimicrobial resistance

\textit{Campylobacter} spp. is likely inherently resistant to cloxacillin, nafcillin, oxacillin, sulfamethoxazole, trimethoprim, and vancomycin.\(^\text{35}\) However, other types of resistance might be a result of mutations or horizontal gene transfer of resistance genes occurring during therapeutic antimicrobial drug use in humans and animals. For instance, fluoroquinolone resistance is conferred by a point mutation in the quinolone resistance-determining region (QRDR) of the \textit{gyrA} gene.\(^\text{70,71}\) The QRDR-\textit{gyrA} point mutation most commonly results in the substitution Thr-86→Ile, but less frequent variants do occur (Thr-86→Ala, Asp-90→Asn).\(^\text{70-72}\) Resistance to macrolides (erythromycin, azithromycin, tylosin) is associated with a mutation in the 23S rRNA gene in position 2074 or 2075,\(^\text{73}\) which is not acquired easily, while tetracycline resistance is achieved through the action of a ribosomal protection protein Tet(O) encoded by the chromosomal or plasmid-carried \textit{tet(O)} gene.\(^\text{74}\) In addition, different efflux pumps act to reduce the intracellular concentrations of antimicrobial substances, thereby increasing their minimum inhibitory concentration (MIC) values.

The emergence of antimicrobial-resistant \textit{Campylobacter} spp. isolates is an increasing global concern. According to the European Food Safety Authority (EFSA), high levels of fluoroquinolone (ciprofloxacin and nalidixic acid) resistance were reported for \textit{C. jejuni} isolated from patients, broilers, and cattle in 2013. However, large differences in resistance levels between member states (MS) were noted, from practically none-existent resistance levels in Finland to high numbers of ciprofloxacin-resistant isolates in Spain. However, most European \textit{C. jejuni} isolates are still susceptible to gentamicin and erythromycin, making erythromycin the first drug of choice in areas with high levels of fluoroquinolone resistance.\(^\text{75}\) In Finland, all broiler-derived \textit{C. jejuni} were susceptible to ciprofloxacin, gentamicin, erythromycin, and tetracycline in 2013,\(^\text{75}\) and low levels of resistant \textit{C. jejuni} were found in broilers between 2007 and 2012.\(^\text{76}\) Of all the livestock tested, bovines were the species most frequently carrying ciprofloxacin-resistant \textit{C. jejuni} (9.2%), while zoo animals often carried tetracycline- and streptomycin-resistant isolates (31.3% and 25%, respectively).\(^\text{76}\) Knowledge about the occurrence of antimicrobial resistance in \textit{C. jejuni} collected from the environment and wild animals is lacking. Wild birds may serve as reservoirs and vectors for antimicrobial-resistant isolates after acquisition of \textit{C. jejuni} from livestock or human waste and
subsequently disperse these over large distances through migration. Further studies on the topic are warranted.

Some studies have been able to show an association between MLST and fluoroquinolone resistance in *C. jejuni*. For instance, a connection between fluoroquinolone resistance and ST-21 CC and ST-1036 was found in *C. jejuni* isolated from chickens in Belgium and Senegal, respectively, while ST-464 was associated with quinolone resistance in Switzerland, irrespective of the host. However, most studies have been unable to connect lineages to specific resistance patterns.

6.2 Subtyping of *C. jejuni*

High numbers of human infections and a variety of reservoirs and vehicles for genetically diverse organisms such as *C. jejuni* necessitates subtyping schemes suitable for a range of different applications. The combined effort to meet this need during the last 30 years have resulted in such methods as biotyping, serotyping, phage typing, metabolic markers, AFLP, multilocus enzyme electrophoresis typing (MLEE), pulsed-field gel electrophoresis (PFGE), gene-sequencing, antimicrobial-resistance profiling, MLST, full-genome MLST and full-genome sequencing (WGS).

6.2.1 Phenotyping

The early phenotyping methods used for *Campylobacter* spp. include biotyping, serotyping, and phage typing, and some of these still find their uses today. However, although these schemes might be suitable for large-scale screenings, their low discriminatory power limits their usefulness. Serotyping was the subtyping method of choice for many years, especially the Penner serotyping scheme. This system, developed by Penner and Hennessy in 1981 relies on the presence of heat-stable antigens. However, several problems have been identified: non-typeable strains, cross-reaction between serological assays, phase variation in antigen genes and horizontal gene exchange resulting in re-assortment of antigens among different genotypes. Summarized, these disadvantages make serotyping a poor tool in epidemiological investigations.

MLEE is the origin of MLST and is based the same principles. MLEE differentiates between isolates according to electrophoretic mobility of their housekeeping (usually metabolic) enzymes. However, due to synonymous base substitutions, posttranslational modification, and isoelectric amino acid substitution, MLEE lacks the ability to infer the true phylogenetic relationship between strains. In addition, MLEE is laborious and difficult to standardize between laboratories. Therefore, MLEE is seldom used in *Campylobacter* research.

6.2.2 Metabolic markers

The use of metabolic markers as subtyping tools in epidemiology relies on the assumption that different niches (i.e. hosts) create different environments to which the bacteria adapt. In addition, the use of metabolic markers can provide additional valuable information on the metabolic capacity of different *C. jejuni* strains. The metabolic traits or genes used are unequally distributed in the *C. jejuni* population, and are thus not essential for growth or survival, but could give a metabolic advantage in certain environments. *AnsB(s)*, *Cj0486/fucP*, and the enzymatic detection of GGT activity are described earlier and have been linked to host, lipooligosaccharide locus (LOS) class, and MLST genotype. However, the use of metabolic markers in *C. jejuni* epidemiology is limited.
6.2.3 Genotyping

Genotyping is used to distinguish between different C. jejuni subpopulations and have been proven to be an irreplaceable working tool in a range of different applications, such as molecular epidemiology and identification of pathogenicity and virulence. No golden standard genotyping technique exists, but according to French and Marshall: “The ideal genotyping method (for source attribution) would be based on genomic markers that not only inform on the animal reservoir (genes that are host associated) but also of pathway (genes that indicate susceptibility or stress related to food production)”.

6.2.3.1 Amplified fragment length polymorphism

AFLP is a based on whole-genome polymorphism and has been used in interspecific and intraspecific differentiation of Campylobacter spp.. It is highly discriminatory and may be considered advantageous since a single protocol can be used to obtain the same resolution as PFGE. However, interpretation of typing results is challenging when isolates yield similar patterns, and the method is expensive and laborious. Nevertheless, AFLP is often used to describe novel taxa.

6.2.3.2 Pulsed-field gel electrophoresis

PFGE is based on electrophoretic banding patterns of the restriction profiles of purified whole-cell DNA. It is a powerful tool in epidemiological studies of C. jejuni and is especially suitable for outbreak investigations. SmaI and SacI/KpnI have been the most commonly used restriction enzymes, with KpnI being the most discriminatory of the three. Difficulties in reproducing the methodology and interpretation of the results among different laboratories make it challenging to use PFGE as a unified typing scheme on a wider scale. However, PFGE is still extremely useful for differentiation of closely related strains and is frequently utilized in research, surveillance, and epidemiology.

6.2.3.3 Nucleotide sequence-based typing

6.2.3.3.1 Sequencing of flagellin genes

FlaA and flaB sequencing can be done by PCR amplification of the flaA or flaB genes, with subsequent sequencing of the PCR product. There is a curated database available, the Pubmed Campylobacter flaA database (http://pubmlst.org/campylobacter/). The fla genes code for flagellin proteins (A and B) and are highly variable, and thus, are able to differentiate between closely related strains. This technique does not allow phylogenetic analysis of strains or differentiation between C. coli and C. jejuni, and is unsuitable for long-term epidemiological studies.

6.2.3.3.2 Multi locus sequence typing

MLST of C. jejuni is based on the sequence variation in fragments of (usually) seven housekeeping genes located evenly around the bacterial genome. The most commonly used loci in C. jejuni are aspartase A (aspA, CJE0082), glutamine synthetase type I (glnA, CJE0798), citrate synthase (gltA, CJE1851), serine hydroxymethyltransferase (glyA, CJE0451), phosphoglucomutase (pgm/glmM, CJE0409), transketolase (tkt, CJE1817), and ATP synthase & subunit (uncA, CJE0100). The sequence variations are indexed and thereafter summarized as a row of numbers assigned a ST, which may further be grouped together with related STs in CCs. These CCs are defined by different methods involving counting allelic differences from a central genotype or to other STs in the CC, but split decomposition, NeighborNet, minimum spanning trees, and eBURST/goeBURST can be used to group STs. The central genotypes are typically high-frequency STs, widely distributed in
space and time, and obtain a central position when analyzing C. jejuni populations using heuristic approaches. Furthermore, the CCs are often associated with specific phenotypes like host association, pathogenicity, and antimicrobial susceptibility.\textsuperscript{100} MLST provides a unified and portable genotyping method for C. jejuni for which a web-based database (http://pubmlst.org) offers a mean to catalog the vast variation of C. jejuni.\textsuperscript{101} Since MLST is based on allele designation rather than nucleotide sequence, MLST adjusts for recombination and can be applied to phylogenetics.\textsuperscript{102} It is a powerful tool to detect groups of related isolates (“clones” or “lineages”). However, MLST lacks the resolution to differentiate between very closely related isolates.\textsuperscript{101} MLST has been the genotyping method of choice during the last decade and has been invaluable in the study of strain diversity within hosts, possible host associations, evolution, pathogenicity, and epidemiology. It is especially suitable for long-term studies.\textsuperscript{82,103-107}

To achieve higher discriminatory power, MLST has been combined with antigen gene sequencing typing.\textsuperscript{108} This method allocates arbitrary numbers to the sequence variable regions (SVRs) of the antigenic genes flaA, flaB (see above), and porA,\textsuperscript{109} encoding an outer membrane protein. This typing scheme has been used in, for instance, outbreak investigation,\textsuperscript{110} but has not achieved common acceptance and use.

6.2.3.3.3 Acquiring full-genomes by next-generation sequencing (NGS)

In 2005, Roche’s launched the 454 platform, which together with the Illumina, PacBio, and Ion Torrent platforms make up the apex of today’s NGS technology. NGS is much faster and more accurate than the original Sanger sequencing\textsuperscript{111} and demands less manpower and fewer costs. The development of parallel analysis has been the foundation for this improvement. The work flow of NGS is common to second- and third-generation sequencing techniques which involves a DNA extraction step, a library preparation step, and finally a sequencing and data recording step.

For second-generation sequencing, the library preparation typically includes enzymatic or mechanical shearing, but DNA can also be sequenced intact. The DNA is thereafter amplified into millions of copies in either an emulsion PCR (Roche 454 platform) or cluster PCR (Illumina platform) before the sequencing step commences. Roche 454 and Illumina are based on sequence-by-synthesis or pyrosequencing; during a second PCR step, all of the amplified DNA fragments (the DNA to be sequenced) act as templates, and every time a new fluorescent labeled dNTP is added to the chain, light emission is created and registered. These light signals are subsequently computationally transformed into the sequence reads (www.illumina.com).

Third-generation sequencing includes PacBio and Nanopore sequencing (MinION\textsuperscript{TM}). No DNA shearing or PCR amplification is needed to sequence on these platforms. The PacBio is also a sequence-by-synthesis technique, but observes the DNA synthesis in real-time thanks to the development of the SMRT Cell (www.pacificbiosciences.com). MinION\textsuperscript{TM} is also real-time sequencing, but is based on a different principle, namely the detection of disruptions in a membrane’s electrical current. These disruptions are caused when the DNA molecule to be sequenced passes through a protein nanopore lodged into a high electrical resistance membrane. These nanopore signals are then used to characterize the type of molecule passing through the pore; for instance, to distinguish between the four standard DNA bases A, C, G, and T. This technique has a much wider use as well and has the potential to recognize modified bases, RNA
without the need for reverse transcription, proteins, and small molecules (www.nanoporetech.com).

6.3 Campylobacter infections in humans

6.3.1 Clinical presentation, pathology, and treatment

The most important Campylobacter species in human gastroenteritis is C. jejuni, accounting for 90-95% of all campylobacteriosis cases. The majority of the remaining cases are caused by C. coli, but the importance of C. coli as an enteric pathogen varies between regions, degrees of urbanization, and age of the patient. In the Finnish campylobacteriosis epidemiology, C. coli plays a minor role (5-10%), and the remainder of this section will therefore focus on C. jejuni.

In the industrialized world, the most common presentation of a C. jejuni infection is acute, self-limiting gastrointestinal illness. The infectious dose is low and as little as 800 cells have been reported to cause disease under experimental conditions. The incubation period is typically two to five days, but up to eight days has been reported. Half of the patients experience a febrile period with fever, malaise, and abdominal pain preceding the diarrhea. Fresh blood in the stool is common, while vomiting occurs in approximately 15% of the cases. Normally, the diarrhea lasts for two to three days, while discomfort can persist for weeks.

The most severe, but rare, complication following a C. jejuni infection is Guillain-Barré syndrome (GBS), a paralytic condition that requires hospitalization. Reactive arthritis is another complication, following 1-5% of campylobacteriosis cases, and mainly affects the bigger joints, but smaller joints can also be involved. Other rare complications include extra-intestinal infections and bacteremia, occurring mainly in immunocompromised patients. Furthermore, studies have found an association between irritable bowel syndrome and C. jejuni infections.

In developing countries, C. jejuni infections are common in very young, causing watery diarrhea, while adults are infrequently affected.

The pathological lesions seen in humans suffering from Campylobacter infection include acute inflammatory enteritis extending to the colon and rectum, with terminal ileitis and cecitis with mesenteric adenitis as prominent features.

6.3.2. Development of immunity

The occurrence of immune and non-immune individuals in a population can obscure epidemiology and risk assessment, influencing surveillance data and leading to misinterpretation of trends, biased predictions and faulty assessments of risks. Even though the role of host susceptibility in Campylobacter infection and development of disease is not fully understood, it is clear that both the innate and adaptive immune responses play a role. Development of antibodies, mainly IgA, IgM and IgG, has received plenty of research interest, and the humoral immune response seems to be able to convey short-lasting protection against disease-development from homologous strains, although infection is not avoidable. Black et al. (1988) reported immunity against two homologous strains 28 days after the first infection, while longer lasting immunity, i.e. asymptomatic carriage, have been reported following repeated occupational exposure. Furthermore, Miller et al. (2005) found evidence for immunity against commonly circulating Campylobacter strains in senior citizens. As for heterologous strains, a strong antig and protection against heterologous strains. Therefore, repeated exposure to different strains seems to be needed to provide at least partial immunity against an arsenal of Campylobacter strains, which might explain the infrequent incident of campylobacteriosis in adults reported from developing
countries, as frequent and multiple exposures at a very young age to a wide range of Campylobacter strains is common in endemic areas.

6.3.2 Epidemiology

6.3.2.1 Incidence and burden of illness
The European Union (EU) notification rate of campylobacteriosis was 64.8 per 100,000 inhabitants in 2013, but varied considerably between the MSs; from <2 to 173.7 per 100,000 inhabitants in Latvia and Czech Republic, respectively. However, the true incidence of Campylobacter is poorly characterized. The national statistics grossly underestimate the actual population incidence because only a fraction of the campylobacteriosis cases are presented to health services, and many of these are not investigated further. For instance in the UK, Tam et al. found that every campylobacteriosis case reported to the national surveillance represented 9.3 community campylobacteriosis cases between 2008 and 2009. Overall, the true incidence is expected to range between 4.4 and 9.3 per 1000 person-years in high-income countries. The global estimate of burden of campylobacteriosis for 2010 was 7.5 million disability-adjusted life-years (DALYs). This was a clear improvement from 1990, when the burden of campylobacteriosis was estimated to be 16.6 million DALY’s. However, Campylobacter enteritis accounted for 8.5% of the total burden of diarrheal disease, ranking fourth after rotavirus, cryptosporidiosis, and Escherichia coli diarrhea (combined enterotoxigenic and enteropathogenic E. coli infections). Thus, Campylobacter remains one of the most frequently occurring bacterial causes of gastrointestinal diseases worldwide.

The epidemiology of Campylobacter infections differs between developing and industrialized countries. In developing countries, symptomatic disease is most commonly seen during the first two years of life, and disease in adults is rare. The clinical picture also differs from that seen in industrialized countries, as watery diarrhea is the most common presentation. The peak in the very young and the lack of clinical illness in adults are believed to be due to immunity, as stated above, as a high percentage of asymptomatic seropositive individuals have been reported in several studies. In addition, co-infection with other enteric pathogens is not uncommon.

In Finland, 4887 campylobacteriosis cases were registered in 2014, equaling an infection rate of 90.1 cases per 100,000 inhabitants. The number of cases has been quite stable from 2005 to 2013, but has increased by almost 20% from 2013 to 2014. The rate was highest among young adults (136.1/100,000), with males slightly more frequently affected (54.4%). The majority of campylobacteriosis cases were from the Helsinki area (rate 133.1/100,000) and imported from abroad (50.3% of the cases with known travel history). However, travel-information is lacking in 32.7% of the Finnish cases. Sweden and Norway report travel-history in 95% and 87.9% of cases, with approximately 50% of these countries’ campylobacteriosis cases being travel-associated. A similar pattern is plausible for Finland due to the comparable demographic patterns, social structure, and culture in Nordic countries.

6.3.2.2 Occurrence and seasonal variation
Most Campylobacter infections occur sporadically, and compared with other enteric pathogens, campylobacteriosis outbreaks (more than two cases of a similar illness resulting from exposure to a common source) are rare. However, outbreaks are relatively more common in Finland, with Campylobacter-contaminated drinking water and raw milk as the most frequently reported sources. Studies of outbreak cases have yielded knowledge on the long-term health
consequence and pathology of Campylobacter infections and the genomic epidemiology of C. jejuni through the use of NGS techniques and comparative genomics.

In temperate regions, the number of campylobacteriosis cases increases during summer, and the effect is accentuated by increasing latitude. However, seasonal variation with a similar summer peak has been reported as far south as in Egypt. In Finland, the seasonal peak occurs in July-August, while in countries with a warmer climate the case-peak typically occurs earlier, in the spring. The reasons for these fluctuations are not clear, but climate, human behavioral changes, and seasonal variation of Campylobacter occurrence in reservoirs have been tendered as plausible explanations.

6.4 Campylobacter jejuni in animal reservoirs and the environment

6.4.1 C. jejuni in animal reservoirs

A reservoir is defined as an animal species or a non-animal substance upon which the pathogen depends for its survival. The growth of C. jejuni is limited by its requirements for a microaerobic atmosphere and its temperature range (30-42°C). It is difficult to envision an extraintestinal environment accommodating these conditions, thus, the intestinal mucosa of mammals and birds serve as the amplification site and natural reservoir in the contamination pathway of this organism.

Reservoirs of C. jejuni include a wide variety of birds and mammals, both domesticated and wild, who are mainly asymptomatic carriers. C. jejuni exhibits a truly ubiquitous nature and has been isolated from everything from the common housefly to Antarctic macaroni penguins and red kangaroos. Here, we describe the reservoirs of special importance to public health.

6.4.1.1 Broiler chickens

Poultry is defined as domesticated avian species, encompassing broiler chickens, hens, and turkey. In the following, only broiler chickens will be discussed.

Chicken was recognized as a potential source for Campylobacter infections already in 1977, and it is still considered to be the most important foodborne source of both sporadic and outbreak-associated campylobacteriosis. C. jejuni colonizes the avian gut and can contaminate the chicken carcass during slaughter. Following survival of C. jejuni through the processing chain, humans become infected through contaminated chicken by either consumption of undercooked meat or due to cross-contamination from raw meat to ready-to-eat foods.

However, the chicken reservoir is attributed (see section on “Source attribution”) more campylobacteriosis cases than the number estimated to be acquired through consumption of chicken meat in risk analysis. Also, some studies have pointed out a lack of a proper temporal link between chicken and human isolates. This indicates the presence of an indirect transmission route for C. jejuni, i.e. not through the direct foodborne pathway, from chicken to human or the occurrence of a third shared source. Indeed, the expert elicitation EFSA Panel on Biological Hazards (BIOHAZ) has attributed 60-70% of campylobacteriosis cases in the EU to the chicken reservoir as a whole, but only a fraction of these (20-30%) was due to consumption of contaminated chicken meat. Alternative transmission pathways to the foodborne route from chickens to humans are currently unknown and warrant further investigation, and increased knowledge on the C. jejuni population in chickens is therefore needed.
6.4.1.1.1 Production of chicken meat and monitoring of C. jejuni in the Finnish chicken food chain

The production scale of the Finnish broiler chicken industry grew between 2004 and 2014, to 106 and 101 million kilos of produced and consumed chicken meat, respectively (http://www.siipi.net/index.php/siliparjalitto/tilastoa). In addition to exporting chicken meat, Finland also imports approximately 10-15 million kilos of mainly processed poultry produce annually (http://www.mmm.fi). The number of rearing farms (= 140) has stayed stable since 2004, while the average farm and flock sizes have increased (=35,000 birds per farm) (epp.eurostat.ec.europa.eu). The farms work on a contractual basis with one of three Finnish chicken slaughterhouses with scheduled setting and slaughter dates, and depopulation strategies, like thinning, are not practiced. Instead, Finnish farmers use the all-in all-out system with an empty period from one to four weeks. The farmers receive continuous training and guidance from advisors from the poultry industry or the district veterinary officer in biosecurity and animal welfare.

Broiler chickens are slaughtered at the age of 35-40 days with a highly automated slaughtering process. There are only three slaughterhouses handling chickens in Finland, of which two use carbon dioxide stunning and one electrical stunning. After stunning, hanging, exsanguination, scalding (water temperature 54-56°C), de-feathering, removal of head/feet, evisceration (automated), cleaning, washing, air chilling (2°C for three hours), meat cutting, margination, and packaging follow, in this order. The first and second meat inspections are after de-feathering and evisceration, respectively. The meat is packed on the day of slaughter, and most products are sold fresh, marinated, and packed in modified atmosphere.

In 2004, the compulsory Campylobacter monitoring program for chicken slaughter batches was implemented. According to this surveillance program, all chicken batches slaughtered between June and October undergo compulsory testing for the presence of C. jejuni and C. coli, while the sampling from January to May and in November and December is randomized. Farms found to deliver positive batches receive additional counselling on biosecurity from the slaughterhouse veterinarian and future batches are scheduled for slaughter at the end of the day until two consecutive batches test negative for Campylobacter. No action for the chicken meat after a positive result is demanded.

6.4.1.1.2 Prevalence of C. jejuni in chicken flocks

C. jejuni is the most commonly isolated Campylobacter spp. from conventionally reared chickens in the Nordic countries, while C. coli occurs more frequently in Southern Europe and in organic and free-range flocks. In 2013, altogether 29.6% of the EU’s chicken batches tested positive for Campylobacter, but this number is heavily biased by an overrepresentation of reported data (73.2%) from the Nordic countries, which historically have had a low prevalence of positive flocks (0.6–13.1% in 2013). During the 2008 EFSA baseline survey prevalence in the EU was 71.2%, CI95% [68.5–73.7], which is a number closer to the real prevalence of Campylobacter in chicken batches on an EU level since the sampling and reporting was representative that year. The annual fraction of C. jejuni-positive chicken flocks in Finland ranged between 3% and 7% from 2004 to 2013, which is considerably lower than the prevalence reported from big broiler producing countries like Poland, Hungary, and the UK (range 74.2-80%). As there is no surveillance data from regions outside the EU and EEC countries, the prevalence reports from
countries like USA (87.5%) and Australia (26.8%) are not updated and are irregular and based on smaller studies,\textsuperscript{168,169} while data from developing countries are missing altogether.

A seasonal variation in the incidence of \textit{C. jejuni} colonization of housed chicken flocks is reported worldwide.\textsuperscript{163,164,170} During summer the number of \textit{C. jejuni} positive flocks increase, and the timing of the peak varies with latitude. In Finland, the peak is normally in July or August. The reasons behind this pattern are poorly defined, but increasing temperature,\textsuperscript{164,171} presence of fly-vectors\textsuperscript{149} and migrating birds,\textsuperscript{172} and seasonal fluctuations in other reservoirs\textsuperscript{146,147} are possible explanations. However, the increase in \textit{C. jejuni}-positive chicken flocks has been reported to occur after the summer peak observed in human cases,\textsuperscript{86,143,144} indicating occurrence of seasonality in a common third, yet unidentified, environmental reservoir.

Once a bird in the flock gets colonized with \textit{C. jejuni}, a rapid bird-to-bird transmission soon results in complete within-flock colonization, though variation in the colonization level exists.\textsuperscript{173,174} Whether the flock is colonized by single or multiple strains varies; in Finland, the chicken flock is usually colonized by one genotype only (Hakkinen and Kaukonen, presented at the 15th International Workshop on \textit{Campylobacter}, \textit{Helicobacter}, and Related Organisms, Niigata, Japan, 2-9 September 2009). However, substantial evidence exists that several strains may colonize a chicken flock simultaneously,\textsuperscript{87,165,173-175} possibly reflecting a higher environmental transmission pressure and gaps in biosecurity.

MLST typing has increased the knowledge about the nature of the \textit{C. jejuni} populations colonizing chickens. The typical chicken-colonizing genotypes are the generalists (ST-45 CC), the farm-animal generalists (ST-21 CC), “chicken-associated” genotypes (ST-257 CC) and singletons, meaning STs represented by a single isolate only.\textsuperscript{104,176-178} ST-257 CC might be a “chicken-associated” genotype in developed countries, and this association transcends geographical location.\textsuperscript{79,80,106,164,175,179,180} In addition, some genotypes are frequently found in both the chicken and ruminant reservoir, possibly representing a shared \textit{C. jejuni} population between these reservoirs.\textsuperscript{58} Chickens rarely share genotypes with isolates of non-agricultural origin, like wild birds or environmental water,\textsuperscript{172,180} probably due to the presence of ecological barriers between these sources or that non-agricultural strains lack the ability to colonize chickens.

\subsection*{6.4.1.1.3 Colonization of chicken}
\textit{C. jejuni} usually colonizes the mucus overlaying the intestinal wall in the ceca and small intestine,\textsuperscript{181} but can also be recovered from extra-intestinal sites such as the liver and spleen.\textsuperscript{165,182,183} Colonization doses as low as 40 colony-forming units (CFUs) have been reported,\textsuperscript{184} and once colonized, the numbers of \textit{C. jejuni} in the ceca rapidly increase to high levels, such as $10^8$-$10^9$ CFU/g,\textsuperscript{183,184} and subsequently spread throughout the flock in a few days via the fecal-oral route.\textsuperscript{87,173} \textit{C. jejuni} is conventionally considered a harmless commensal in the chicken gut, but recent evidence that the organism induced disease in certain bird breeds may come to challenge this paradigm.\textsuperscript{185}

Newly hatched chicks are free from \textit{C. jejuni}, and continue to test negative for \textit{C. jejuni} until approximately two or three weeks of age.\textsuperscript{87,175} The reason for this delay in detection (known as the lag phase) is not completely understood, but age-dependent transmission of \textit{C. jejuni} and the presence of maternal antibodies and commensal organisms in the gut of young chicks may be involved.\textsuperscript{181,186,187} However, since day-old chicks are susceptible to \textit{C. jejuni} colonization under experimental conditions, the lag phase could reflect lack of exposure to \textit{C. jejuni} during the early phase of rearing and/or lack of sufficiently sensitive detection methods for \textit{C. jejuni} under natural
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AK Llarena

Even so, broiler chickens usually carry *C. jejuni* until slaughtered, which is approximately at 35 days of age, while a gradual reduction in the within-flock prevalence has been demonstrated in chickens under experimental conditions and in the longer living breeders.

6.4.1.1.4 Transmission of *C. jejuni* to conventionally reared chicken flocks

Major research efforts have been directed to determine sources and routes of transmission to chickens with the ultimate goal of identifying effective intervention strategies to reduce the number of *C. jejuni*-positive chicken flocks. Transmission of *C. jejuni* is best described as the route the bacteria use to reach chickens inside the farmhouse, and this is accomplished either vertically (i.e. from parent to offspring) or horizontally (i.e. from the surrounding environment). The role of vertical transmission is still debated, and even though some studies support this theory, unified evidence for this transmission route is lacking. Therefore, the primary transmission route is most probably horizontal acquirement of *C. jejuni*.

*C. jejuni* from the farm environment may contaminate chicken flocks if biosecurity is breached. Since *C. jejuni* does not replicate in the environment, the presence of *C. jejuni* outside of a host must be the result of prior fecal contamination from an animal reservoir. The previous chicken flock is an unlikely source of *C. jejuni* colonization, as no evidence for carry-over contamination inside or in the environment surrounding the chicken house has been found, confirming the effectiveness of the cleaning and disinfection procedures used during the empty period. Livestock, pets, pests, and wild birds present on or adjacent to the farms can carry *C. jejuni*, but the relative importance of these reservoirs as a contamination source to the chickens remains unclear. Similar genotypes have been isolated from adjacent swine and cattle herds both before and after the chicken become *C. jejuni*-positive; thus, the direction of the spread is unknown. Furthermore, the majority of genotypes found in livestock have not been recovered from chickens. This situation also applies to wild birds as a source, since wild birds and wildlife in general are colonized with a *C. jejuni* population distinct from that in chickens. The role of pests is not fully understood either, as few exclusive studies exist on this topic, but small mammals appear to be infrequently colonized by *C. jejuni*. Horizontal transmission through rodents cannot, however, be excluded.

Transmission from the surrounding environment or other animal reservoirs to the chickens can also occur indirectly via such vehicles as personnel, equipment, and insects. Especially houseflies and equipment used during thinning have received abundant research attention. For instance, flies carried *C. jejuni* in Denmark, and similar genotypes of *C. jejuni* have been recovered from equipment, personnel and slaughtered chickens. Furthermore, by installing fly screens, Hald et al. were able to reduce the *C. jejuni* prevalence in Danish chicken flocks by 35%, thereby showing the importance of this transmission route. There is no evidence for the introduction of *C. jejuni* via feed, litter, or drinking water, but all play a major role in the within-flock spread of *C. jejuni*.

Risk factors associated with *C. jejuni* colonization of chickens reflect colonization capacity, epidemiology, and transmission routes. Through risk analysis studies, the most important risk factors for chicken colonization have been identified as follows: increasing age of birds, summer season, geographic location in an animal-dense area, poor hygiene, or lacking biosecurity practices and depopulations strategies such as thinning. Furthermore, a recent systematic review highlighted the risk for contaminating a new flock with the presence of a contaminated barn.
environment due to inadequate disinfection and cleaning, an insufficient empty period, and the presence of an adjacent broiler flock.  

What then are the major reservoirs and transmission pathways leading to \textit{C. jejuni} colonization in poultry? Unfortunately, even 30 years of \textit{C. jejuni} research has been unable to determine an unequivocal answer to this question. The answer probably lies somewhere along the line of a bit of everything at all times; \textit{C. jejuni} in chicken probably originates from multiple reservoirs through a variety of sources and vehicles.

\subsection*{6.4.1.1.5 \textit{Campylobacter} at slaughter}

A positive association exists between \textit{C. jejuni}-colonized flocks and \textit{C. jejuni} prevalence and counts on carcasses.\cite{207-209} The strength of this association varies between countries, probably due to differences in slaughter processes and in-flock prevalences of \textit{C. jejuni}.\cite{163} However, direct or indirect contact between \textit{C. jejuni}-positive and -negative carcasses in the slaughterhouse can even lead to contaminated carcasses originating from \textit{C. jejuni}-free flocks,\cite{210} although at low levels.\cite{209}

The prevalence and concentration of \textit{C. jejuni} on carcasses vary in the slaughter chain; an increase is seen after de-feathering and evisceration and a decrease after scalding, chilling, and washing (only concentration).\cite{211} More specifically, a tendency exists that higher scalding temperature (\textgreater{} 55°C) and water chilling reduce the \textit{C. jejuni} concentrations more than lower scalding temperatures and use of air chilling despite the increased risk of cross-contamination associated with water chilling.\cite{208,211}

\subsection*{6.4.1.1.6 Intervention strategies for broiler production}

Reducing \textit{C. jejuni} in the food chain, particularly in chicken products, has been and still is a major strategy for combating campylobacteriosis. Hindering the colonization of chicken flocks on farms is suggested to be the most cost-efficient measure,\cite{212} but preventing \textit{C. jejuni} contamination and survival is addressed on all levels of the production chain.

Strategies to reduce or eliminate environmental exposure of the chicken flocks require proper biosecurity measures. Even though the best approaches constituting good biosecurity are currently unknown, minimum measures includes boot dips or changes of footwear, hand washing, and physical barriers.\cite{212} In addition, maintaining good hygiene practices around thinning and transport\cite{204} and increasing farmers’ motivation\cite{213} are essential. Hindering access of insects, especially flies, to the chicken house by use of fly screens has been efficient in lowering \textit{C. jejuni} prevalence of chickens in Denmark.\cite{205}

However, in many regions it has been difficult to prevent the transmission of \textit{C. jejuni} to the chicken house.\cite{214} thus, approaches to reduce within-flock prevalence and bacterial load have been developed. The addition of various feed or water additives, like organic and fatty acids,\cite{215} probiotics,\cite{216} and bacteriocins,\cite{217} has had various effects in different studies, mainly by reducing the \textit{C. jejuni} concentration in the ceca or increasing chickens' resistance to colonization. Vaccination of chickens, use of colonization-resistant chicken breeds or lineages, and administration of bacteriophages are currently hypothetical approaches under intensive study.\cite{214,218-220}

The shedding rates and colonization levels of \textit{C. jejuni} increase during transport, resulting in heavily contaminated transport crates.\cite{181} Inadequate washing and disinfection of crates may lead to contamination of the residual flock after thinning\cite{202} or cross-contamination of a \textit{C. jejuni}-
negative flock.\textsuperscript{210} To prevent this, measures targeted at reducing the amount of \textit{C. jejuni} in transport crates, such as minimizing transport and holding times,\textsuperscript{221} are necessary.\textsuperscript{222} To decrease the prevalence of \textit{C. jejuni}-contaminated carcasses in the slaughterhouse, the focus is on preventing fecal spilling and cross-contamination of meat and chickens.\textsuperscript{223} To achieve this, good hygiene practices, Hazard Analysis Critical Control Point,\textsuperscript{224} correctly adjusted slaughter equipment,\textsuperscript{208} and logistic slaughter are essential. However, a limited effect of scheduling of flocks has been shown, mainly due to problems of sensitive and sensible testing of chicken flocks on-farm.\textsuperscript{214,225}

To reduce the level of contamination on the carcass, chemical and physical interventions are available. Chemical carcass treatment (e.g. lactic acid, chlorine dioxide, and acidified sodium chlorite) can reduce \textit{C. jejuni} concentration with 0.47 to 1.8 log\textsubscript{10} at the point of application.\textsuperscript{212} Some chemical decontamination methods are in use in New Zealand and USA, but are currently not authorized in the EU. Cooking, irradiation, and freezing are the most effective methods to decrease the levels of \textit{C. jejuni} on carcasses,\textsuperscript{214} and freezing is in use in some countries.\textsuperscript{208} A significant portion of the chicken meat sold in Finland is marinated, which has been found to decrease the survival ability of \textit{C. jejuni}.\textsuperscript{226} Furthermore, the Finnish practice of packing chickens in consumer-friendly portions minimizes the need for handling prior to heat treatment and reduces the risk of cross-contamination in the kitchen.

In addition, increasing consumer awareness of safe food hygiene practices during preparation and cooking is necessary to properly control the campylobacteriosis epidemic, but changing the public’s behavior has proven to be challenging and inefficient.\textsuperscript{214}

6.4.1.2 Wild birds
\textit{C. jejuni} is one of the most important avian zoonotic agents, and wild bird carriage of \textit{Campylobacter} spp. has received much research interest. According to a recent review, a total of 2187 of the 11,357 studied wild birds of 205 different species were colonized by a \textit{Campylobacter} species.\textsuperscript{227} The most commonly colonized species were ducks, pigeons, shorebirds, covies, doves, thrushes, and starlings, while passerines, other than thrushes and starlings, were infrequently colonized. Except for the shorebirds and thrushes, the majority of the high carriage birds were associated with human activity in one way or another.\textsuperscript{227} Temporal variation in prevalence has been reported from black-headed gulls,\textsuperscript{228} geese,\textsuperscript{172} thrushes,\textsuperscript{229} herring gulls,\textsuperscript{230} and starlings,\textsuperscript{231} possibly reflecting the presence of seasonality. Rapid turnover of \textit{C. jejuni} genotypes and age-dependent colonization have been reported for starlings, with young birds being more frequently colonized by \textit{C. jejuni}.\textsuperscript{231} Waldenström \textit{et al.} found that an already wild bird-adapted \textit{C. jejuni} strain colonized European robins more frequently than a human-derived strain, suggesting the presence of currently unknown host-specific factors in \textit{C. jejuni} necessary for colonization of at least the European robin.\textsuperscript{232}

Several studies have attempted to attribute human isolates to the wild bird reservoir using serotyping, PFGE, and MLST. From these studies, it is evident that the \textit{C. jejuni} population found in wild birds is genetically distinct from the \textit{C. jejuni} populations in agricultural host species and humans,\textsuperscript{58} as the vast majority of \textit{C. jejuni} geno- or serotypes seen in wild birds have not been reported in human cases or animal reservoirs, although some overlap exists.\textsuperscript{228,230,233-235} In addition, some of the geno- or serotypes may be especially host-adapted, e.g. the ST-1020 in starlings.\textsuperscript{231} However, the occurrence of shared geno- or serotypes in the wild bird and farm animal niche is probably a result of wild bird-acquisition of these strains from anthropogenic
sources, rather than vice versa, since the birds often reside close to human settlements or farm activity when sharing is observed.\textsuperscript{172}

The \textit{C. jejuni} community in the wild bird is determined by bird taxonomy, meaning that each bird species has its own typical \textit{C. jejuni} population.\textsuperscript{229} This feature is not observed among agricultural animals, which usually harbor a \textit{C. jejuni} population shared by several animal species.\textsuperscript{58} Actually, host species has been suggested to be a stronger driver than geography for \textit{C. jejuni} in wild birds, resulting in low genetic subdivision between \textit{C. jejuni} collected from a silver gull in Australia and black-headed gulls in Sweden.\textsuperscript{229}

For wild geese, carrier rates between 0 and 50.2\% are reported.\textsuperscript{172,236,237} The few studies assessing \textit{C. jejuni} genotypes in geese hypothesized that their \textit{C. jejuni} are distinct from \textit{C. jejuni} in humans and agricultural hosts.\textsuperscript{172,237} More research on different wild bird reservoirs is warranted.

### 6.4.1.3 \textit{C. jejuni} in other domestic animal reservoirs

Bovines are a well-acknowledged reservoir for \textit{C. jejuni}, which is frequently isolated from healthy cattle. Carriage rates between 4.6\% and 89.4\% are reported through slaughterhouse surveys, with \textit{C. jejuni} as the predominant species.\textsuperscript{147,238,239} Carriage and excretion rates show temporal and age-dependent patterns; a peak in shedding rates is seen in late spring and early summer.\textsuperscript{147,240} and calves are more commonly colonized than adult cattle.\textsuperscript{147,239,241} According to MLST studies, ST-61 CC and ST-48 CC may possibly be adapted to ruminants and are among the most commonly found genotypes in sheep and cattle in England, Scotland, Luxembourg, and Finland.\textsuperscript{107,179,242-244} Despite frequent isolation of \textit{C. jejuni} from the intestines of bovines, the organism is rarely recovered from bovine meat.\textsuperscript{238,245-247} making foodborne exposure of \textit{C. jejuni} through meat unlikely. Rather, campylobacteriosis attributed to the bovine reservoir is assumed to be the result of direct contacts during farm visits or occupational activity in rural areas.\textsuperscript{105,248} in addition, consumption of unpasteurized milk is a well-described risk factor for acquiring campylobacteriosis, and is often linked to outbreaks.\textsuperscript{124,136}

\textit{C. jejuni} is also recovered from other farm animals, including sheep\textsuperscript{146,179,244} and swine, but \textit{C. coli} is more commonly isolated from both.\textsuperscript{233,244} However, neither of these reservoirs has been associated with significant numbers of campylobacteriosis cases.\textsuperscript{104,176,249} Contact with pets, such as dogs and cats, especially puppies or dogs with diarrhea, are often described as risk factors for contracting campylobacteriosis in case-control studies.\textsuperscript{250,251} \textit{C. upsaliensis} is far more common in dogs than \textit{C. jejuni},\textsuperscript{252} but the \textit{C. jejuni} genotypes isolated from pet dogs are often similar to human disease isolates.\textsuperscript{253,254} Whether dogs act as an infection source to humans or whether dogs and humans share a common source is currently unknown.

### 6.4.2 Environmental contamination by Campylobacter

\textit{C. jejuni} has been recovered from various environmental matrices and survives well in water, especially cold water, and soil despite being considered fragile and oxygen-sensitive.\textsuperscript{255} As \textit{C. jejuni} lacks the ability to multiply outside a host,\textsuperscript{256} the finding of the bacteria in the environment must be due to fecal contamination through, for instance, sewage spill or direct fecal deposit by animals. In fact, the environment is often treated as proxy for wildlife in source attribution, even though run-off from agricultural or anthropological sources also can lead to \textit{C. jejuni} contamination, rendering this proxy inaccurate.\textsuperscript{249}

The fate of \textit{C. jejuni} in different milieus is influenced by extrinsic factors, like temperature, water activity, amount of nutrients, UV-light, and nature of the microbial community,\textsuperscript{257} and intrinsic...
strain-dependent factors.\textsuperscript{258} \textit{C. jejuni} lacks many of the commonly used stress responses of Gram-negative bacteria\textsuperscript{34} and has therefore developed alternative mechanisms to cope with life outside the host. For instance, \textit{C. jejuni} has an array of anti-oxidative activities and several stringent responses, conserving the viability of the organisms in nutrient-poor and oxygen-rich environments. [reviewed in \textsuperscript{259}] Also, some strains form biofilms in mono- or co-culture on abiotic surfaces and in water systems to increase \textit{C. jejuni}'s ability to survive.\textsuperscript{260,261}

Water, sewage, and beach sand are the environmental milieus most commonly investigated for the presence and survival of \textit{Campylobacter}. Beach sand may provide a sun-protected environment for \textit{C. jejuni} and retain the bacteria during low tide, thereby increasing the survival potential of \textit{C. jejuni}. In line with this finding, Khan \textit{et al.} detected the highest percentage of \textit{C. jejuni} positive samples from wet beach sand (36%), while the occurrence of \textit{C. jejuni} declined when sampling beach (27%) and offshore water (0%).\textsuperscript{262} Yamahara \textit{et al.} detected \textit{Campylobacter} spp. in dry sand collected from 13% of the marine beaches in California, even though water activity in sand and \textit{Campylobacter} spp. concentration and occurrence were positively associated.\textsuperscript{255}

The intrinsic factors partially determining the fate of \textit{C. jejuni} in different environmental niches might be evident along different \textit{C. jejuni} lineages. For instance, it has been suggested that certain STs are more adapted to survive in the environment than others,\textsuperscript{49,58,263} but this hypothesis is controversial. Our group has been unable to show different survival abilities in sand and water between several tested lineages like ST-677 and ST-45 (unpublished material). Concordant with our findings, most genotypes from abiotic environmental samples have no coherent genetic structure and are attributed to other wild bird or farm animal sources, suggesting that these \textit{C. jejuni} are of fecal origin from a mixture of reservoirs.\textsuperscript{58}

The role of the environmental pathway in the complex epidemiology of \textit{C. jejuni} is not fully understood. Apart from generalists like ST-45 CC, the genotypes found in the environment are distinct and diverse and only infrequently isolated from human patients\textsuperscript{263} and most studies have been unable to show a direct link between environmental sources and acquisition of campylobacteriosis.\textsuperscript{199,264} However, the role of the environment as a transmission pathway to other animal reservoirs, including our agricultural species, should not be underestimated.

6.4.2.1 Water

Even though \textit{Campylobacter} spp. seems to survive best in cool, dark water, like well-water,\textsuperscript{265} \textit{Campylobacter} spp. have been recovered from a wide variety of water bodies. For instance, \textit{C. jejuni} was present in 8.4-37% of water samples collected from marine and freshwater beaches and rivers in Canada and USA.\textsuperscript{266,267} ST-45 CC is often isolated from environmental waters, which might reflect the increased environmental survival capacity of this genotype in combination with the widespread distribution of this clonal complex.\textsuperscript{268} Also, a coincidental seasonal peak of ST-45 in water samples and human patients has been noted.\textsuperscript{265} However, large diversities occur, and in a longitudinal American study of river water the most common ST and CC was the ST-61 and ST-179 CC, respectively, but in general the population was extremely diverse and most STs occurred only once.\textsuperscript{267} In addition, Meinersmann \textit{et al.} stated that the isolates recovered and characterized through their study represented the bare minimum of the \textit{C. jejuni} actually present in the river, as the organisms probably were released into water pulse-wise and subsequently diluted to extinction within a short distance from the source.\textsuperscript{267}
A number of *Campylobacter* outbreaks have been linked to consumption of contaminated drinking water\(^{136-138,269-272}\) and consumption of untreated drinking water and swimming in seas, lakes, and rivers are risk factors for contracting sporadic *C. jejuni* infection.\(^{142,145,251,273}\) However, isolation of the causative *C. jejuni* when tracking outbreaks and sporadic cases is challenging. Large water masses and low infectious dose coupled with rapid water exchange makes isolation after detection of cases difficult. In addition, risk activities like drinking well-water and swimming in natural water usually occur during summer when the amount of UV radiation and water temperature are at their highest. This contributes to a rapid death of *C. jejuni* cells, further complicating the recovery of the causative agent.\(^{266}\) Therefore, most *C. jejuni* isolates originating from water are recovered outside of an epidemiological context, but serve as the best proxy for the *C. jejuni* causing human cases.

### 6.5 Source attribution of *Campylobacter* infections

Identification of the most frequent transmission routes and foodborne sources of campylobacteriosis is of utmost importance for prioritizing food safety interventions and setting public health goals.\(^{274}\) *C. jejuni* has a complex epidemiology, and transmission can occur in numerous ways, including contaminated food, water, and raw milk, and direct animal and environmental contact. To overcome this problem, methods to quantify the relationship between human patient data and possible infection reservoirs, transmission routes, and risk factors have been developed.

**Source attribution of *Campylobacter* infections**

Source attribution, defined as the process of assigning the burden of a given disease due to a specific source or pathway, uses several approaches, and different authors classify them according to different schemes. Pires et al. uses the terms microbial, epidemiological, and expert elicitation approach and intervention studies,\(^{274}\) while French and Marshall\(^{91}\) classify the approaches according to their target level in the risk chain; reservoir and pathway attribution, exposure/risk assessment, and epidemiological risk factor modeling. An array of these approaches or, more often, combinations thereof have been crucial in the work with source attribution of campylobacteriosis cases in Finland and abroad.\(^{177,199,275}\)

Epidemiological approaches include tools like case-control or cohort studies describing either sporadic or outbreak cases, of which the former is by far the most commonly used.\(^{91}\) Case-control studies have identified risk activities like travel, dining in restaurants (chicken, beef, and pork), and swimming in natural waters for contracting sporadic *C. jejuni* infection. Children are particularly at risk of acquiring campylobacteriosis through direct contact with farm animals or pets.\(^{145,276,277}\) To quantify the relationship, many epidemiological studies include a calculation of population-attributable factors or risk (PAF or PAR). Chicken consumption stands out as the number one cause of campylobacteriosis, with PARs varying from 4.9% to 31% worldwide.\(^{250,251,278-280}\)

Compilations of outbreak investigations can also give valuable information on the attribution of different reservoirs, pathways, risk factors, and exposures to *Campylobacter* infections. However, one needs to bear in mind the different epidemiology for outbreaks and sporadic cases, questioning the value of using such compilations for source attribution since the vast majority of campylobacteriosis cases are sporadic. Nonetheless, alongside other epidemiological studies they are highly useful. Greig et al. attributed 29.3% of all *Campylobacter* outbreaks to chicken,\(^{281}\) and Domingues et al. reported consumption of undercooked chicken, unpasteurized dairy products, and drinking water as risk factors for campylobacteriosis outbreaks.\(^{277}\)
Intervention studies can provide compelling evidence of the burden of illness attributed to a specific source by showing a drop in campylobacteriosis cases after an intervention. Source attribution is based on quantification of this case reduction (apparently) due to the removal of the exposure. For instance, in the year 2000, Iceland implemented several broiler-targeted control measures, including increased biosecurity on broiler farms, scheduling of broiler slaughter, and freezing of Campylobacter-positive broiler carcasses. Coupled with an increase in consumer awareness and Campylobacter monitoring, a 40% drop in campylobacteriosis cases from 116 to 33 per 100,000 inhabitants, was reported in Iceland from 1999 to 2000.\textsuperscript{222} In Denmark, the implementation of freezing chicken carcasses from Campylobacter positive batches in 2001/2002 may have led to a reduction in campylobacteriosis cases by 5% and 19% in 2002 and 2003, respectively.\textsuperscript{276} A more impressive effect of intervention strategies aimed at Campylobacter in the poultry industry was seen in New Zealand, which experienced a 54% decline in campylobacteriosis notification rates and a 74% reduction in cases attributed to poultry.\textsuperscript{282} Furthermore, the use of a disaster as an epidemiologic tool offers especially good opportunities to observe changes in the occurrence of campylobacteriosis. For instance, the withdrawal of domestic poultry products due to the dioxin crisis in 1999 led to a 40% drop in campylobacteriosis cases in Belgium.\textsuperscript{283}

A microbiological approach to source attribution is based on the analysis of Campylobacter isolates, with attribution done by comparing subtypes of Campylobacter isolates from various sources and pathways with those collected from patients. Sources are attributed a proportion of human cases relative to the amount of “indicator subtypes” found among the human isolates.\textsuperscript{91,274} MLST has been the subtyping method of choice during the last decade,\textsuperscript{82,103-107} but other subtyping schemes like PFGE and serotyping have also been adopted in this field of research.\textsuperscript{235,284} More recently, NGS and whole-genome comparisons are starting to stand out as the genotypic tool of choice to trace both outbreaks and sporadic cases and were, for instance, used in real-time outbreak investigations during a Canadian listeriosis outbreak in 2008.\textsuperscript{285}

Different methods are used to attribute genotypes of human isolates to possible sources. For instance, simple comparison, like the proportional similarity index (PSI), estimates the area of intersect between two frequency distributions.\textsuperscript{233} Poultry was most similar to human isolates according to a New Zealand and Norwegian study,\textsuperscript{177,233} while a Finnish study found decreasing overlap between C. jejuni of human and chicken origin from 1997 to 2003 by simple comparison of MLST types.\textsuperscript{106} More advanced mathematical models, like the Dutch model\textsuperscript{286} and the Hald\textsuperscript{287}/ modified-Hald model,\textsuperscript{249} estimate the number of human cases that can be attributed to each source by comparing subtypes from human cases with the relative occurrence of that subtype in each source. The Hald and modified-Hald use Bayesian inference to estimate the uncertainty around parameters, which is an improvement from the Dutch model. Wilson \textit{et al.} developed the Asymmetric Island model, based on Wright’s Island model,\textsuperscript{288} to infer the source of human infections using a genetic approach that models DNA sequence evolution and zoonotic transmission.\textsuperscript{176} By estimating the rate of genomic migration, recombination, and mutation, the model assigns probabilistically each human case to one of the source populations, thereafter calculating the total number of campylobacteriosis cases attributable to each source.\textsuperscript{176} This model is advantageous in its ability to attribute human cases infected with subtypes without an identified reservoir.\textsuperscript{91} In Lancashire, England, 56.5% and 39.3% of the human cases were attributable to chicken and ruminant sources, respectively,\textsuperscript{176} while 78% of the Scottish campylobacteriosis cases were attributed to chicken meat\textsuperscript{104} by use of this model.
In the same study, Sheppard et al. also ran the Bayesian-based software program STRUCTURE on the same material, finding 70% of the human cases to be attributed to the chicken reservoir. De Haan et al. could track 45.4% and 44.3% of Finnish campylobacteriosis cases to the chicken and bovine reservoir using Bayesian Analysis of Population Structure (BAPS), also a Bayesian-based software program (see below for more detailed information). Furthermore, combinations of some or all of these approaches are applied to improve the estimates at the same or all levels.

The choice and sample sizes for databases included in source attribution are important. According to Smid et al. (2013), the self-attribution degree decreases when the sources vary across time and space. For instance, the importance of chicken as a source for human infections is negatively correlated with the increase in 1) time between the samplings and 2) geographical distance between the sampling points. According to Smid et al. (2013), a lower critical limit of 100 isolates per food source is recommended to achieve satisfactory statistical power and confidence in the asymmetric island model.

Collectively, all of these source attribution studies have emphasized the importance of chicken, followed by cattle, as a source of human sporadic campylobacteriosis, and have revealed regional variation in the origins of Campylobacter infections.

6.5.2 Outbreak investigations

The goal of an outbreak investigation is to identify the cause and provide the best means to control the epidemic. This requires several different steps that often occur simultaneously: 1) preliminary investigations, 2) identification of cases, 3) collection and analysis of data, 4) implementation of control measures, 5) dissemination of findings, and 6) follow-up studies.

Therefore, resolution of an outbreak relies on a good surveillance system for detection and cooperation between epidemiologists, sanitarians, and microbiologists, usually spread over a wide geographical area.

In the field of microbiology, the focus is especially on the third stage, the collection and analysis of data, although knowledge about the pathogen at hand is important during all stages (i.e. identification of cases, possible propagation methods, long-term sequelae for follow-up studies). Elucidation of an outbreak with a C. jejuni etiology requires the use of high-resolution typing methods. Earlier, serotyping was used to routinely screen outbreak-related strains, although this method might disguise the clonal relationship of the strains. Significantly more suitable subtyping methods are available today, for instance, the genotyping methods PFGE, MLST, fla-sequencing, and more recently, NGS. However, genomic events can take place that alter the clonal relationship between strains, occluding their true epidemiological relationship.

6.6 Analysis of population structure

A population is considered structured when a single, simple population is disrupted by so-called subpopulations, demens, or local populations. These subpopulations together make up the metapopulation or the "Population of populations". The distinction between the subpopulations can be discrete or continuous. Genetic drift and selection can lead to structuring, while genetic migration and random mating homogenize the gene frequencies.
6.6.1 Measures of diversity
Diversity is the presence of different organisms in an ecosystem, for instance, different genotypes in a C. jejuni population. A diversity index measures quantitatively how many different genotypes there are in a dataset (abundance) and simultaneously takes into account how evenly the isolates are distributed among these genotypes (evenness). Two popular methods are the Shannon Index of Diversity (SID) and the Simpson Diversity Index (SDI). However, these are just diversity indices and not true measures. The number of equally common species required to give a particular value of an index is called the "effective number of a species". This is the true diversity of the community in question, and algebra is used to arrive at this number. 296

6.6.2 Non-phylogenetic Bayesian clustering methods
These methods work within a Bayesian framework to determine the number of genetic groups present in a system and estimate the fraction of an isolate's genotype that originated from the identified groups, i.e. the admixture. 297 Many different kinds of software exist, but two of the most frequently used tools in Campylobacter research have been BAPS 290 and STRUCTURE. 289 In addition to performing population structure analysis, these software programs can probabilistically assign human isolates to the putative origin population and have therefore been used in source attribution (see above). Both of these programs work on minimizing the Hardy-Weinberg and linkage disequilibrium that would result if isolates from different populations were to be incorrectly assigned to a population. They do, however, differ in their methods to derive the number of subpopulations (K); STRUCTURE utilizes a stochastic approach, while BAPS employs an optimization algorithm to arrive at the most likely K based on the data provided (posterior). 298 Both programs allow the isolates to be of mixed origin and are able to infer the correct number of clusters even when clusters were not well differentiated (FST=0.02–0.03). 298 However, STRUCTURE does not adjust for linkage between the loci, while BAPS does. 101

6.6.3 Genetic distance and phylogenetic models
Phylogeny is the history of an organism's lineages as they change through time, and inferring this history using genomic data makes up the basis of phylogenetic analysis. In its broad sense, phylogeny covers a wide variety of methods like evolutionary distance, genetic networks, analysis of comparative genome data, evolutionary rates, and diversification analysis. 299

Traditional population genetic analyses, such as F-statistics (FST: Fixation index in the Subpopulation relative to the Total population) or genetic distances, remain one of the most commonly used approaches for characterizing population differentiation. The FST describes the heterozygote deficit due to population subdivision and therefore the proportion of genetic variation found between, as opposed to within, each population.

A phylogenetic tree is most commonly used to reconstruct the evolutionary relationship between isolates in a dataset. Available approaches to construct such a tree are either distance-based, like Unweighted Pair Group Method using Arithmetic Averages 300 and the neighbor-joining method, 301 or based on the sequence data directly, such as in maximum parsimony 302 and maximum likelihood. 303 Distance-based methods calculates a distance matrix in which the pairwise difference between every isolate in the database is computed, and the tree is constructed based on this. Sequence-based methods attempt to find the tree most suitable for the available DNA sequences. To validate the phylogeny achieved, bootstrapping 304 or Bayesian approaches 305,306 can be used. All sequence-based models assume a specific sequence evolution, and if the evolutionary model is violated the reconstruction may be incorrect.
Thus, when data do not follow a tree-like evolution due to, for instance, recombination, reconstruction of the phylogeny using the methods above is not biologically relevant. Such data will usually support several trees, and instead of choosing a specific one, building a split network in which incompatible and ambiguous signals are presented as parallel edges makes more sense. Networks can be built using different methods\textsuperscript{307} and are particular useful in describing large data affected by recombination.

ClonalFrame, developed by Didelot and Falush,\textsuperscript{308} is a sequence-based Bayesian method estimating a tree genealogy, but the difference from the former methods lies in its evolutionary model, which assumes recombination. ClonalFrame identifies each tract of polymorphism originating from horizontal gene transfer as one single event and therefore determines the clonal frame for each branch, i.e. the subset of the genome that did not undergo recombination. This approach is superlative to other genealogy methods in estimating the extent of the isolate’s true clonal frame and is therefore much used in estimating the phylogeny of \textit{C. jejuni}.
7. Aims

1. To evaluate the three metabolic traits GGT production, \( \text{ansB}(s) \), and \( \text{fucP} \) as candidate host adaption elements compared with MLST (I).

2. To investigate the population structure of \( \text{C. jejuni} \) in birds, as exemplified by wild geese and broiler chickens (II and IV).

3. To investigate the similarity between \( \text{C. jejuni} \) in barnacle geese, humans and agricultural animals, with a special focus on chickens (I, II, and IV).

4. To investigate the applicability of whole genome sequencing to \( \text{C. jejuni} \) outbreak investigations (III).

5. To characterize the \( \text{C. jejuni} \) population found on Finnish chicken farms over a decade and to identify factors of importance for the transmission of \( \text{C. jejuni} \) to chicken flocks (IV).

6. To identify the most cost-efficient genotyping method to trace \( \text{C. jejuni} \) from reservoirs to humans in different epidemiological settings (I-IV).
8. Materials and methods
An overview of the methods used in Studies I-IV is shown in Figure 1 (p. 72).

8.1 Sampling (II and IV)

8.1.2 Barnacle geese (Branta leucopsis) (II)
A serial cross-sectional survey during 2011 and 2012 was performed to determine the proportion of barnacle geese positive for Campylobacter spp.. The sample size necessary to estimate a proportion of 0.5 in an infinite population with a confidence level of 0.95 and desired precision of 0.05 was 385 (http://epitools.ausvet.com.au).

Freshly voided fecal droppings were collected with transport swabs (TS0001, Oxoid, Thermo Fisher Scientific, Vantaa, Finland) from 12 different sites in the Helsinki area (1-12); of which five sites (1, 2, 3, 4, and 12) were sampled both years. Between nine and 97 samples were collected on each occasion, reflecting variation in flock sizes, from birds mainly considered Finnish breeders (all sites at all times, except site 12 at sampling point 6). This resulted in a total of 924 samples (n=496 in 2011 and n=428 in 2012) collected during 27 different time-points in the high peak season from June until September.

8.1.1 Broiler chickens (IV)
The 380 chicken C. jejuni isolated at slaughter in 2004, 2006, 2007, 2008, and 2012 were collected in the Finnish Campylobacter monitoring program for poultry. Compulsory testing for the presence of C. jejuni and C. coli in chicken batches took place at all Finnish slaughterhouses (n=3). All chicken batches slaughtered between June and October, were tested while the sampling from January to May and in November and December was randomized using an expected target prevalence of 5% (1% since 2008), confidence level of 95%, and desired precision of 5% (1% since 2008). In 2004, no sampling took place between January and May, but the described randomized sampling above was utilized between November and December.

8.2 Isolation and species determination of Campylobacter spp. (II and IV)

8.2.1 Barnacle geese (II)
For the isolation of Campylobacter spp. from barnacle geese feces, each transport swab was enriched in 5 ml of selective Bolton broth (CM0983 and SR0183, Oxoid) supplemented with 5% defibrinated horse blood within 12 h of collection and incubated under microaerobic (5% O2, 10% CO2, 85% N2) conditions at 37°C for 48 h. A loop-full (10 μl) of Bolton broth was subcultivated onto mCCDA (CM0739 and SR0155, Oxoid) and incubated under the above-mentioned conditions.

Suspected Campylobacter spp. colonies were subcultivated onto two nutrient agar plates supplemented with 5% defibrinated horse blood (hereafter referred to as NBA) (CM0309, Oxoid) and incubated under both microaerobic conditions for 18–20 h and aerobic conditions at 28°C for 24 h. Cultures with typical colony and Gram-staining morphology and lack of aerobic growth were further subjected to species confirmation by PCR (see below).

8.2.2 Chicken batches (IV)
The detection of C. jejuni in chicken cecal samples between 2004 and 2012 was done according to the method of the Finnish Food Safety Authority (Evira) number 3512/5 and carried out by the slaughterhouses themselves. Briefly, one pooled sample consisted of 10 intact ceca per slaughter batch, and these cecal contents were dispersed into 5 ml of sterile peptone water (0.1%). A 10-μl loop-full of this suspension was cultured onto Campylobacter blood-free selective agar (mCCDA) (media supplier varies between different slaughterhouse laboratories) and incubated under
microaerobic conditions at 41.5 ± 0.5°C for 24–48 h. In the event of typical *Campylobacter* growth on the mCCDA plates, one colony was subcultivated and sent to Evira for confirmation tests (ISO 10272–1:2006). The isolates were stored in Brucella broth (Cat. no. 211088, BD Biosciences, Vantaa, Finland) supplemented with 15% glycerol at -70°C to await further analysis. During 2004, 2006-2008, and 2012, a total of 423 chicken batches were positive for *C. jejuni*, yielding a mean *C. jejuni* prevalence of 3.0%, CI 95% [1.8%, 4.2%]. The majority of isolates were collected during summer (June till October prevalence: 5.8%, CI 95% [4.9%, 6.7%]).

### 8.2.3 DNA isolation and species identification by PCR (II, III, and IV)

The *C. jejuni* isolates from chickens were subcultivated twice on NBA under microaerobic conditions at 37°C for 24-72 h. DNA was extracted from the presumptive *Campylobacter* colonies collected from barnacle geese feces (II), the three *C. jejuni* isolates collected from humans (n=two) and water (n=one) during a waterborne outbreak (III), and the *C. jejuni* isolated from chickens (IV) by the use of a commercial DNA isolation kit (A1120, Wizard® Genomic DNA Purification kit, Promega, Fisher Scientific, Vantaa, Finland). The DNA from barnacle geese isolates was used as a template in a species-determining PCR as described earlier, and *C. jejuni* from both chickens and barnacle geese were subjected to MLST typing using the methods described below. DNA of seven chosen barnacle geese isolates and the waterborne outbreak-related strains were subjected to WGS as described under “7.4.4. Next-generation sequencing”.

### 8.3 The *C. jejuni* isolates (I-IV)

The isolates included in this thesis are presented in Table 1.

#### 8.3.1 Human isolates (I-III)

The human isolates included in Study I and the BAPS analysis in II (n=355) were from domestically acquired, sporadic infections collected during the summer peak in the Helsinki region. A more detailed description of their collection and MLST typing is provided. The two isolates used in the comparative genomics Study III (IHV116260 and IHV116292) were collected from human patients during a community outbreak of *C. jejuni* gastroenteritis linked to drinking water contamination in 2000 and have been characterized earlier by Hänninen et al. and Kuusi et al. The human isolates were chosen on the basis of PFGE profile similarity and identical MLST type (ST-45) to the presumed source isolate collected from tap water (4031) and were re-analyzed by NGS in Study III. These human-derived isolates were of 77 STs and 22 CCs, and 16 isolates were left unassigned to clonal complex (UA).

#### 8.3.2 Poultry isolates (I-IV)

The poultry isolates included are mostly collected from broiler chickens at slaughter (n=380), while 33 and four isolates were obtained from retail chicken and turkey meat, respectively. The retail meat sampling in 1996 (n=4, solely chicken, in I) and 2003 (chicken, n=30, in I and II, and turkey, n=3, in I) was done and MLST-typed as described earlier. The *C. jejuni* isolated from chicken ceca during slaughter were included in Study I (1999, 2006, 2007, n=142,), a BAPS analysis in II (1999, 2004, 2005, 2006, 2007, 2008, n=253,), III (2012, n=4, all ST-45) and IV (2004, 2006, 2007, 2008, 2012, n=380). MLST typing of the poultry isolates included in Study I resulted in altogether 77 STs and 10 CCs, while 18 isolates were UA, while the distribution of MLST types included in the BAPS analysis of Study II is described in Study IV and elsewhere.
8.3.3 Bovine isolates (I and II)
The 119 bovine-derived isolates included in Study I and the BAPS analysis in II originated from a Finnish slaughterhouse survey and were MLST typed in two earlier studies. The bovine isolates were assigned to 49 STs that clustered in nine CCs, leaving 15 isolates UA.

8.3.4 Wild bird isolates (I and II)
Most of the wild bird isolates were of barnacle geese origin (n=170), but some isolates from Canadian geese, Herring gulls, and Common gulls were included in Study I and the BAPS analysis in II (n=23). The latter 23 isolates divided into 15 STs and 7 CCs characterized elsewhere. The 42 wild bird isolates included in Study I were classified as "Environmental". Study II included isolates collected from barnacle geese during 2011 and 2012 (n=156) in addition to the above mentioned isolates included in the BAPS analysis.

8.3.5 Water isolates (I, II and III)
Study I and the BAPS analysis in II included 52 isolates collected from water at swimming beaches and water bodies (river/lakes) during 2002, 2005, 2006, 2007, and 2009 of 22 and nine different ST and CCs, respectively. The water isolates included in Study I were classified as “Environmental”. The tap water isolate in Study III (named 4031) was associated with the same waterborne outbreak from which two patient isolates originated (as described under “8.3.1 Human isolates”).

8.3.6 Zoo animal isolates (Study I and II)
The 19 zoo isolates in Study I and the BAPS analysis in II were collected from seven and nine different mammalian and bird species, respectively, including barnacle geese and black-headed gulls present in the zoo during sampling, and were of seven different STs and five CCs. These isolates were classified as “Environmental” in Study I.
### 8.3.7 Table 1
Isolates included in this thesis with year of collection, analysis used, and references on collection and/or original MLST sequencing

<table>
<thead>
<tr>
<th>Origin of isolate</th>
<th>Background/source</th>
<th>Year of collection</th>
<th>Subtyping</th>
<th>Study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (all feces samples)</td>
<td>Waterborne outbreak (n=2)</td>
<td>2000</td>
<td>PFGE, MLST, NGS</td>
<td>III</td>
<td>270,311</td>
</tr>
<tr>
<td></td>
<td>Sporadic and domestic cases (n=454)</td>
<td>1996, 1999, 2002, 2003, 2006</td>
<td>MLST, metabolic markers</td>
<td>I (n=355), II (n=454)</td>
<td>103,106</td>
</tr>
<tr>
<td>Poultry</td>
<td>Chicken (n=33) and turkey (n=4) meat</td>
<td>1996, 2003</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>103,83,312</td>
</tr>
<tr>
<td>Bovine</td>
<td>Fecal samples at slaughter (n=119)</td>
<td>2003</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>103,107,238</td>
</tr>
<tr>
<td>Wild birds</td>
<td>Fecal samples, Canadian goose (n=2)</td>
<td>2005</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Fecal samples, gulls (n=7)</td>
<td>2007</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Surface water, swimming beaches (n=22)</td>
<td>2005, 2006, 2007</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Tap water, outbreak related (n=1)</td>
<td>2000</td>
<td>PFGE, MLST, NGS</td>
<td>III</td>
<td>270,311</td>
</tr>
<tr>
<td>Zoo animals</td>
<td>Mammals and exotic birds (n=19)</td>
<td>2008</td>
<td>MLST, metabolic markers</td>
<td>I and II</td>
<td>199</td>
</tr>
</tbody>
</table>
8.4 Subtyping

8.4.1 Metabolic markers $\text{GGT}$, $\text{ansB(s)}$, and $\text{fucP}$ (I)

In Study I, 710 $\text{C. jejuni}$ isolates from various hosts and sources with known MLST types (Table 1) were tested for the presence of three putative metabolic markers according to the following instructions: The production of GGT was tested with the method described by Chevalier et al. Briefly, $\text{C. jejuni}$ was harvested and added to 200 µl of distilled water after a 24-h growth on NBA, and 200 µl of reagent (100 mM Tris [pH 8.25], 2.9 mM $\gamma$-glutamyl-carboxy-3-nitro-4 anilide, and 100 mM glycylglycine) was added. After 30-min incubation at 37°C, GGT activity changed the solution color from clear to yellow. $\text{C. jejuni}$ 81-176 and NCTC 11168 were used as a positive and negative controls, respectively. A PCR was used for the detection of $\text{ansB(s)}$ and $\text{fucP}$. The PCR conditions for the detection of $\text{ansB(s)}$ were as follows: 1× PCR buffer (Thermo Fisher Scientific), 1 U DyNAzyme DNA polymerase (Thermo Fisher Scientific), 250 µM of each dNTP (Thermo Fisher Scientific), 0.5 mM of MgCl$_2$ (Sigma-Aldrich Finland Oy, Helsinki, Finland), 10 pmol of each primer, and 50 ng of genomic DNA. Primers used were ansBF: GGGGAATGGTAACTCCACAA and ansBR: CCTGCTATCCTTCCACCTGT. The cycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min. The products of $\text{ansB}$ with and without a secretion signal differed by only 40 bp; therefore, a 3% MetaPhor (Lonza, Fisher Scientific Oy) gel was used for visualization. $\text{C. jejuni}$ strains 81-176 and NCTC 11168 were used as positive and negative controls, respectively. The PCR conditions for $\text{fucP}$ were the same as those described previously.

8.4.2 Multilocus sequence typing (II and IV)

The MLST profiles of all of the barnacle geese isolates included in Study II and 48 of the chicken isolates (IV) were determined using a published PCR amplification-based MLST protocol. The sequences were analyzed with BioNumerics version 5.1 (Applied Maths, NV, Sint-Martens-Latem, Belgium), and STs and CCs were assigned in the $\text{Campylobacter}$ MLST database (http://pubmlst.org/campylobacter/). The MLST profiles of the remaining 233 chicken isolates were determined by NGS as described below. The assembled contigs were uploaded to the above-mentioned $\text{Campylobacter}$ MLST database, which automatically annotated loci and numbered alleles available in the bacterial isolates genome database (BIGSdb).

8.4.3 Pulsed-field gel electrophoresis (III and IV)

A total of 366 (IV) $\text{C. jejuni}$ chicken cecal isolates were subtyped using PFGE with $\text{SmaI}$ and/or $\text{KpnI}$ and named S1, S2, etc., and K1, K2, etc., respectively, as described by Hakkinen et al. The four chicken cecal isolates from 2012 included in Study III were only typed with $\text{KpnI}$ and interpreted by the method of Tenover et al.

8.4.4 Next-generation sequencing of $\text{C. jejuni}$ (II-IV)

The outbreak isolates (III: two human isolates IHV116292 and IHV116260 and one water isolate 4031) were subjected to WGS using Illumina HiSeq sequencing technology with 100 cycles paired-end reads and >200x coverage. In addition, a 5-kb mate-paired library was created for the water isolate 4031, also by Illumina HiSeq (BaseClear B.V., Leiden, the Netherlands). Finally, the seven barnacle geese isolates of the CCs ST-1034 CC and ST-702 CC and 233 chicken isolates were sequenced by Illumina HiSeq technology, paired-end reads, and ≈40x coverage (FIMM Institute for Molecular Medicine, Helsinki, Finland).

For all reads, the ConDeTri Perl script with default settings and a minimum read length of 75 nucleotides was used for trimming and filtering. For assembly in Study III, ABySS 1.3.5., with default settings, and MIRA were used, while Studies II and IV made use of ABysS only. The outbreak isolates were closed, and virtual genomes for the four chicken strains from Study III (6538/12, 6237/12, 6236/12, and 6497/12) were generated using Mauve with 4031 as a scaffold. The remaining genomes of Studies II and IV were kept as draft genomes (assembled contigs).
8.5 Antimicrobial sensitivity analysis (II)
A subsample (n=61) of the barnacle geese isolates, representing both years, all collection sites, and each ST identified at that site, was tested for antimicrobial susceptibility against ciprofloxacin, erythromycin, and tetracycline using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.\textsuperscript{322} \textit{C. jejuni} ATCC 33560 was used as a control. Epidemiological cut-off values (ECOFFs) from the European Committee on Anti-microbial Susceptibility Testing (EUCAST) for erythromycin (MIC $\leq 4$ mg/l), ciprofloxacin (MIC $\leq 0.5$ mg/l), and tetracycline (MIC $\leq 1$ mg/l) were used to classify the isolates as wild-type and non-wild-type (EUCAST; www.eucast.org).

The sequence of the QRDR region in the \textit{gyrA} gene was determined by PCR amplification followed by subsequent Sanger sequencing using the protocol of Griggs et al. (2005) for isolates (n=15) with a MIC of 1.0 mg/l for ciprofloxacin.\textsuperscript{72} Mutations in the \textit{gyrA} gene were detected by comparison with the published \textit{gyrA} sequences of \textit{C. jejuni} (GeneBank accession no. L04566.1). Isolates lacking these mutations were classified as sensitive for ciprofloxacin in the discussion, regardless of their MIC=$1.0$ mg/l for ciprofloxacin.

8.6 Population structure

8.6.1 Population diversity and differentiation (II and IV)

SID (II) and SDI (IV) were calculated to determine the ST diversity for each year and collection site (II) and the adjusted database (IV). Both index’s account for the abundance and evenness in a population, but the SID varies from 0 (no variation) to high values (even number of multiple STs), while the SDI lies between 0 (no variation) and 1 (maximum diversity). A diversity t-test was performed to evaluate significant difference in diversity indices, with $P \leq 0.05$ regarded as significant. Furthermore, the number of effective genotypes were calculated by the method described by Jost et al.\textsuperscript{296} All computations of diversity were performed in PAST version 2.17c (II) and 3.01 (IV).\textsuperscript{323}

In Study II and for the purpose of this thesis, Arlequin suite version 3.5\textsuperscript{324} was used to calculate the $F_{ST}$ for population differentiation between barnacle geese isolates collected in different years, and between chicken and barnacle geese populations collected during all years and separately for 2012. $P$-values $\leq 0.05$ were regarded as significant.

8.6.2 Bayesian Analysis of Population Structure (II)

BAPS version 5.3 was used to analyze the genetic population structure,\textsuperscript{290,325,326} using linkage clustering with the corresponding admixture model (100 Monte Carlo runs, 100 Monte Carlo reference samples using 10 iterations). Minimum population size was set to three, and $K$ was assumed to lie between two and ten to which STs were assigned after the highest posterior probability. An admixture inference threshold for possible recombination was set at $\leq 0.05$.

The input consisted of the concatenated allelic profiles of MLST data from Studies I, II, and IV in addition to earlier published material. The resulting database represented the total MLST-characterized \textit{C. jejuni} population in Finland and contained 210 \textit{C. jejuni} STs collected from 454 human patients, 283 chickens, 120 bovines, 57 natural waters, 19 zoo animals, and 185 wild birds (Table 1). The association between source and cluster was assessed by Pearson Chi-square or Fishers exact test as appropriate, and $P \leq 0.05$ were considered significant.

8.7 Comparative genomics

8.7.1 Building of database and core genome (II-IV)

In Study II, a database consisting of the full-genome sequences of seven barnacle geese and 78 \textit{C. jejuni} subsp. \textit{jejuni} isolates (GenBank and own collection), one \textit{C. jejuni} subsp. \textit{doylei} (GenBank), and one \textit{C. coli}
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AK Llarena

(own collection) strains isolated from humans, farm and wild animals, and surface water was created (Supplementary Table S1). The core genome of 583 genes was generated by gene findings and annotation using RAST, followed by determination of homologous groups by OrthoMCL version 2.0.2. MAFFT-FFT-NS-I version 7.0 and TranslatorX perl script were used to create a core genome multiple alignment, which was used in all comparative genomic analyses in Study II.

In Study III, the outbreak and chicken isolates were compared with the publicly available genomes of five additional ST-45 CC isolates, one ST-267 (ST-283 CC), and one ST-354 (ST-354 CC) originating from humans and poultry. The genomes were aligned using progressive Mauve. NCTC 11168 genome was used as a basis for assigning locus designations.

8.7.2 Phylogenetic analysis

8.7.2.1 Trees and dendrograms (I and II)
Dendrograms of the allelic profiles of the 710 isolates in Study I and the aligned core genomes of Study II (see Supplementary Table S1) were constructed using a distance-based matrix (neighbor-joining) with the tree-drawing tools PHYLIP and Phylodendron (I, available at http://pubmlst.org/analysis) or FastTree2 (II), applying the generalized time-reversible model. The iTOL online tool (http://itol.embl.de/itol.cgi) was used in both studies.

8.7.2.2 Networks (II and III)
Neighbor-Net graph and split decomposition were generated using SplitsTree4 from the core genomes of Study II (Supplementary Table S1) and for the core genome of the outbreak and chicken strains in Study III. In addition, a consensus network was constructed from ClonalFrame (see below) in Study II, also by using SplitTree4.

8.7.2.3 ClonalFrame (II and III)
ClonalFrame was used to reconstruct the clonal genealogy of 1) the aligned core genomes of the *C. jejuni* ssp. *jejuni* reported in Supplementary Table S1 in Study II and 2) the aligned core genome of the outbreak and chicken strains in Study III. In Study II, the ClonalFrame was run with 60,000 iterations, of which the first 50,000 were discarded and every following 10th iteration sampled. Two independent ClonalFrame Markov Chain Monte Carlo runs were performed, resulting in a posterior distribution of 2,000 samples (two runs of 1,000 samples each). This distribution was analyzed with a consensus network with mean edge weights at a threshold of 0.2 and an equal-angle split as implemented in SplitsTree4. In Study III, the clonal genealogy based on whole genome was reconstructed using ClonalFrame, with 10,000 burn-in iterations followed by 10,000 data collection iterations. The consensus tree represents data from three independent runs with 75% consensus required for inference of relatedness.

8.7.3 Comparative genomic analysis (III)
All genome comparisons were done using BLAST, usually the BLASTN function, and all atlases were generated using BLAST Ring Image Generator version 0.95 (BRIG). Synteny was checked using Mauve and Artemis Comparative Tool (ART). Polymorphisms were investigated by progressive Mauve, and classified into single-nucleotide polymorphisms (SNPs) and cluster of nucleotide polymorphisms (CNPs) based on their likely origin from mutations and recombination, respectively. A SNP was defined as a polymorphism occurring >200 bp from the next polymorphism in both directions. The definition of CNP in Study III was the occurrence of a group of ≥2 polymorphisms within the same range (i.e. 200 bp), also separated from the next polymorphism with >200 bp.
8.8 Statistical analysis (II-IV)

8.8.1 Descriptive statistics (II and IV) and test for independence (I, II and IV)
All descriptive statistics and cross-tabulations were done in IBM SPSS version 21 (International Business Machines Corp., Armonk, NY, USA). In Studies I and IV, Pearson Chi-square or Fisher’s exact test was used to assess the relationship between ST and CC with the three metabolic markers (independently or in combinations) and PFGE types, respectively. The same tests were used to test for independence in *C. jejuni* prevalence in barnacle geese from the variable year (including only sites tested both years: 1, 2, 3, 4, and 12) and between sites within one year (including all sites of collection) in Study II. P-values below 0.05 were regarded as significant.

8.8.2 Creating the adjusted database (IV)
In Study IV, an adjusted database was created to account for clustering of flocks within farms. Isolates with similar MLST and PFGE profiles collected from chicken batches slaughtered during the same rearing cycle (time interval = one week) were merged to account for one isolate. When no PFGE profile was available for two isolates of the same rearing cycle with similar MLST types, both isolates were included. This adjusted database was used in all analyses in Study IV, except the farm-associated description in which the full database was used (includes all isolates).

8.8.3 Logistic regression (IV and this thesis)
We used logistic regression to determine whether the occurrence of the most common CCs (ST-45 CC, ST-21 CC, and ST-677 CC) and ST-45 in chicken flocks was dependent on year and season of collection or the area in which the chickens were raised. The effect of season could be measured on ST-45 CC and ST-45 only, due to lack of sufficient isolates in the two remaining CCs.

In the logistic regression, the variable "Year" had five categories, one for each study year, while season was transformed into concatenated months, namely the variable "Period" dividing into five epidemiological relevant categories ("January to June", "July", "August", "September", and "October to December"). The different abattoirs were used as proxies for "Area" (three categories, "A", "B", and "C") since Finnish farms are geographically concentrated around their respective slaughterhouse.

The reference category was chosen to simplify the interpretation of the Odds Ratios (ORs). The model was fitted using a scale parameter estimated under the full model (procedure LOGISTIC, scaled using William’s method), and Hosmer and Lemeshow was applied to test goodness of fit. Interaction between variables was not assessed due to the low number of observations. Only results from logistic regression models that were statistically significant ($P < 0.05$) are reported.

In this thesis, logistic regression was applied to determine the effect of MLST and source on the occurrence of individual metabolic markers. Three models were built, with the presence or absence of GGT production, *ansB*(s), and *fucP* as dependent variables, and "CC" and "Source" as independent variables. The categories within each independent variable with enough isolates to be included in the models were ST-21 CC, ST-45 CC, and ST-677 CC and "Human", "Poultry", and "Bovine". All independent variables were entered simultaneously, and a cut-off value of $> 0.5$ for probability for group membership was used. The reference category was chosen to ease the interpretation of the ORs, and outliers (standard errors $> 2.0$) were dropped if they created significant associations in the model. Only models performing significantly better than the intercept-only model are reported, with the utility assessed by classification accuracy (25% classification improvement for the full vs. the intercept-only model).

8.9 Data deposition
The seven barnacle geese and outbreak-related isolates (human and chickens) were deposited to the European Bioinformatics Institute (EMBL) under the project numbers given in Supplementary Table S1 (II)
and PRJEB4165 (III). The genome of C. jejuni 4031 was submitted to EMBL with the accession number HG428754 (III).
8.10 Figure 1
Overview of the materials and methods used in this thesis. A) Study I, B) Study II, C) Study III and D) Study IV.
9. Results

9.1 Prevalence of C. jejuni (II and IV)

9.1.1 Barnacle geese (II)
Of the 924 samples collected in Study II, altogether 156 barnacle geese samples were positive for C. jejuni, resulting in an overall isolation rate of 16.9% CI[14.5%, 19.3%]. Significantly more C. jejuni was isolated in 2012 (23.1% CI[19.1%, 27.1%]) than in 2011 (11.5% CI[8.6%, 14.4%]) (Figure 2). In addition, the occurrence of C. jejuni varied significantly between collection sites in both 2011 and 2012 (P = 0.004 and P = 0.003, respectively), from no Campylobacter spp. feces at site 3 in either year to 38.5% occurrence on site 12 in 2012. Only three of 27 samplings were negative for Campylobacter spp..

9.1.2 C. jejuni on chicken farms and flocks (IV)
During the five study years a strong seasonal variation was seen in the frequency of C. jejuni isolation in chickens; the summer prevalence of C. jejuni-positive slaughter batches ranged between 5.0% and 6.6%, while no Campylobacter spp. were detected during the winter and spring months until 2008, after which the winter and spring prevalence ranged between 0.3% and 2.7%. This corresponds to an overall weighed average of 3.0% CI[1.8%, 4.2%] for the five study years (Figure 2).

Figure 2: C. jejuni prevalence of chickens and barnacle geese in the six study years. The barnacle geese were sampled only in 2011 and 2012. The 2011 chicken prevalence is included in this graph, but is not discussed further.

An average of 135.7 CI[125.9, 145.4] farms delivered chickens to slaughter each study year, and 37.0 CI[29.8, 44.2] of these delivered C. jejuni positive flocks, leaving the majority (72.2%) C. jejuni negative (Figure 3). A total of 118 farms delivered at least one positive batch during the five study years, and Study IV included 114 of these farms (96.6%). Most farms (53.1%) had only one positive batch during a year or was positive at one time-point during the study period (59.6%, Figure 3). Approximately half of the farms (48.2%) that delivered more than one positive batch within a year did so during the same rearing cycle (i.e. within one week), meaning that these farms had several halls in which C.
*jejuni* positive chicken flocks were raised simultaneously. Ten farms were responsible for delivering >2% of the *C. jejuni* positive flocks to slaughter (Farms A11, A12, A19, B1, B5, C4, C5, C8, C28, C38), and were referred to as “high-frequency farms”, even though no knowledge about the production volumes of these farms was available.

**Figure 3:** Clustered column graph for the number of farms delivering chicken batches to slaughter. The number of farms delivering *C. jejuni*-positive batches during each study year and the frequency of farms according to their rate of positive batches (X) delivered annually are also given. Numbers are from the full database.

9.2 Prevalence of *C. coli* in barnacle geese (II)
One *C. coli* isolate was recovered in each study year, which equals an annual *C. coli* prevalence of 0.2%. These isolates were not analyzed further.

9.3 GGT production, *ansB*(s), and *fucP* (I)
Of the 710 Study I isolates, 31.1% produced GGT, 49.3% carried *ansB*(s), and 30.3% were positive for *fucP*.

9.3.1 Association with multilocus sequence types (I)
The studied metabolic traits showed patterns of association with certain STs and CCs (Figure 4). The most common combination was production of GGT and *ansB*(s) presence and *fucP* absence. Exceptions to this pattern included *ansB*(s)-positive ST-677 CC, and *fucP*-positive ST-21 CC and ST-48 CC. ST-61 CC, ST-692 CC, ST-1034 CC, and ST-1332 did not carry any of studied metabolic traits.
The acquisition of metabolic traits split the ST-22 CC into two groups; ST-22 had both GGT production and *ansB*(s), while ST-1971 was positive for *ansB*(s) only. The ST-45 CC was rather complex, mainly due to the different metabolic profiles seen inside ST-45. Three metabolic profiles were evident for this ST: 1) GGT-production and *ansB*(s) presence, 2) *ansB*(s) presence, and 3) absence of all studied metabolic markers.
GGT production was highly linked to the presence of ansB(s), but the presence of ansB(s) did not require GGT production. Very few isolates were positive for all three metabolic markers, and the combination of fucP-positive together with the two other traits was rare.

9.3.2 Association with source (I)
Poultry and bovines were positively (P = 0.012) and negatively (P < 0.001) associated, respectively, with C. jejuni carrying the combination of GGT production, ansB(s) presence, and fucP absence (Figure 5). The environmental isolates, i.e. water, wild bird, and zoo animal isolates, were associated with ansB(s) presence only. Human isolates were evenly distributed across a variety of metabolic markers and combinations thereof. All associations were tested with the Pearson Chi-square or Fisher’s exact test.

Figure 4: 100% stacked bar chart illustrating the occurrence of three metabolic markers GGT production, ansB(s), and fucP according to the eight major CCs. No bar for ST-61 CC reflects the absence of all studied metabolic markers in this CC. See text for details.

Figure 5: Clustered bar chart showing the distribution of the three metabolic markers in human (H), bovine (B), poultry (P), and environmental (E) sources. Significant associations are marked with an asterisk. See text for details.
9.3.3 Association with CC and source (I)
To further assess the relationship between the metabolic markers, MLST, and source, logistic regression was applied to account for possible confounding effects due to population structure. One model per metabolic marker was built, and only models enhancing the accuracy of prediction by 25% are reported. No outliers were dropped in the model for GGT production or \( \text{ansB} \). In the \( \text{fucP} \) model, seven outliers fulfilled the criteria for elimination.

As seen in the naïve independency tests, ST-45 CC was strongly associated with the production of GGT and isolates of this clonal complex were 83.2% and 70.6% (both \( P < 0.0001 \)) more likely than isolates of the ST-21 CC and ST-677 CC to be GGT-positive. No difference between isolates of the ST-21 CC and ST-677 CC with regard to GGT production was noted.

The presence of \( \text{ansB} \) was highly associated with ST-677 CC, which was 25.7% (\( P < 0.0001 \)) more likely to be positive for this trait than ST-45 CC, which in turn had 85.5 (\( P < 0.0001 \)) higher odds of being \( \text{ansB} \)-positive than ST-21 CC. Safe to say that most ST-21 CC was \( \text{ansB} \)-negative, with the exception of three \( \text{ansB} \)-positive isolates.

The presence of \( \text{fucP} \) was separated (almost) completely between the CCs, so the estimate bears evidence of numerical problems and the results of the logistic regression should be interpreted with care. However, the ST-21 CC was almost completely \( \text{fucP} \)-positive (100% more likely than ST-45 CC and ST-677 CC to be \( \text{fucP} \)-positive). The source categories were equally distributed and did not significantly influence the presence or absence of the studied markers.

9.4 MLST typing of \textit{C. jejuni} populations (II and IV)

9.4.1 \textit{C. jejuni} in barnacle geese (II)
Of the 154 \textit{C. jejuni} isolates, a full MLST profile was achieved for 150 (96.2%). These were assigned to 33 STs, clustering into seven CCs, of which ST-1034 CC and ST-702 CC were most common (Figure 6). Over half of the isolates (57.3%) were assigned to four STs, while 15 STs (45.5% of the STs and 10% of the isolates) were observed only once.

The ST-based diversity as measured by SID was significantly higher in 2012 than in 2011 (2.57 vs. 1.98), although the two \textit{C. jejuni} populations were highly similar (\( F_{ST} = 0.005 \)). A site-dependent variance in diversity was also observed; for instance, site 12 was significantly more diverse in 2012 than in 2011. Despite the diversity, ST-702 CC and ST-1034 CC were consistently observed, and these CCs were isolated from all sample sites. Furthermore, the two central STs of these CCs, ST-702 and ST-1034, were, together with ST-692 and ST-1269, the only STs repeatedly isolated from the same sample site. However, most isolates behaved rather sporadically, occurring only once during a season and with no reappearance at the following sampling time.
9.4.2 *C. jejuni* in Finnish chickens (IV)

The Finnish chicken isolates were assigned to 63 STs, 28 of which were UA (Figures 6 and 7). The remaining STs and isolates (88.7%) clustered to 12 CCs. The adjustment of the database resulted in 273 isolates approximately equally distributed between the study years (range 47-58). The SDI was 0.87 CI\(_{95}\%\) [0.86, 0.91] for all years, based on ST distribution, even though the frequency of genotypes was highly skewed, with three and four of the CCs and STs accounting for 73.5% and 52.5% of the isolates, respectively. Of the 63 STs, 39 (61.9%) were observed only once, indicative of the existence of a highly volatile *C. jejuni* population segment in Finnish chickens.

Logistic regression was carried out to investigate the effect of year, season, and site of slaughter on the occurrence of ST-45 CC, ST-21 CC, ST-677 CC, and ST-45 (Table 2). According to this analysis, the odds of an isolate being of ST-45 CC was higher in Area A and C compared to Area B, but this CC was evenly distributed among the study years and seasons. On the other hand, the odds of isolating a ST-21 CC were higher in 2008 and Areas B and C than in 2004 and Area A respectively. No effect of any variable was seen on ST-677 CC, while ST-45 was more common from September to December than in spring.

9.4.3 Comparison of *C. jejuni* population in chickens and barnacle geese (II and IV)

The *C. jejuni* population in the barnacle geese population was less diverse than the one in chickens (SID\(_{barnacle\ geese}\) = 2.62, SID\(_{chicken}\) = 2.97, \(P = 0.015\)). The effective numbers of *C. jejuni* STs in barnacle geese and chickens were 19.5 and 13.7, which means that *C. jejuni* in barnacle geese were approximately 30% less diverse compared to the isolates collected from chickens. However, when only considering 2012 (when both species were sampled), the barnacle geese *C. jejuni* were more diverse than chicken *C. jejuni* (SID\(_{barnacle\ geese}\) = 2.53, SID\(_{chicken}\) = 1.98, \(P = 0.008\)). The genetic distance was noticeable, \(F_{ST} = 0.363\), when all isolates were included in the analysis, and even higher when just considering 2012 (\(F_{ST} = 0.470\)). On CC level, most CCs seem to be fairly separated by source (Figure 7). One exception was the ST-1332 CC, which was equally divided between chickens and barnacle geese.

The majority of STs were found in one source only (77 STs). Eight STs were shared between the two animal populations, namely ST-45, ST-230, ST-583, ST-692, ST-993, ST-1326, ST-1332, and ST-4307 (Figure 8), four of which clustered in the ST-45 CC. The shared ST constituted 23% and 43.6% of the isolates and 26.7% and 12.7% of the STs collected from barnacle geese and chickens, respectively. In 2012, only ST-45, ST-230, and ST-583 were shared between the two reservoirs.
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AK Llarena

Figure 6: 100% stacked bar chart of the CC distribution in barnacle geese and chickens during all study years. The dominance of ST-45 CC in chickens and ST-702 CC and ST-1034 CC in barnacle geese is clearly illustrated.

Figure 7: 100% stacked bar chart comparing the proportions of isolates with different CCs originating from chicken and barnacle geese (II and IV). The numbers are based on all years of collection (2004, 2006-2008, 2011, and 2012), and for the chicken isolates the adjusted database was used to account for clustering of flocks within a farm.

9.5 PFGE types of the *C. jejuni* population (III and IV)

In Study III, the outbreak and chicken-derived strains, all ST-45, were PFGE typed with *KpnI*. The human isolate IHV116260 and the water isolate 4031 had identical PFGE profiles, while the human isolate IHV116292 differed from the other two outbreak strains by three bands. According to the classification criteria of Tenover *et al.*, the IHV116292 isolate was considered closely related and part of the outbreak in the original
The four chicken isolates included had either an identical PFGE profile to the water isolate 4031 (6538/12; 6237/12; 6236/12) or differentiated by two bands (6497/12).

In Study IV, C. jejuni from chickens assigned to 226 and 32 Smal and KpnI types, respectively, as assessed from the adjusted database. The most common types were K36, S54, S64, S7, S4, S66, S12, S55, S78, and S74, but none of these types accounted for more than 7.8% of the isolates. This was due to the high discriminatory power of PFGE, which subdivided the CCs and STs. For instance, ST-45 CC accounted for 40 different PFGE types. Some patterns of association between MLST and PFGE were also noted: chicken ST-45 CC isolates associated with PFGE types S4, S54, S55, S66, and S74, while ST-677 CC was associated with S64 and S78. The ST-45 was highly diverse, and accounted for 23 PFGE types, of which S4, S55, S66, and S7 were significantly associated with this ST.

Figure 8: 100% stacked bar chart showing the contribution of barnacle geese and chicken to the eight shared STs (ST-45, ST-230, ST-583, ST-692, ST-993, ST-1326, ST-1332, and ST-4307) collected during the study years.

9.6 Antimicrobial susceptibility of C. jejuni isolated from barnacle geese (II)

In Study II, altogether 61 C. jejuni isolates were tested for their MICs to ciprofloxacin, tetracycline, and erythromycin. According to their ECOFF values, all isolates were erythromycin wild-types, while 75.4% and 95.1% were ciprofloxacin and tetracycline wild-types, respectively. The isolates (n=15) that were non-wild-types for ciprofloxacin all had MIC=1.0 mg/l, but lacked the typical mutation in the QRDR of the gyrA gene.72 The three isolates (ST-45, ST-1268 and ST-1332) of non-wild-types for tetracycline all had MIC>32 mg/L.
Table 2: Results of the multiple regression analysis used to predict the probability that a *C. jejuni* isolate belonged to the ST-21 CC, ST-45 CC, ST-677 CC, and ST-45. Significant ORs ($P \leq 0.05$) are depicted in boldface.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>CC</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ST-45 CC</td>
<td>ST-21 CC</td>
</tr>
<tr>
<td>Year</td>
<td>OR w IC95%</td>
<td>P</td>
</tr>
<tr>
<td>2004</td>
<td>0.15 [0.03, 0.86]</td>
<td>0.03</td>
</tr>
<tr>
<td>2006</td>
<td>0.65 [0.28, 1.50]</td>
<td>1.30 [0.31, 5.42]</td>
</tr>
<tr>
<td>2007</td>
<td>0.44 [0.19, 1.03]</td>
<td>1.12 [0.27, 4.75]</td>
</tr>
<tr>
<td>2008</td>
<td>0.67 [0.29, 1.46]</td>
<td>4.58 [1.37, 15.3]</td>
</tr>
<tr>
<td>2012</td>
<td>1.14 [0.50, 2.61]</td>
<td>1.07 [0.30, 0.86]</td>
</tr>
<tr>
<td>Area</td>
<td>0.03</td>
<td>0.001</td>
</tr>
<tr>
<td>A</td>
<td>2.74 [1.30, 5.76]</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>13.5 [3.30, 58.2]</td>
</tr>
<tr>
<td>C</td>
<td>2.17 [1.03, 4.32]</td>
<td>4.77 [1.23, 18.5]</td>
</tr>
<tr>
<td>Period</td>
<td>0.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Jan-June</td>
<td>1</td>
<td>2.18 [0.64, 7.39]</td>
</tr>
<tr>
<td>July</td>
<td>2.22 [0.75, 6.55]</td>
<td>2.18 [0.64, 7.39]</td>
</tr>
<tr>
<td>August</td>
<td>1.24 [0.41, 3.68]</td>
<td>0.58 [0.15, 2.14]</td>
</tr>
<tr>
<td>Sept</td>
<td>2.01 [0.61, 6.60]</td>
<td>4.09 [1.09, 15.3]</td>
</tr>
<tr>
<td>Oct-Dec</td>
<td>1.81 [0.48, 6.81]</td>
<td>5.29 [1.24, 22.6]</td>
</tr>
</tbody>
</table>
9.7 Analysis of population structure (I-III)

9.7.1 Bayesian Analysis of Population Structure (II)
In Study II, BAPS was performed to investigate the relationship between the isolates of barnacle geese origin and those of other sources. A total of 1,118 isolates were included in the analysis, and the concatenated MLST profiles were used as input. These isolates clustered into five groups, hereafter referred to as BAPS clusters. The majority of the isolates from barnacle geese and other wild bird species grouped into BAPS cluster 4 together with half of the bovine isolates. The human isolates grouped into BAPS cluster 1 (solely human isolates) and 2 (together with the chicken-derived isolates). Isolates of STs collected from human and barnacle geese rarely shared a cluster, and when they did most were of the ST-45 CC (87.5%).

9.7.2 Phylogenetic analysis (I-III)
In Study I, the phylogenetic relationship between the 710 isolates was investigated in a neighbor-joining unrooted dendrogram based on their MLST allelic profiles. The production and presence of GGT, \(\text{ansB}(s)\), and \(\text{fucP}\) were plotted on their respective ST together with the sources to reveal potential patterns of association. In this dendrogram, the metabolic markers reconstructed the clonal frame in the majority of cases, which is often seen when a trait is vertically inherited. This correlates well with the notion that these metabolic markers are associated with CCs and STs.

Three separate methods were used to reconstruct the phylogenetic relationship between the isolates from barnacle geese and other sources: a maximum likelihood dendrogram, a neighbor-net graph, and ClonalFrame. In the maximum likelihood dendrogram, the barnacle geese formed a monophyletic group together with a chicken isolate of ST-4001. This barnacle geese group and its sister group (ST-573 and ST-6555) shared a recent ancestor with a bigger clade containing CCs frequently associated with farm animals and humans, including ST-21 CC, ST-61 CC, and ST-48 CC. However, conflicting signals and inconclusiveness about recombination necessitated the building of a neighbor-net graph. The splits attained a reasonable tree-like structure, and the barnacle geese isolates were separated from the other strains by a long split supporting their monophyletic relationship. Even so, conflicting signals remained and left the clonal relationship of ST-573 and ST-6555 uncertain. Further attempts to clarify this relationship with the use of ClonalFrame and a consensus network were unsuccessful. However, the clonal frame of the barnacle geese isolates was consistent with that inferred by maximum likelihood.

The clonal relationship between the outbreak isolates and the chicken isolates was further investigated by ClonalFrame in Study III. The water strain 4031 and the human isolate IHV116260 and all of the chicken strains were monophyletic, while the IHV116292 was of a separate branch by the root. In the split decomposition analysis, the genetic distance
between the chicken isolates and 4031 was smaller than between the chicken isolates and IHV116292 (0.0061 vs. 0.0175).

9.8 Comparative genomics (III)
The use of a paired-end and 5 kb mate-paired library resulted in a complete assembly of the water isolate 4031. The human isolates IHV116260 and IHV116292 were sequenced by paired-end only, assembled and mapped against 4031. The contigs of the human IHV1162060 isolate matched those of the water isolates perfectly, and these isolates are therefore, for all intents and purposes, the same isolate and will be referred to as only 4031 in the following.

The IHV116292 was highly similar to the water isolate. Significant variation was detected inside and directly downstream of the CJIE2. The CJIE2 of IHV1116292 had 84.3% nucleotide similarity with the CJIE2 of 4031. The central 22 kb region was the most divergent section, and contained an extra KpnI restriction site responsible for the divergent PFGE pattern observed. A further difference between the IHV116292 and the water 4031 included the region downstream of ybbB; 4031 had a duplicate of Cj0967-Cj0975 inserted here, while IHV116292 had an ammonium transporter instead. Furthermore, the number of SNPs and CNPs between these two isolates was more abundant than that observed between the IHV116260 and 4031 (see Table 3).

The four chicken ST-45 isolates were included in Study III to calibrate the interpretation of the comparative genomics results obtained between the outbreak isolates. The chicken isolates were isolated 12 years later than the outbreak isolates. In addition, three of the chicken isolates (6236/12, 6237/12, and 6538/12) had similar KpnI PFGE type as 4031, while one differed by two bands (6497/12). The chicken and outbreak strains were all very similar in BLASTN comparison. Differences were located in the known variable genome regions; LOS, flagellar loci, and CJIE2. Furthermore, the chicken strain 6497/12 lacked CJIE2 entirely and the SNPs analysis between 6236/12 and 4031, excluding CJIE2 and CNPs, revealed 64 SNPs.

CJIE2 sequences of the chicken isolates were also compared with each other and the outbreak strains. 6236/12 and 6237/12 originated from the same farm and rearing cycle and had an identical CJIE2 sequence. The CJIE2 in a third isolate (6238/12) differed from the two former isolates in three positions. Furthermore, CJIE2 of the chicken strains was more similar to that of IHV116292 than 4031. Such a finding, despite a closer inferred relationship between chicken strains and 4031, implies the occurrence of recombination in CJIE2, disrupting the clonal frame.
### Table 3: Overview of genomic differences between the outbreak strains included in Study III

<table>
<thead>
<tr>
<th>Isolate</th>
<th>4031</th>
<th>IHV116260</th>
<th>IHV116292</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromosome (bp)</strong></td>
<td>1, 669, 329</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- coding area</td>
<td>94.28%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CDSs (n)</strong></td>
<td>1, 697</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- function predicted (%)</td>
<td>73%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MLST</strong></td>
<td>ST-45, ST-45 CC</td>
<td>ST-45, ST-45 CC</td>
<td>ST-45, ST-45 CC</td>
</tr>
<tr>
<td><strong>PFGE</strong></td>
<td>Same</td>
<td>Same</td>
<td>Different</td>
</tr>
<tr>
<td><strong>Metabolic markers</strong></td>
<td>No GGT production ansB(s)-neg fucP-neg</td>
<td>No GGT production ansB(s)-neg fucP-neg</td>
<td>No GGT production ansB(s)-neg fucP-neg</td>
</tr>
<tr>
<td><strong>LOS class</strong></td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td><strong>Integrated elements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Vestigial Mu-like phage</td>
<td>4807 bp upstream of cipA</td>
<td>4807 bp upstream of cipA</td>
<td>4807 bp upstream of cipA</td>
</tr>
<tr>
<td>- CJIE2</td>
<td>36,567 bp 52 ORFs (three with function predicted)</td>
<td>36,567 bp 52 ORFs (three with function predicted)</td>
<td>36,058 bp 49 ORFs Extra KpnI- restriction site</td>
</tr>
<tr>
<td>Ammonium transporter downstream of ybbB</td>
<td>Lacking</td>
<td>Lacking</td>
<td>Present</td>
</tr>
<tr>
<td>Cj0967-Cj0975-homolog cluster</td>
<td>Present</td>
<td>Present</td>
<td>Lacking</td>
</tr>
<tr>
<td>SNP analysis**</td>
<td>Original</td>
<td>+ three SNPs</td>
<td>+69 SNPs*</td>
</tr>
<tr>
<td>CNP analysis**</td>
<td>Original</td>
<td>None</td>
<td>+ nine CNPs affecting nine genes</td>
</tr>
</tbody>
</table>

CDS: coding DNA sequences
Same: IHV116260 and 4031 were of the same KpnI PFGE type
Different: KpnI PFGE profile differed by three bands from 4031 and IHV116260.
Vestigial Mu-like phage: CJIE1
bp: base pairs
*Excluding the CJIE2 and CJ0967-CJ0975 duplicate downstream of ybbB in 4031 and IHV116290
**See Materials and methods for definition
10. Discussion

10.1 Evaluation of the three metabolic traits of GGT production, \textit{ansB}(s), and \textit{fucP} as candidate host adaption elements compared with MLST (I)

Successful source attribution is dependent on genotyping methods that carry a strong host signal and are characteristic of the transmission pathway considered. In Study I, the potential host association of three metabolic attributes (GGT production, \textit{ansB}(s), and \textit{fucP}) was investigated and compared with MLST types using three different methods; univariate analysis (Fisher's exact test and Pearson's Chi-square), neighbor-net dendrogram, and further extended by multivariate analysis performed with simultaneous binary logistic regression. We found a strong link between the metabolic markers and their combinations, and MLST types, as also reported in other studies. No effect of host or source was seen in the multivariate analysis including isolates of ST-21 CC, ST-45 CC, and ST-677 CC (67.3\% of our isolates).

The \textit{ggt}-gene is part of the accessory genome of \textit{C. jejuni} and is located close to what is believed to be a recombination hotspot. A recent paper by Skarp-de Haan \textit{et al.} (2014) attempts to reconstruct the evolutionary history of this gene in \textit{e}-proteobacteria, suggesting that its origin is a horizontal gene transfer (HGT) event occurring between the ancestors of \textit{Helicobacter} and \textit{Campylobacter} species. The \textit{ggt}-gene was subsequently vertically transmitted to \textit{C. coli} and \textit{C. jejuni}.

The evolution of the \textit{ggt}-gene in \textit{C. jejuni} was illustrated in a rooted maximum likelihood tree and corroborates well with the topology of our neighbor-joining dendrogram (although the latter is unrooted), offering a possible explanation for the observed distribution pattern of GGT seen in Study I. A gradual loss of \textit{ggt} during evolution was reflected by the reduced occurrence of this gene in lineages further from the root. Indeed, the GGT producers of our study, ST-42 CC, ST-45 CC, ST-283 CC, and ST-1275 CC, were all located close to the root of the maximum likelihood tree, while GGT-negative strains, such as ST-21 CC, ST-48 CC, and ST-206 CC, were located in a different cluster further away from the root. This phenomenon is especially well demonstrated for ST-22 CC, divided into the GGT-positive and GGT-negative ST-22 and ST-1947, respectively, indicating \textit{ggt}-loss in the ST-1947.

GGT production was associated with the presence of \textit{ansB}(s) \textit{(P < 0.001)}, and \textit{ansB}(s)-negative GGT producers were rarely found (6.3\% of isolates). However, GGT-negative and \textit{ansB} (s)-positive isolates were common (40.6\% of isolates). Possibly, GGT and \textit{ansB}(s) were acquired together from a common ancestor, but due to stronger selective pressure against \textit{ggt}, \textit{ansB}(s) was preserved while \textit{ggt} was exposed to the above-described gene loss. This is demonstrated nicely in the ST-45, in which 38.2\% of isolates are positive for both GGT production and \textit{ansB}(s), 19.7\% carry \textit{ansB}(s) only, and 37.6\% are negative for all traits.
Another striking pattern is the global occurrence of the *fucP*-gene in the ST-21 CC. One possible explanation of the *fucP* origin could be an HGT event in the ST-21 CC ancestor, with subsequent vertical transmission of the gene to its descendants. In contrast to the *ggt*-gene, there is no evidence of gene loss along this lineage. This CC is, together with ST-45 CC, a generalist,\textsuperscript{57,59} which is reflected by the recovery of this CC from all studied reservoirs, implying that *fucP* is more associated with the lineage (ST-21 CC) than the host. *FucP* is present in several other lineages outside the ST-21 CC, for instance, in the ST-4919 and ST-2611, but not in the monophyletic way as in ST-21 CC. The same is also evident for GGT-production, which is present in, for instance, ST-3809. An HGT event of *fucP* and *ggt* from ST-21 CC or ST-45 CC, respectively, could explain the sporadic occurrence of these genes in these isolates. Indeed, ST-21 CC and ST-45 CC recombine frequently with other specialist lineages, enabling these strains to acquire the metabolic markers when needed in their niche.\textsuperscript{53}

The isolates were either GGT- or *fucP*-positive, but rarely both (1.3% of the isolates), as noted in earlier studies.\textsuperscript{67,336,338} ST-45 CC and ST-21 CC readily recombine \textit{in vitro}, but no evidence for homologous recombination between these lineages in nature has been found.\textsuperscript{53} ST-45 CC accounted for 71.0% of the GGT-positive isolates, and the cryptic ecology of these isolates could explain the exclusive nature of these two traits.

Earlier studies have characterized the metabolism-associated markers examined here as important traits in colonization,\textsuperscript{65,68} invasion,\textsuperscript{66,67} and clinical outcome,\textsuperscript{338} and have implied that they might be essential for colonization of certain hosts.\textsuperscript{69} For example, Gonzalez \textit{et al.} noted that *ggt*-positive isolates were more common in chickens and humans than in bovines, reflecting the possible necessity of this gene product for chicken colonization.\textsuperscript{89} Zautner \textit{et al.} proposed *fucP* to be bovine-associated due to the linking of this trait to the livestock-associated gene cluster CJ1321-CJ1326.\textsuperscript{258,336} However, we found no effect of source on the occurrence of the studied genetic markers in our multivariate analysis where the effect of population structure (MLST) was accounted for, which is probably the reason behind the discrepancy between our results and those of Gonzalez \textit{et al.} (2009) and Zautner \textit{et al.} (2012). In fact, we noted the presence of all combinations of the studied metabolic traits in all hosts, implying that no specific metabolic factor determines colonization or infection for at least ST-45 CC, ST-21 CC, and ST-677 CC, concurring with earlier findings.\textsuperscript{57,90} Feodoroff \textit{et al.} (2010) found that GGT production was more common in human-derived \textit{C. jejuni} acquired in Finland than in imported \textit{C. jejuni} isolates, which tended to be *fucP*-positive. No MLST data were available in the study of Feodoroff \textit{et al.} (2010), but McCarthy \textit{et al.} showed that ST-45 CC is more common in Finnish human domestic campylobacteriosis cases than in \textit{Campylobacter} cases abroad\textsuperscript{339} so the linkage between metabolic markers and origin of infection is probably through the MLST association.
In conclusion, we found no evidence for a host association of the three studied metabolic markers and these are therefore unsuitable as the sole genotypic method in determining source attribution. However these metabolic genes may still play a role in the metabolism and host adaption of C. jejuni, especially in more specialized lineages not investigated in the multivariate analysis.

10.2 Population structure of C. jejuni in wild and domestic birds, as exemplified by wild geese and broiler chickens (II and IV)

The barnacle goose population in the Helsinki area is big, consisting of both Arctic and Baltic breeders during spring, summer, and autumn. Barnacle geese began to breed in Finland in the 1990s, and today, approximately 1300 couples nest in the Helsinki area (www.ymparisto.fi). In addition, the number of birds temporarily increases to approximately one million during the spring and autumn migrations. Wild birds are considered to be a natural reservoir for Campylobacter spp., and since barnacle geese reside in urban areas, recreational parks, and public beaches, investigation of the potential threat posed by these birds to public health is warranted. During our study we found that a significant proportion (16.9%) of the barnacle geese in Helsinki was colonized by C. jejuni. Nevertheless, the C. jejuni population that colonized barnacle geese was in most cases both genotypically and phylogenetically different from the C. jejuni population found in human patients and agricultural animals. However, as some ST types, such as ST-45, were similar, fecal contamination from barnacle geese might be an underreported source for human infections.

The prevalence of C. jejuni in barnacle geese concatenates well with earlier reports on Campylobacter spp. occurrence in wild geese. Furthermore, the overrepresentation of C. jejuni relative to C. coli seems to also be quite typical for geese, and it is also noted in other wild bird species. We found more C. jejuni in 2012 than in 2011, possibly reflecting a temporal variation in prevalence due to the earlier sampling time in 2012 (started in June vs. July). Sampling of younger birds and differences in prevalence due to seasonal variation in C. jejuni carriage could have generated more C. jejuni-positive samples in 2012. Furthermore, the summer of 2011 was drier and warmer than in 2012 (http://en.ilmatieteenlaitos.fi/statistics-from-1961-onwards), facilitating rapid environmental die-off of C. jejuni, with subsequent decreased exposure of geese to the C. jejuni organisms. Since population diversity is expected to increase as more isolates are obtained, the increased diversity of the 2012 samples relative to 2011 may be a consequence of the larger study population.

The prevalence of C. jejuni in chicken batches was constantly low during the study years, ranging from 5.0% to 6.6% during summer and from 0.0% to 3.2% during winter. The seasonal variation in C. jejuni occurrence in chickens is well established, and various
theories have been presented as explanations (see Introduction). The low prevalence in Finland is most likely due to a combination of good biosecurity, including the ban on thinning, and a favorable cold climate. 171,341

The chicken and barnacle geese isolates consisted of 63 and 33 STs, respectively. The population structure of C. jejuni in these host species had similar features; the populations in both species were divided into species-specific, generalist, agricultural, and “singleton” subpopulations. However, the relative sizes of these subpopulations within their respective reservoirs varied. In barnacle geese, ST-702 CC and ST-1034 CC constituted the majority of the isolates (59.8%) and were repeatedly isolated at different sample sites at most time-points (14 and 13 of 18 time-points, respectively), suggesting a possible adaption of these CCs to the barnacle geese reservoir. In addition, representatives of these CCs formed a monophyletic clade in all phylogenetic analysis, consistent with a host-specific and separate evolution in the barnacle geese reservoir. These CCs have also been linked to geese earlier. 172,236,237,342 Summarized, ST-702 CC and ST-1034 CC are probably a species-specific subpopulation in barnacle geese, constituting the largest groups of C. jejuni recovered from the geese. This concatenates well with the findings of Griekspoor et al. (2013) that the bird species itself determines the resident C. jejuni population. 229 In chickens, no isolates of the proposed chicken-specific ST-257 CC (see Introduction) were recovered in our data set. However, the ST-677 CC was the second most common CC (10.6%), and this lineage might be especially well adapted to Nordic chickens, 106,180 and could therefore be interpreted as a species-specific subpopulation. In addition, Sheppard et al. (2014) identified seven possibly chicken-adapted CCs (ST-257 CC, ST-283 CC, ST-353 CC, ST-354 CC, ST-443 CC, ST-573 CC, and ST-661 CC) 53 of which ST-283 CC and ST-353 CC were represented with 13 isolates in our chicken collection. However, taken together, these CCs represented a smaller proportion of the chicken isolates than the geese-associated CCs in barnacle geese.

The major subpopulation in the chicken isolates was the generalists, including the agricultural generalist. On average, 63% of the isolates were of the ST-45 CC or ST-21 CC, and 33% were assigned to ST-45. These lineages are often recovered from different hosts and environmental sources, which is typical for generalists. 53,57,176,343 Such a subpopulation was also identified in the barnacle geese as ST-45 CC, but only 12% of the geese isolates assigned to this clonal complex, and no ST-21 CC was recovered.

The occurrence of singletons was approximately the same among chicken and barnacle geese isolates (9.2% and 6%, respectively). The infrequent detection of these STs in their respective reservoirs might be due to their rarity in the environment or their lower colonization potential.

The exact reasons behind the high frequency of host-specific and generalist C. jejuni lineages in geese and chickens, respectively, are not known. The simplest explanation is the hypothesis that C. jejuni has colonized chickens more recently than geese, and that the
organism has not yet had time to adapt sufficiently to chicken. This would lead to the lack of a host signature in chicken-derived C. jejuni, with subsequent dominance of generalist lineages. However, Sheppard et al. (2014) rejected this hypothesis based on the following two findings: 1) The generalist lineages were just as genetically diverse as the more specialist ones and 2) The generalists seemed to be as good colonizers, if not better, than the specialist lineages, reflecting an independent ecological strategy. Earlier, Sheppard et al. proposed and has subsequently been supported by Dearlove et al. that the generalist lineages came about through extensive horizontal gene transfer between C. jejuni both within the same and different host species. Due to the clear advantages for a generalist lifestyle in the agricultural niche, where animals of different species live in high numbers and in close proximity to each other, these lineages quickly gained dominance. However, such HGT events are probably infrequent in geese since wild birds live in completely different environments and exhibit dissimilar lifestyles to chickens. This may have resulted in wild birds exhibiting a stronger host signature, as described by Sheppard et al. and Griekspoor et al. as well as in the present study.

10.3 Similarity between C. jejuni colonizing barnacle geese and agricultural animals and infecting humans, with a special focus on chickens (II, and IV)

The C. jejuni population in chicken (19.5 effective genotypes) was significantly (\(P = 0.015\)) more diverse than the C. jejuni population in barnacle geese (13.7 effective genotypes). However, the diversity is generally higher in a bigger sample, and when comparing the time-period both species were sampled (year 2012), the C. jejuni population of barnacle geese was more diverse than that of chickens (eight vs. four effective genotypes, \(P = 0.008\)). This correlates well with other studies finding a higher level of diversity in wild birds than in domesticated birds. Higher C. jejuni exposure from various sources and more frequent carriage of multiple strains in wild birds coupled with the use of genetically similar birds raised in high-density flocks in a closed environment in chicken production might help explain some of the observed differences in diversity.

We found little evidence for potential cross-contamination between chickens and barnacle geese. A limited number of shared genotypes was noted; of the 85 different STs collected during Studies II and IV, only eight were shared between chickens and barnacle geese (ST-45, ST-230, ST-583, ST-692, ST-993, ST-1326, ST-1332, and ST-4307), and in 2012, only ST-45, ST-230, and ST-583 were common between the two reservoirs. The high F\(_{ST}\) values, grouping in different BAPS clusters, and clustering of typical C. jejuni from geese in a monophyletic clade on the ClonalFrame argue against a common C. jejuni population mixing freely between chickens and barnacle geese.

We also compared the barnacle geese isolates with C. jejuni of human, water, bovine, and zoo animal sources using BAPS. Human and barnacle geese isolates grouped into two different BAPS clusters and were also separated on the ClonalFrame, implying that barnacle geese are not a major source for human campylobacteriosis. However, the
barnacle geese isolates did share a BAPS cluster with isolates from the bovine reservoir, indicating the possibility that one species acts as a reservoir for the other. But the ClonalFrame topology makes the occurrence of gene flow or horizontal gene transfer or the existence of a shared source between the bovine and barnacle geese hosts unlikely. Rather, the shared BAPS cluster is probably a result of the small genetic distance existing between isolates collected from geese and bovines. For instance, isolates of the ST-48 and ST-61 (typical bovine STs) were separated from the geese clade by only 4.6 substitutions per 100,000 bp in the maximum likelihood dendrogram. It is therefore plausible that BAPS lacked sufficient power to differentiate between barnacle and bovine isolates based on MLST, and whole-genome sequencing (WGS) was therefore necessary to infer the correct population structure.

The shared genotypes between barnacle geese and other reservoirs warrant consideration. The shared STs between chickens and barnacle geese during 2012 imply barnacle geese as a possible source for *C. jejuni* transmission to chicken flocks that year. Indeed, studies have indicated migrating birds as a risk factor for transmission of *C. jejuni* to chicken flocks\(^{206}\), but since Finnish chickens and Helsinki barnacle geese do not reside in the same region of the country, this risk of transmission is probably minimal. Also, 18 barnacle geese isolates assigned to two STs commonly found in human patients (ST-45 and ST-230), indicating barnacle geese as a possible campylobacteriosis source. However, the lack of geese-specific genotypes like ST-702 CC and ST-1034 CC in the chicken reservoir or human patients argues for transmission from the agricultural and human niche to the wild birds, rather than vice versa. Furthermore, all of the shared genotypes were of the ST-45 CC, a known generalist, and the origin of this CC in chickens and humans is especially difficult to trace due to the eroded host-signal and ubiquitous nature of this lineage.\(^{343}\) Summarized, even though barnacle geese could be the origin of *C. jejuni* transmission and infection to chicken flocks and humans, this reservoir probably plays a minor role in the chicken and human *C. jejuni* epidemiology.

To describe a potential correlation between the antimicrobial susceptibility patterns of barnacle geese and agricultural animals, a representative collection of barnacle geese isolates was tested against a panel of commonly used antimicrobials. All isolates were wild-types for the tested antimicrobials, except for three non-wild-types against tetracycline. Since barnacle geese are migratory to central and northern Europe, strain acquisition in the winter harboring areas could have resulted in a higher level of resistance than that seen in Study II.\(^{77}\) The lack of transfer of resistant *C. jejuni* from the agricultural niche to barnacle geese during migration could be a result of ecological separation between the two or deficient colonization capacity of agricultural strains in barnacle geese\(^{232}\). However, the three tetracycline resistance isolates might be a result of such an acquisition since tetracycline resistance is much more common in continental Europe\(^{344}\) than in Finland.\(^{76}\) Overall, the antimicrobial patterns are consistent with the remaining of
results in Study II and support the existence of a separate barnacle geese-adapted *C. jejuni* population.

**10.4 Applicability of whole-genome sequencing to *C. jejuni* outbreak investigations (III)**

The primary goal of Study III was to investigate the applicability of WGS to an outbreak investigation, and to compare this WGS data with the genotypic and epidemiological information already available from the investigation executed in 2000. We found that the earlier resolved water outbreak was caused by at least two different *C. jejuni* strains, as shown by reconstruction of a phylogenetic tree, in contrast to the conclusions drawn from the original outbreak investigation relying on PFGE. Alternatively, one of these human strains was mistakenly classified as part of the outbreak when it was in fact a sporadic case occurring in the same area and time-period. Therefore, PFGE overestimated the clonal relationship between the patients and water strains, and WGS was needed to infer the correct epidemiology.

Even though campylobacteriosis outbreaks are rarer than sporadic cases, they do happen, especially in the Nordic countries. Knowledge of the character and microevolution of *C. jejuni* genomes during outbreaks is crucial before WGS can become a useful and reliable tool in real-time outbreak investigations. Study III was the second study to apply WGS to retrospectively solve a campylobacteriosis outbreak. However, while WGS has not yet been utilized to solve *C. jejuni* outbreaks in real-time, studies have demonstrated sufficiently high turnaround time to enable WGS data to influence the course of an outbreak for other pathogens. For instance, WGS was utilized to solve a foodborne outbreak of listeriosis in Canada and a nosocomial outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) in a neonatal ward in Cambridge.

The origin of the observed PFGE differences between the outbreak strains was an extra *KpnI* restriction site in the 22 kb central region of CJIE2 in IHV116292. Isolates with slightly different macrorestriction patterns are still classified as related, and the genetic diversity observed in the CJIE2 was most likely a result of a single recombination event (as described in Section 8.8), which can create high genetic diversity between related isolates. This effect of integrated elements on PFGE profiles has also been reported in the above-mentioned listeriosis outbreak, in which bacteriophage insertion in the *L. monocytogenes* genome created different macrorestriction patterns among the outbreak-related strains. Therefore, the finding of this central divergent region alone does not mean that the IHV116292 isolate was unrelated to the outbreak. According to some studies, the use of a simple cut-off value for SNPs to decide whether a set of isolates is related is controversial, as variations in the number of SNPs between outbreak strains occur and have been reported for both *L. monocytogenes* and
However, this might not be the case for *C. jejuni*. If IHV116292 and 4031 are assumed to be related and part of the outbreak, the 69 SNPs observed between IHV116292 and 4031 could theoretically be a result of 1) host-specific conditions during infection or 2) adverse environmental stress. But for *C. jejuni*, only a few SNPs are expected to be produced during human passage, and SNPs cannot be generated in the environment since *C. jejuni* is unable to replicate outside a host. Therefore, the 4031 and IHV116292 appear to be unrelated based on analysis of SNPs.

To further verify this hypothesis, we reconstructed the genealogy of the outbreak and chicken isolates and estimated the number of SNPs between an unrelated chicken isolate (6236/12) and water strain 4031. The underlying assumption to include the chicken isolates as calibrators was that since the chicken and outbreak isolates shared an ST and PFGE type, they must have had a common ancestor. Combined with the time separation of 12 years between the outbreak and collection of the chicken isolates, estimation of the expected number of substitutions over time was possible. In this analysis, we found that the human-derived outbreak strains were paraphyletically related and that the numbers of SNPs in the IHV116292 and chicken strain relative to 4031 were comparable (62 and 69 SNPs, respectively). If the genetic diversities seen between the IHV116292, IHV116260, and 4031 were generated during the outbreak, the relationship between these strains would have been monophyletic and we would have expected more substitutions in the chicken isolate than in IHV6292 relative to the 4031 isolate. However, since our analysis showed the opposite, there must have been at least two strains circulating in the community during the occurrence of this water outbreak (4031 and IHV116292). So, by interpreting the topology of the phylogenetic tree while simultaneously considering the effect of genetic drift (SNPs), as also described by Koser et al., we were able to infer the correct relationship between the outbreak isolates; IHV116292 was different from 4031 already prior to the outbreak. As only ten patient isolates were originally characterized by PFGE, the frequency at which the water isolate occurred among the outbreak patients is not known, and further analysis of human strains from the same outbreak and time-period from that region could reveal whether IHV116292 was part of the outbreak or a single sporadic case.

### 10.5 Characterization of the *C. jejuni* population found on Finnish chicken farms over a decade and identification of factors of importance for the transmission of *C. jejuni* to chicken flocks (IV)

One aim of Study IV was to investigate the effect of year, site of origin, and season on the dynamics of the *C. jejuni* population on Finnish chicken farms. In addition, we wanted to investigate the possibility of the occurrence of a persistent colonization source. We found a stable occurrence of three dominant lineages, ST-45 CC, ST-677 CC, and ST-21 CC, over nine study years, verifying the population stability found earlier by Gonzalez *et al.* as
measured by genetic markers. Additionally, we found that ST-45 CC was overrepresented in Finnish chickens with chicken in other countries. Similar to other studies, we did not find any evidence of a persistent contamination source on farms stretching over several years. However, we did identify possible cross-contamination between chicken halls on the same farms, most probable due to a breach in biosecurity.

Although the population was generally consistent through space and time, lineage distribution did vary marginally between years, site of collection, and season. ST-21 CC peaked in 2008, but otherwise the distribution of three investigated CCs was similar between the years. Furthermore, ST-45 CC and ST-21 CC were more commonly isolated from slaughterhouse A and C and from B and C, respectively, which could reflect regional differences in C. jejuni exposures to the chickens or indicate such management differences as farming practices, feed, water, hygiene, and biosecurity between producers. In our study, however, all CCs were collected from every Finnish slaughterhouse, indicating a national distribution of these lineages.

The extraordinarily high occurrence of ST-45 CC is a continuation of a phenomenon already observed in Finnish chicken meat in 2003 and warrants further discussion. The reasons behind this dominance are unknown, but the cold climate might exaggerate the seasonal effects already described for this clonal complex. However, we did not see any effect of season on occurrence of ST-45 CC in our multivariate analysis, but the ST-45, the main ST of ST-45 CC, was more commonly found in September and winter than in spring. This might reflect an increased survival capacity of ST-45 compared with other STs at time-points outside the July-August peak.

Additional explanations for the high isolation rate of ST-45 CC in chickens include this CC’s widespread occurrence in different hosts and the existence of a “self-enforcing prevalence circle” in Finland, resulting in a high relative environmental exposure to the chicken flocks. Since chickens often carry ST-45 CC, they function as gigantic incubators for this CC, resulting in higher environmental contamination with ST-45 CC. In addition, Finnish campylobacteriosis cases are dominated by ST-45 CC which cannot in most cases be linked to the consumption of chicken meat. The chickens and human patients might have been colonized or infected, respectively, by each other indirectly or through sharing of other reservoirs or sources. Clearly, more research on this matter is warranted to reduce the amount of ST-45 CC in chicken flocks and human cases.

One central finding was that Finnish chicken flocks and farms were only rarely and sporadically colonized by C. jejuni. Within a given year, most farms did not deliver any C. jejuni-positive batches, and of the positive farms, most were positive in only one of the study years. Also, half of the farms that did deliver more than one positive batch did so in the same rearing cycle from several halls on the premises. In addition, we found no
indications of a persistent colonization source, as *C. jejuni* of most farms, including the ten “high-frequency farms”, experienced a genotype switch in either MLST or PFGE or both if they were positive over several years. Summarized, our findings argue for an infrequent and non-persistent horizontal transmission from the environment to Finnish chicken flocks, which further testifies to the effectiveness of the biosecurity measures implemented. However, two farms (B10 and C14) did have the same ST and PFGE profile over several years, but this was interpreted as the introduction of a different strain of the same genotype; the strains in question were the generalist ST-45 with its associated PFGE types *Sma*I S7 and S66 being probably widespread in the environment.

Similar to others,\(^\text{210}\) we did find evidence of house-to-house transmission during a rearing cycle, as many of these flocks carried *C. jejuni* of the same MLST and PFGE types. The transmission between chicken houses on the same farms is mostly due to workers and vehicles moving from one house to another or transmitting *C. jejuni* into one house from the other houses’ surroundings. The transmission risk increases with the number of houses on the farms, probably due to accumulation of *C. jejuni* in the farm environment.\(^\text{221,349}\)

### 10.6 Identification of the most cost-efficient genotyping method to trace *C. jejuni* from reservoirs to humans in different epidemiological settings (I-IV)

This thesis investigated *C. jejuni* collected from various sources, such as birds, human cases, several mammalian reservoirs, and water, in different epidemiological settings, including both sporadic and outbreak-related *C. jejuni* infections. The studies have been conducted over short periods, such as the study on *C. jejuni* related to a waterborne outbreak, to longer periods, like the nine-year longitudinal study of *C. jejuni* on Finnish chicken farms. We have used a plethora of subtyping methods, from phenotyping by GGT production and antimicrobial sensitivity, to genotyping by PFGE and MLST and further to advanced WGS by NGS. To analyze the output of these subtyping results, we have utilized statistically naïve tests, such as Pearson’s Chi-square and Fisher’s exact tests, to more elegant multivariate regression analysis and Bayesian clustering methods. Comparative genomics has been a cornerstone in three of four studies, as is time-typical for the genomic era in which we are situated. Through these experiences, we have tried to utilize the most sensible and cost-efficient genotyping method in each epidemiological setting, and the experiences with these will be discussed below.

There is still a need for the relatively simple and traditional subtyping methods in the epidemiologic work with *C. jejuni*, especially where WGS is not yet possible. PFGE, when using *KpnI*, is highly discriminatory and in concordance with the epidemiology, and is just as discriminatory as some sequence based-methods, e.g. comparative genomic fingerprinting.\(^\text{350}\) PFGE has long been the gold standard in outbreak investigations,\(^\text{351}\) and
althought it overestimated the clonal relationship of the outbreak isolates in Study III, the method performed well for the majority of strains recovered during the outbreak.\textsuperscript{270,311} Furthermore, the addition of PFGE to the MLST in Study IV allowed the necessary differentiation to infer the correct relationship between the isolates collected during a rearing cycle. However, interpretation of PFGE patterns over longer time-periods warrants caution, as typing over time requires methods that are adequately stable genetically to allow interpretation. PFGE might be sensitive to genomic instability due to genomic rearrangements, and isolates' PFGE profiles may shift even during chicken passage,\textsuperscript{352} obscuring their clonal relationship. In Study IV, we interpreted the PFGE patterns over several rearing cycles, and chose to classify different PFGE patterns as unrelated despite earlier suggestions.\textsuperscript{317} This conservative approach might have led to an underestimation of the level of recycling of isolates on chicken farms, but the opposite interpretation might have caused overestimation of existing of isolate persistence since \textit{C. jejuni} with related PFGE patterns might be unassociated, as shown in Study III.

We found that MLST performed well in describing the population structure and defining the clonal frame in all of our long-term analyses in Studies I, II, and IV. While the high discriminatory power of PFGE resulted in data with no obvious trends when compared over several years (IV), MLST revealed persistent lineages in concordance with earlier Finnish studies,\textsuperscript{103} and a similar population structure as described abroad in chickens (IV) and geese (II),\textsuperscript{104,172,180,229} thereby verifying the accuracy of our results. However, the MLST yielded similar STs for unrelated isolates as derived by PFGE in Study IV, and the human, water, and chicken isolates included in Study III were of the same ST, even though they were unassociated by WGS. Thus, MLST is not sufficiently discriminatory to be used as the sole subtyping method in short-term epidemiology, as has also been stated earlier.\textsuperscript{351}

The metabolic markers used in Study I followed the population structure and only increased the discriminatory power inside ST-45, which is already known as a heterogeneous ST based on Penner heat-stable serotypes, \textit{flaA SVR}, microarrays, stress response analysis, and LOS class distribution.\textsuperscript{33,84,110,353,354} The genomic heterogeneity of the generalist ST-45 is not surprising, as this ST is recovered from different hosts and sources. The lacking host association of metabolic markers independent of MLST indicates that these markers are unsuitable as the sole subtyping method for source attribution, and their sensitivity for recombination and gene loss makes them less suitable for studies on population structure and evolution. Thus, the metabolic markers did not enhance the analysis in Study I.

WGS provides an unambiguous and powerful typing method for epidemiological investigations in both outbreak situations and clinical practice. Different needs for discriminatory depth are easily achieved by varying the number of genes included, from
extraction of the traditional seven-gene MLST, via in silico construction of PFGE profiles by various restriction enzymes and extended MLST schemes, to a pan-genome approach using the full complement of genes in a species. In addition to its use in traditional epidemiological applications, WGS can determine phenotypic characteristics such as antimicrobial resistance patterns, toxin production, and virulence. WGS is therefore the ultimate method for bacterial typing, but its use is limited by high costs and time-consuming procedures in combination with the need for skilled scientists to compute and interpret the sequences.

We found that WGS was needed to infer the correct population structure between bovine and barnacle geese isolates, which was not unambiguously resolved by MLST. Without access to WGS, we might have concluded that bovine and geese shared a C. jejuni reservoir. Furthermore, WGS inferred the correct epidemiology in the waterborne outbreak in Study III, similar to experiences gained in other outbreaks.

In conclusion, utilization of an arsenal of subtyping methods in the epidemiological work with C. jejuni may be necessary due to its enormous genetic variation. However, as WGS becomes increasingly affordable and interpretation of sequences more standardized, we are convinced that bench-top WGS will conquer the field of C. jejuni epidemiology in the future.
11. Conclusions

1. The metabolic markers of GGT production, \textit{ansB(s)}, and \textit{fucP} follow the clonal frame and are not suitable in source attribution as they are not solely host associated (I).

2. Barnacle geese generally carry \textit{C. jejuni} of a different population than the one colonizing agricultural animals, especially chicken, and infecting humans, and is therefore not a major source of human campylobacteriosis (II and IV).

3. WGS is needed to unambiguously solve outbreaks of \textit{C. jejuni} and infer the correct relationship between closely related strains. It is superior to MLST in resolving clusters of isolates from different reservoirs (II and III).

4. Chickens in Finland carry a highly skewed \textit{C. jejuni} population, which was dominated by ST-45 CC and ST-45 over a decade (IV).

5. The chicken farms are sporadically and infrequently colonized by \textit{C. jejuni}, and no persistent colonization source is evident (IV).

6. Due to its superb applicability to all epidemiological settings and time-frames, WGS is the ultimate typing tool for \textit{C. jejuni}. However, traditional typing-methods, such as PFGE and MLST, are still valuable and remain able to infer the correct epidemiology in situations where WGS is not yet feasible (I-IV).
12. References


31. Kokotovic B, On SL. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *campylobacter coli* by analysis of amplified fragment length polymorphisms.


57. Gripp E, Hlahla D, Didelot X, et al. Closely related *Campylobacter jejuni* strains from


64. Flint DH, Smyk-Randall E, Tuminello JF, Draczynska-Lusiak B, Brown OR. The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its fe-S cluster, but the enzyme remains in the cell in a form that can be reactivated. *J Biol Chem*. 1993;268(34):25547-25552.


81. Kittl S, Heckel G, Korczak BM, Kuhnert P. Source attribution of human Campylobacter isolates by MLST and fla-typing and association of genotypes with quinolone


107. de Haan CP, Kivistö RI, Hakkinen M, Corander J, Hänninen ML. Multilocus sequence types of finnish bovine...


149. Hald B, Skovgard H, Pedersen K, Bunkenborg H. Influxed insects as vectors for
Population genetics and molecular epidemiology of *Campylobacter jejuni*
AK Llarena

*Campylobacter jejuni* and *campylobacter coli* in danish broiler houses. *Poult Sci.*


158. EFSA BIOHAZ (Panel on Biological Hazards). Scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA J.* 2010;8(1):1437.


160. Perko-Mäkelä P. *Campylobacter jejuni* and *C. coli* in finnish poultry production.
University of Helsinki; 2011.


163. EFSA (European Food Safety Authority). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008, part A: *Campylobacter* and *Salmonella*


Population genetics and molecular epidemiology of *Campylobacter jejuni*

AK Llarena


212. EFSA BIOHAZ (Panel on Biological Hazards). Scientific opinion on *Campylobacter* in broiler meat production: Control options and performance objectives and/or targets at different stages of the food chain. *EFSA J*. 2011;9(4):2105.


226. Gonzalez M. Characterization of Campylobacter jejuni strains from different hosts and modelling the survival of C. jejuni in chicken meat and in water. University of Helsinki, Faculty of Veterinary Medicine, Department of Food Hygiene and Environmental Health; 2012.


312. Hänninnen ML, Perko-Mäkelä P, Pitkala A, Rautelin H. A three-year study of Campylobacter jejuni genotypes in humans with domestically acquired infections and in


Population genetics and molecular epidemiology of *Campylobacter jejuni*

AK Llarena

**2008;9:539.**


339. McCarthy ND, Gillespie IA, Lawson AJ, et al. Molecular epidemiology of human *Campylobacter jejuni* shows association...


