Molecular epidemiology and comparative genomics of *Campylobacter jejuni* isolates from wild birds

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Contents

Abbreviations

A ATP: adenosine tri phosphate

B BRIG: blast ring image generator

C CC: clonal complex

CDS: coding sequence

CJIE: *Campylobacter jejuni* inserted elements

Cjj: *campylobacter jejuni* subsp. *jejuni*

D DNA: deoxyribonucleic acid

E E-MEM: Eagle's Minimum Essential Medium

F FBS: fetal bovine serum

G G2 phase: growth 2 phase

GBS: Guillain Barré syndrome

I ISO: international organization for standardization

M M phase: mitotic phase

mCCDA: modified charcoal cefoperazone desoxycholate agar

MLEE: multi locus enzyme electrophoresis

MLST: multi locus sequence type

MOI: multiplicity of infection

P PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFGE: pulsed-field gel electrophoresis

R RAST: rapid annotations using subsystems technologies

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

S spp.: species

ST: sequence type

subsp.: subspecies

T T3SS: Type 3 secretion system

T4SS: Type 4 secretion system

T6SS: type 6 secretion system

tRNA: transfer ribonucleic acid

U UV: ultraviolet

General introduction

1. General facts on *Campylobacter* spp.

Campylobacter infections, then named "*Cholera infantum*" were first observed in 1886 by Theodor Escherich, but due to inability to isolate them, he dismissed their probable etiological involvement in reported enteric diseases in infants. In the following years, vibrio-like organism were established as veterinary pathogens following multiple cases of abortion in ewes and dysentery in cattle, and were referred to as "*Vibrio jejuni*" [147]. In 1963, those vibrio were assigned to the new genus *Campylobacter*, from the Greek *kampylos* (curved) and *baktron* (rod), distinctly characterized by their low DNA base composition and microaerophilic growth requirement [143]. The filtration technique by Dekeyser et al. [46] that allowed the first isolation of *Campylobacter* from stool and the development of a selective medium by Skirrow [146] have allowed the discovery of more species of the genus.

Currently, the genus *Campylobacter* belongs to the family *Campylobacteraceae*, Order *Campylobacterales*, Class of *Epsilonproteobacteria* and Phylum *Proteobacteria*. It includes 17 recognized species and 6 subspecies, the most frequent being; *C. coli*, *C. concisus*, *C. curvus*, *C. fetus* (which has two subspecies *fetus* and *venerealis*), *C. gracilis*, *C. helveticus*, *C. hominis*, C. *hyointestinalis* (subsp. *hyointestinalis and* subsp. *lawsonii*), *C. insulaenigrae*, *C. jejuni* (subsp. *doylei* and subsp. *jejuni*), *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum* (subsp. *bubulus* and subsp. *sputorum*) and *C. upsaliensis*. The most commonly associated with human diseases are *C. jejuni* (subsp. *jejuni*) and *C. coli* [164]. Other species such as *C. lari* and *C. upsaliensis* have also been isolated from patients with diarrhoeal disease, but are reported less frequently. Of veterinary importance, mainly, *C. fetus* and *C. jejuni* and at a minor level *C. coli*, are important causes of reproductive failures in ruminants [83, 148].

Members of the genus *Campylobacter* are Gram negative, catalase and oxidase positive, motile by the means of a polar flagellum at one or both ends though some species, such as *C. gracilis*, are non-motile. They are non-spore-forming, have a capsule and are curved rods of 0.2-0.9

µm of wide and 0.5-5 µm of long (Fig.1). Campylobacters can show a coccoid form when in viable but non-culturable state [114].

Campylobacters are fastidious, microaerophilic bacteria, necessitating 3-15% O_2 and 3-5% CO_2 [96]. For this reason, an artificial atmosphere of 5% O_2, 10% CO_2 and 85% N_2 is commonly used to culture campylobacters. The growth temperature range for *Campylobacter* spp. is 30-44 ºC [24] with an optimum of 42ºC which reproduces its adaptation to the temperatures found in intestines of birds where *Campylobacter* finds a usual habitat. However, it is common to grow *Campylobacter* at 37ºC when they are suspected or known to be pathogenic.

C. jejuni has a respiratory metabolism and is unable to ferment or oxidize carbohydrates, reason why it acquires needed energy from amino acids and metabolites of the citric acid cycle [113]. The genome size of *C. jejuni* is relatively small (1.6-2.0 megabases), it was first sequenced in 2000 [124] and since then the number of complete and draft genomes have been increasing.

Campylobacter spp. is a commensal of livestock, birds and many other animals [117]. Moreover, it is mainly transmitted through food chains through handling or consumption or contaminated water and symptomatically characterized by relatively acute enteritis. Therefore, its definitive diagnosis can only be made by detecting the bacteria in feces of a patient or by isolating it from food or water.

So far, two ISO (International Organization for Standardization) procedures developed for optimal detection of *Campylobacter* spp. are available: a horizontal method for detection of thermotolerant *Campylobacter* in food and animal feeding stuffs [85] and a procedure for the isolation of *Campylobacter* from water [86]. However, neither of these two may be optimal for the isolation of campylobacters from live animals. From fecal samples, the isolation method embraced by many laboratories worldwide is based on using cephalotin-containing medium and a growth temperature of 42º C. However, this technique may not support the isolation of species other than *C. jejuni* and *C. coli* because only some strains of *C. fetus* subsp. *fetus* and *C. hyointestinalis* can grow at 42ºC [141].

Basic biochemical tests for the differentiation of *Campylobacter* species are available and can be used by most clinical laboratories. However, *Campylobacter* species are biochemically inert and the discrimination between two species may rely on a single biochemical test (Table 1). Moreover, biochemical variants of well-known species have been reported. *C. jejuni* hydrolyze hippurate, while *C. jejuni* subsp. *doylei* varies in its ability to do so [156]. Consequently, hippurate hydrolysis has become a golden biochemical test widely used to identify *C. jejuni* and differentiate it from *C. coli* that are phenotypically and genotypically identical. The growth characteristics and biochemical tests are summarized in Table 1.

Given the limitation of tests associated with the biochemical inertness of *Campylobacter* spp., genotype-based species identification methods have been developed. PCR methods, both conventional and quantitative, based on a variety of species-specific genes have been established for species confirmatory identification. For *C. jejuni*, these methods include*cadF* [40], *ceuE* [81], 23S *rDNA* [58] and *cdtA* [10], among others. The PCR test for *C. jejuni* show variations in sensitivity and specificity probably due to the high heterogeneity of the *Campylobacter* species, implicating that since a single PCR cannot identify all species, a polyphasic strategy should be taken for anyone intending to identify *Campylobacter* spp. [122]. Partial 16S rDNA sequencing is widely used for species identification of *Campylobacter jejuni* [162].

2. Epidemiology and pathogenesis of *C. jejuni*

Campylobacteriosis is a significant burden to public health services in developed countries. It has been estimated that nearly 1% of the US population suffer from campylobacteriosis per year [145]. In Canada, the rate of campylobacteriosis was found around 37.7 to 48.7 per 100,000 populations [129], whereas in the EU notification rate was 55.49 cases per 100,000 persons [57]. In Japan, *Campylobacter* has overtaken *Salmonella*, to be a leading cause of bacterial foodborne pathogen since 1998, Campylobacteriosis accounts for 300 to 600 cases each year according to the statistics of the Ministry of Health, Labour and Welfare [111].

In developing countries, figures are difficult to find but campylobacteriosis has been reported to be hyperendemic [167]. In the WHO report, the incidence of campylobacteriosis in developing countries ranged from 5000 to 20000 cases per 100,000 of population of children [167], which makes the situation in developed countries look insignificant.

Until early 1990s, raw milk and water were undeniably considered the main sources for human campylobacteriosis. However, between 1998 and 2004 with unresolved outbreaks, led to a search for additional transmission routes [45]. From that moment, the importance of food chains particularly those involving poultry were recognized. Since then, campylobacters have now been recognized to colonize the intestinal mucosa of a wide range of avian, animals and humans [18, 117].

In comparison to other *Campylobacter* species, *C. jejuni* has been recognized as a successful commensal in chicken and cattle [18, 20] whilst its sister species *C. coli*, is more linked with sheep and pigs [125]. As for *C. fetus subsp. fetus*, is a cause of reproduction-associated campylobacteriosis in sheep and cattle, but it can also cause disease in humans [83, 148]. Other well established pathogens are *C. helviticus* and *C. upsaliensis* in cats and dogs [32] and *C. hyointestinalis* and *C. mucosalis* in pigs [125]. Cats and dogs, whether sick with diarrhea or healthy, are frequent carriers of *Campylobacter* spp. [70, 77] with predominance of *C. upsaliensis* although *C. jejuni*, *C. coli* and *C. helveticus*.[32].

Environment and environmental water are taken as potential sources whilst environmental contamination is likely to be initiated by fecal dropping by domestic livestock, wild mammals, wild birds and humans [117]. Specifically, wild birds such as geese and mallard ducks were reported to be implicated in outbreaks of campylobacteriosis arising from water supply contamination in Norway [157] and of river contamination in England [120]. Lastly, human sewage as well as drinking water are other well established sources of human campylobacteriosis [89].

The body temperature of birds corresponds to the optimal growth temperature for thermophilic *Campylobacter* spp. Therefore, *Campylobacter* spp. is well adapted to their gut where they thrive as commensals. For this reason, birds have been widely regarded as natural hosts of these organisms [39] and *Campylobacter* spp. have been isolated in both domesticated and wild birds. A

high-prevalence of *C. jejuni* and *C. coli* are often reported in crow [162], ducks [140], geese [12], ostriches, parrots, pigeons, quails, sparrow, waterfowl [82, 123], turkeys [27] and European starlings [39]. Wild birds are recognized as a potential hazard in the transmission of infection to human or livestock [21, 65]. The prevalence rate vary from 0% to 100% [36], with variances associated with the ecology of birds species, feeding habits of different birds, sample size, sensitivity of culture and detection method and lastly migratory patterns [158]. In wild birds, *C. jejuni* and *C. coli* are the most prominent species found. However, *C. lari* can also be found to a lesser extent [158].

In Japan, *Campylobacter* spp. colonization of wild birds was also reported in several studies [67, 87, 98, 107, 108]. In these studies, *Campylobacter* spp. were isolated from feces, intestinal content or fecal swabs and from a wide range of wide birds, including pigeons, crow, sparrow and turtledove. However, despite their in findings, no genotyping was done to know the source of these pathogens and probable relationship to genotypes involved in human or livestock infection.

Although *C. jejuni* has been recognized as an important food-borne pathogen, the mechanisms of its pathogenicity are still not well-elucidated. The pathogenesis of *C. jejuni* has been extensively reviewed by Dasti *et al*. [44], Epps *et al*. [56], Ketley [96], and Young *et al*. [169]. *C. jejuni* infection in humans requires as low as 500 cells to cause disease [23] and enters the host intestine by passing through the stomach acid barrier and colonizes the mucus layer covering the epithelia of the distal ileum first and then that of the colon [96]. Although the pathogenesis of *C. jejuni* is unknown, a sequence of events appears to be indispensable. Those are: chemotaxis and motility, adhesion, invasion, intracellular survival and toxin production.

Chemotaxis is crucial for an effective colonization of *C. jejuni* [171]. To sense the variation of concentration from various chemoeffectors, *C. jejuni* uses generally a cytoplasmic RR (CheY) and a membrane-associated histidine autokinase (cheA) [171]. Effective motility of *Campylobacter* spp. needs the production of a functional flagellum. The flagellum and the “curved” shape of confer a high level motility to *Campylobacter* spp. in viscous environment [96].

In addition, the flagella have been shown to be necessary for infection and/or colonization in many model of infection [115]. The flagellum of *C. jejuni* is composed of two highly homologous

flagellins, FlaA and FlaB, the first one being the major and the second the minor component[73]. The FlaA and FlaB are under regulation of promoters σ28 and σ54 respectively [90]. The FlaA is required for invasion of epithelial cells, since mutation in this gene led to reduced-motility phenotype and a truncated flagella composed of only FlaB [73]. The polar flagellum of *Campylobacter* is composed of O-linked glycosylated flagellin. Moreover, a two-component system comprised of the sensor FlgS and the response regulator FlgR is central for the regulation of the *Campylobacter* flagellum [90].

The aptitude of *C. jejuni* to both, attach to, and enter human and experimental animal model intestinal epithelial cells *in vivo* and *in vitro* is well recognized, although the mechanisms behind these processes are not well characterized [22, 169]. The adhesion between *C. jejuni* and host cells per se, is a critical step for it to initiate an infection [161] and cause disruption of the normal absorption function [166].

Several surface structures have been associated with a role in *C. jejuni* adhesion to host targets. These include; LOS, CPS, flagella, specific adhesin proteins and glycoproteins [93, 169].

Of the two molecular mechanisms used by other entero-invasive bacteria to invade host cells as described by O Croinin and Backer [119], none fits exactly the pathogenesis characteristics of *C. jejuni*. In regard to the first mechanism involving adhesins-host cell receptor interaction, studies were carried out to find important adhesins of *C. jejuni*. CadF protein is the well characterized *C. jejuni* factor that binds extracellular matrix protein fibronectin. Studies have revealed the usefulness of CadF for an efficient colonization of cells *in vitro* [99] in addition to infection of chickens [174]. Other surface proteins involved in *C. jejuni* attachment to host cell are PEB1 and JlpA. Jin *et al*. [88] have reported the implication of JlpA protein in binding Hep-2 cells. However, more recent studies tend to negate the effect previously reported [118]. Study on PEB1 also showed affinity of this protein to epithelial cells [60]. Following studies showed that PEB1 was surface exposed and that when purified, it bound to HeLa cell membranes *in vitro*.

The second mechanism requires an involvement of a Type-III and type-IV secretion systems (T3SS and T4SS). Since the available genomes of *C. jejuni* do not show any T3SS or T4SS,

researchers hypothesized on the role of the flagellum as a secretion tool. Early studies exposed the requirement of *C. jejuni* motility for an efficient invasion of epithelial cells in vitro [115]. In that regard, studies have revealed the importance of a functional flagellum apparatus for the secretion of virulence-associated proteins including the Cia (*Campylobacter* invasion antigen), with the well-known CiaB protein [100] which is involved in invasion.

The most well characterized toxin of *C. jejuni* is the tripartite cytotoxic Cytolethal distending toxin (CDT). The CDT protein is encoded by the three highly conserved genes, *cdtA*, *cdtB* and *cdtC* [163]. CdtA and CdtC are thought to be involved in binding and internalization of the toxin into host cells whereas the CdtB subunit is the toxic compound [102]. CDT toxin has been perceived to permanently arrest cell division at either the G2 phase or early M phase and consequently cell death [163].

It has been postulated that the toxins are responsible of watery diarrhea as opposed to inflammatory. However, an epidemiological survey has observed that patient symptoms do not necessarily correlate with toxin positive versus toxin negative strains [160].

Campylobacteriosis is the common name given to illness caused by pathogenic *Campylobacter spp.*, which is the leading cause of foodborne gastroenteritis caused by a bacterium. This illness is, in most cases, self-limiting and normally lasts for a few days sometimes up to two weeks. The incubation time varies in length, from 2 to 5 days but may be prolonged to 11 days [23]. The symptoms of infections caused by *Campylobacter* are: abdominal cramps and/or pain, a watery or bloody diarrhea, fever, headache, nausea and vomiting. In some patients the diarrhea is minimal and abdominal pain and cramps are dominant characteristics. The fever is reported in most patients (over 90%), it can be low-grade or > 40ºC and lasts up to one full week. Generally, by that time the infection has resolved even without specific treatment. Yet, in some cases, some patients can develop a longer degenerating diarrhea that can last several weeks [91]. The mortality rate for *Campylobacter* infection is 0.05 per 1000 infections and especially in young children or elderly patients where it may cause death or develop into more severe complications.

The infamously known Guillain-Barré Syndrome (GBS), sometimes Guillain-Barré-Strohl syndrome, Landry's paralysis or polyradiculitis, was described for the first time in 1916, and is the most significant complication in *Campylobacter* infections [8, 170]. It has been confirmed that *C. jejuni* is a major precedent in patients affected by the GBS, in at least 1/3 of them [8]. In fact, the molecular mimicry and cross-reactive immune responses to *C. jejuni* lipooligosaccharide induces the development of GBS [84, 170]. This disease is characterized by a rapid-onset weakness of the limbs resulting from an acute neuromuscular paralysis targeting the peripheral nervous system. The symptoms may progress into respiratory distress and severe neurological dysfunction and death in some cases [6]. Although death is rare in case of GBS-affected patients, the most vulnerable patients are elderly and those suffering from other immunocompromizing diseases. There are other possible complications of *C. jejuni* infections such as Miller Fisher syndrome which is a variant of GBS syndrome, characterized by lack of voluntary coordination of muscle movement (ataxia) and paralysis of eye muscles (Ophthalmoparesis).

3. Molecular typing of *C. jejuni*

Molecular typing can be defined as methods used to identify different types of organisms within a species [137]. The objective of bacterial typing is to determine taxonomy, establish phylogenetic relationships, scrutinize evolutionary mechanisms and conduct epidemiological surveys [19]. Traditional, phenotype-based typing methods have successfully been applied to *Campylobacter* spp. screening. These include phenotyping, phage typing and Multi-locus enzyme electrophoresis (MLEE). These methods have variable success and are still used in some laboratories. However, they have many drawbacks including; lack of discrimination, cross reactivity and need high level of technical expertise that most laboratories cannot be afford. [94]. For these reasons, and given an immense progress in DNA-based technologies, detection of DNA variations to compare nucleotide sequences have become commonplace. Typically, the emergence of a new genotyping technology was inspired by an existing one (the Multilocus Sequence Typing [MLST] by MLEE for

example). Most of these relatively new genotyping methods are computer-assisted which allow an easy analysis, interpretation and data sharing between laboratories globally.

The *flaA* and *flaB* genes show 92% homology and exhibit approximately 95% nucleotide variation among various isolates which constitute the basis for flagellin typing schemes. This typing cannot differentiate *C. jejuni* and *C. coli*, because of the gene pools that are shared between these two species [49, 62]. It has been reported that the level of discrimination for *flaA* short variable region typing is greater than serotyping but way less than Flagellin typing and pulsed field gel electrophoresis (PFGE) [131] and should be used in combination with other typing methods mostly MLST [49].

PFGE utilizes rare cutting restriction enzymes that digest the chromosomal DNA resulting in five to fifteen DNA fragments (ranging from 1 to 1000 kb pairs,) depending on the restriction enzymes used and the chromosome digested [153]. The resulting restricted fragments can be separated on an agarose gel by "pulsed-field" electrophoresis in which the orientation of the electric field across the gel is changed periodically. Owing to its high discriminatory power, PFGE is considered as "gold standard" for epidemiological investigations [138]. The advantage of this method is that it analyzes a large portions of a genome (>90%) allowing to detect large recombination events and insertions or deletions of mobile genetic elements within genomic DNA [137]. However, PFGE is very sensitive to small amount of nucleotide variations which can cause the investigator to miss the true relationship between strains. In fact, cases of misinterpretation have been documented [16, 25]. These reasons, coupled with the cumbersome procedures of the method, have made PFGE not be a first choice for many laboratories thus preferring to use MLST. However, PFGE still remains highly discriminatory and is able to detect micro-evolution in bacterial species that may be indistinguishable by MLST [150].

MLST was first developed in 1991, and the technique uses comparative DNA sequencing of conserved housekeeping genes to characterize organisms [104]. In MLST, internal nucleotide sequences of approximately 400-600 of seven housekeeping genes are chosen for analysis. The length of 400-600 is chosen to give a reliable single run on sequencing facilities. In MLST, each

allele is assigned to a unique number according to the order of its discovery. Thus, *glnA*-255 would be the 255th MLST allele identified for *glnA* locus. Thus, each isolate is labeled with seven numbers constituting an allelic profile which in turn is given a sequence type (ST) also called a genotypic number. When isolates share four allelic positions or more, they are grouped under a common central genotype, referred to as the founder ST or the clonal complex (CC) genotype. MLST have many great advantages. In fact, data produced by this technique are unambiguous due to its standardized nomenclature and are highly reproducible. Moreover, the allelic sequences and ST profiles are available in online databases that can be accessible everywhere for comparison with other results.

MLST system for *C. jejuni* was developed by Dingle et al. [51] and it is increasingly used in epidemiological studies and phylogenetic analysis of *Campylobacter* spp. MLST is reported to provide a level of discrimination at least equivalent to 15 to 20 loci as examined by other methods such as MLEE [49]. Table 2 displays the housekeeping genes that were selected from the *C. jejuni* MLST scheme [51]. It has been extensively applied in epidemiological investigations to link *C. jejuni* cases to sources of contaminations. In New Zealand, a combination of MLST and a modified Hald mathematical model showed that 80% of human campylobacteriosis may be attributed to poultry chain contamination [116]. In UK, another study used MLST for a mixed population of geese, starlings, lambs and free-range chickens in the same farm ecosystem [36]. Their findings were compared with human campylobacteriosis-causing genotypes and found that the CC-21 and CC-45 were the most frequent CCs found in geese, but also shared in starlings and chickens. Based on the close relatedness between isolates from geese and those from chickens, they concluded that geese were the source of infection contamination for chickens. Hughes et al. [82] have found that wild birds can carry both poultry and livestock genotypes but also novel genotypes that are unique to wild birds. Also, the existence of unique genotype in wild birds was indicative of genetic recombination *in vivo* [82]. Finally, MLST was applied to assess the risks of wild birds fecal contamination in kids' playgrounds in New Zealand [65]. In this study, half isolates recovered from wild birds fecal material belonged to ST-45, a common genotype in humans and animal campylobacteriosis. The

authors could link two genotypes (ST-177 and ST-682) to the introduction of European birds in New Zealand in the 19th century.

4. Whole genome sequencing of *C. jejuni*

Several studies have sought to attribute and determine the prevalence of specific clones among *C. jejuni* isolates from diverse sources by the use of MLST [9, 37, 49, 51, 64, 105]. This has allowed getting information on different genotypes found in different hosts and specific niches. However, for a better understanding of the detail of evolution and adaptation, the analysis of whole genome for single species or genera provides a better opportunity [133]. Bacteria reproduce asexually; they usually recombine within and between different species, therefore molecular evolution can be a result of recombination, horizontal gene transfer, mutation, deletion and duplication. This result in a re-assortment of variants in an existing natural population [133, 152].

The ability of *C. jejuni* and *C. coli* to undergo chromosomal DNA and plasmid transformation has been reported and laboratory experiments usually use shuttle vectors to prove the natural competency of *campylobacter* spp. [159]. Nowadays, following the use of MLST and the easy accessibility of isolates database, it has been easy to demonstrate the extended *C. jejuni* diversity through MLST dataset from various hosts. In fact, the genes pattern composite of MLST method overlap in different host species [38, 105, 116] which demonstrate the recombination potential for *C. jejuni*. A part from recombination reported for genes of the MLST method, other genes have been reported, including the gene encoding for the resistance against tetracycline (*tetO*) [11] but also in virulence-related flagellin genes [94].

Campylobacter has significant levels of difference at whole genome level, potentially suggestive of evolution process directing to the niche specialization [78]. At this stage, the degree of discrimination of various typing methods including MLST becomes restricted. Indeed, MLST and many typing techniques focus on the central part of a genome, the “core genes” that provide significant phylogenetic signals about inter-species and intra-species phylogenies [133]. In that case,

differences in “accessory genes” that may contribute to specialized host colonization are ignored. Whole genome sequencing are believed to be able to address this limitation.

Hitherto, 20 complete genomes *C. jejuni* and more than one hundred genomes at different level of completion have been sequenced and deposited at GenBank. The genomes completed are from various sources and each genome has its own features that make it unique. For example, *C. jejuni* NCTC 11168 has a genome that does not show any insertion of exogenous DNA, plasmids or transposons [124]. *C. jejuni* RM1221 has four genomic islands designated as “*Campylobacter jejuni* integrated elements” (CJIEs) which make its genome bigger than that of NCTC 11168. Moreover, it is composed of 94% coding sequences [63]. The genome sequence of *C. jejuni* strain 81-176, designated as “hypervirulent”, carries two large plasmids named p*Vir* and p*Tet* [14, 17]. As for the human-origin of *C. jejuni* strain 81116, its genome is a duplication of a 6.5 Kb region which is not present in the genome sequence of the strain NCTC11168 [124].

There are many other, complete / incomplete, genomes of *Campylobacter* in the database. However, considerably, our appreciation of genomic diversity in *C. jejuni* is based solely on human or food chain associated isolates. Thus, it is possible that we do not have the true representation of this diversity, since *C. jejuni* is ubiquitous. More precisely, whole genome sequences of *C. jejuni* from other than human or human food-chain are limited, if any. To my knowledge, there is no sequence available of *C. jejuni* from wild birds that had been deposited by the time of writing this dissertation.

Given the above background therefore, to fully understand the epidemiology, ecology and evolution of *C. jejuni*, appropriate genome sequences must be available. This may contribute to future studies in areas establishing on how the evolution of *C. jejuni* is driven by the adaptation to various niches.

5. Aims of the current study

There are many reports on the prevalence of *Campylobacter* spp. in wild birds in Japan and overseas [34, 67, 87, 98, 107, 108, 162]. However, many gaps of knowledge are still open and

require systematic researches to unravel the contribution of wild birds in the transmission of campylobacteriosis. For example, no similar study has been conducted in the agricultural area of Hokkaido, northern Japan, to find out the occurrence of *Campylobacter* spp. in wild birds residing near farms and human houses. Also there are no reports on the evaluation of the virulence of *Campylobacter* spp. isolates from wild birds either *in vitro* or *in vivo*. Lastly, to characterize the distinct virulence features of isolates from wild birds, whole genome sequences from that niche would be more suitable to carry out comparative genomics. Therefore, the current study aimed to achieve the following three objectives: (i) Establish the infection rate of *Campylobacter* spp. in resident wild birds in Tokachi area, eastern Hokkaido, Japan; (ii) Apply the use of MLST to infer relationship with genotypes infecting human and livestock, as main pathway for the transmission to humans; and, (iii), Produce the genomes of isolates from wild birds and perform a comparative genomics to characterize virulence properties comparatively to the complete genomes of *C. jejuni*.

Table 1 Biochemical properties of various species of the genus *Campylobacter*

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
α-Haemolysis	-	-	(-)	(-)	+	(-)	-	V	-	+	NA	V	V	NA	+	+	+	V	-	+	+	+	+
Oxidase	+	+	+	V	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	(-)	+	V	-
Catalase	W	V	+	-	+	-	+	(+)	V	-	-	+	+	+	V	+	+	+	-	(-)	+	V	-
c-glutamyltranspeptidase	-	(+)	-	-	-	NA	-	NA	NA	-	NA	-	-	NA	-	-	NA	-	NA	NA	NA	-	-
Urease production	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	V*	-
Hippurate hydrolysis	+	-	-	-	-	(-)	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Indoxyl acetate hydrolysis	+	-	+	-	+	V	-	-	V	+	-	-	-	-	+	+	-	-	-	+	-	-	+
Nitrate reduction	+	V	+	(-)	+	+	+	+	(+)	+	-	+	+	+	-	+	+	+	-	+	+	+	+
Selenite reduction	-	NA	V	(-)	-	-	(+)	-	-	-	-	+	+	NA	-	+	+	+	-	+	+	+	+
TTC reduction	-	NA	+	-	V	V	-	-	-	-	NA	-	-	NA	V	+	NA	+	-	-	-	-	V
Trace H-S on TSI agar	-	V	-	-	-	(-)	-	-	-	-	-	+	+	-	-	-	-	-	+	-	V	+	-
Growth at/in/on																							
-5 C (microaerobic)	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37 C (microaerobic)	+	+	+	+	+	V	+	+	-	+	+	+	+	+	+	+	+	+	+	-	V	+	+
4 C (microaerobic)	+	+	+	(+)	(+)	V	(+)	-	V	+	(-)	+	+	-	-	+	+	+	+	(-)	V	+	+
37 C (anaerobic)	-	+	-	+	-	+	(-)	V	+	-	+	-	+	-	-	-	+	-	+	+	+	+	-
Nutrient agar	-	-	+	(-)	+	+	+	+	+	(+)	NA	+	+	NA	+	+	NA	+	+	(-)	V	+	+
CCDA	-	+	+	(-)	(+)	(+)	+	+	V	+	NA	+	+	NA	+	+	NA	+	+	-	+	(+)	+

MacConkey agar	-	+	V	-	-	(+)	(+)	V	(+)	-	-	V	V	NA	-	-	+	-	(+)	-	+	V	-
1% Glycerine	-	V	+	(-)	-	+	+	-	+	V	+	+	V	+	(-)	+	-	+	V	+	V	+	+
-% NaCl	-	NA	-	(-)	-	V	-	-	V	-	+	-	-	-	-	-	-	(+)	+	V	+	+	-
1% Bile	V	NA	(+)	-	V	-	+	+	-	+	NA	+	(+)	NA	+	+	NA	+	+	-	-	V	+
Requirement for H2	V	-	-	+	-	+	-	-	+	-	+	V	V	NA	-	-	-	-	+	+	+	-	-
Resistance to:																							
Nalidixic acid	-	V	-	(+)	V	+	+	V	V	-	V	+	+	+	-	-	+	V	(+)	(+)	-	(+)	-
Cephalotin	+	-	+	-	(+)	-	-	-	-	-	-	(-)	-	+	-	+	+	+	-	-	-	-	(-)

Taxa: 1, *C. avium sp. nov.*; 2, *C. canadensis*; 3, *C. coli*; 4, *C. concisus*; 5, *C. cuniculorum*; 6, *C. curvus*; 7, *C. fetus* subsp. *fetus*; 8, *C. fetus* subsp. *venerealis*; 9, *C. gracilis*; 10, *C. helveticus*; 11, *C. hominis*; 12, *C. hyointestinalis subsp. hyointestinalis*; 13, *C. hyointestinalis subsp. lawsonii*; 14, *C. insulanigrae*; 15, *C. jejuni subsp. doylei*; 16, *C. jejuni subsp. jejuni*; 17, *C. lanienae*; 18, *C. lari*; 19, *C. mucosalis*; 20, *C. rectus*; 21, *C. showae*; 22, *C. sputorum*; 23, *C. upsaliensis*. Data for the biochemical properties were taken from [135]. All taxa are negative for aerobic growth at 37 ºC. +, 90-100% of strains positive; -, 0-10% of strains positive; (+), 75-89% strains positive; (-), 11-25% of strains positive; V, 26-74% of strains positive; W, weakly positive; NA, no data available. CCDA, Charcoal cefoperazone deoxycholate agar (Oxoid); TTC, triphenyl tetrazolium chloride [135]

Table 2 Housekeeping gene locus used for *Campylobacter* MLST

Genes	Name	Function	Gene position [1]
aspA	Aspartase	Amino acid metabolism	96074...97480
glnA	Glutamine synthetase	Amino acid metabolism	658331...656901
gltA	Citrate synthase	citric acid cycle	1605251...1603983
glyA	serine hydroxyl methyl transferase	Energy metabolism	367219...368463
pgm	phosphor glucosamine mutase	Amino acid metabolism	327143...328480
tkt	transketolase	Energy metabolism	1569190...1571088
atpA/*uncA*	ATP synthase a subunit	Energy metabolism	111488...112993

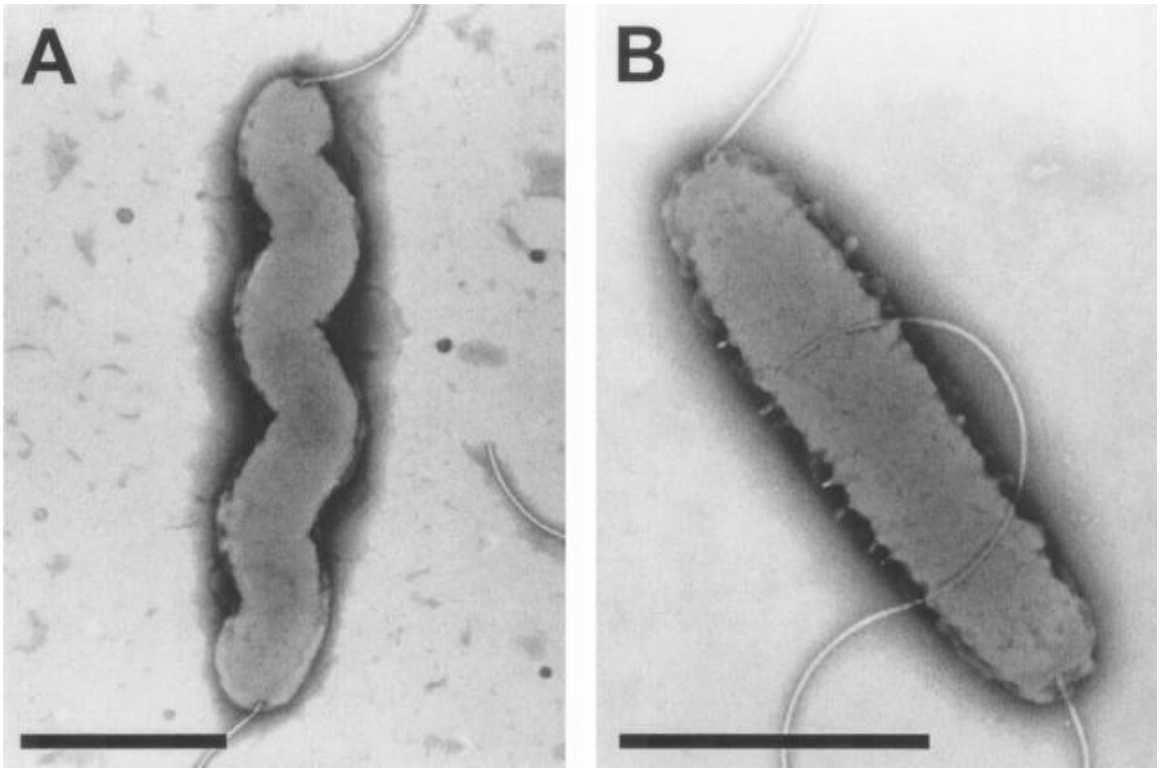


Figure 1 Morphology of *C. jejuni* strain 11168 on electronic microscope with negative staining. (A) is wild-type 11168-O; (B) is the variant of 11168 after multiple passage on agar. Image taken from [68]

Chapter I

Isolation and virulence characterization of *Campylobacter jejuni* from wild birds

1. Introduction

The genus *Campylobacter* is microaerophilic, spiral-shaped, motile Gram-negative bacilli with unipolar or bipolar flagella [145]. It is widely distributed in multiple animal hosts including livestock, wild and companion animals, and also, it is found from environmental sources [26, 89]. Among 17 species of the genus, *C. jejuni* is the leading cause of human bacterial gastroenteritis in developed countries and accounts for ~90% of cases of campylobacteriosis [164]. Patients present with watery and bloody diarrhea accompanied by fever and abdominal pain after a latency period of 2 to 5 days, as well as headaches and nausea [23]. *Campylobacter* infection has also been suggested to be associated with acute polyradiculitis (GBS) [7, 112].

In US, 13,000 hospitalizations attributed to *C. jejuni* including 100 deaths are reported each year [145]. On the other hand, 34 cases per 100,000 inhabitants and 300 cases per 100,000 inhabitants were reported annually in Canada [129] and New Zealand [15] respectively. In Japan, *C. jejuni* is also recognized as the prominent foodborne bacterium since 1998, it has accounted for 300 to 600 cases each year according to the statistical data from Ministry of Health, Labour and Welfare [111].

Humans are infected mainly through consumption of contaminated raw or undercooked food and drinking water [5, 30]. Other potential routes of infection have been documented such as contact with reservoir animals including farm and companion animals, and environmental exposures including wild birds [65], since this pathogen has the ability to colonize a range of environmental reservoirs and multiple animal hosts[117].

C. jejuni is considered to be a commensal bacterium in poultry where its infection is high, with a rate ranging from 50% to 80% [18, 144]. Bird intestines provide optimal conditions for the growth of thermophilic *Campylobacter*, allowing these avian species to serve as asymptomatic carriers [7, 18, 28].

Wild birds are also hosts to *Campylobacter* species, and because of their mobility, they may cause widespread contamination. *Campylobacter* has been isolated from birds, such as crows and

pigeons, sharing the same living area and habitat with humans [34, 67, 82, 87, 107]. Certain cases of contaminated food and drinking water have also been caused by transmission via wild birds [1, 7]. These reports suggest that wild birds may have an impact on *Campylobacter* infection in humans. Further, scavenging birds such as sea gulls and crows hunting for food in areas with raw garbage, are of concern as they may disseminate the bacteria to humans and livestock through contact or environmental contamination.

Previous studies in Japan have reported the occurrence of *C. jejuni* in wild birds [34, 67, 87, 98, 107, 108]. However, the potential virulence of wild bird isolates to humans has not been investigated. In this study, we isolated *Campylobacter* from resident wild birds and examined their virulence characteristics to evaluate the potential health risk to humans and animals.

2. Material and methods

Collection of samples

A total of 173 cloacal swab samples were collected from individual dead birds including 139 crows (*Corvus corone, C. macrorhynchos*), 24 pigeons (*Columbia livia, Streptopelia orientalis*), and 10 Eurasian tree Sparrows (*Passer montanus*). Wild birds were captured by a bird trap for pest control in Tokachi area, Hokkaido, Japan during May 2010 to May 2011. Overall, Four area neighboring livestock farm were sampled.

The birds were sacrificed with gas euthanasia. We also collected samples from officially-sanctioned hunted birds during the above period. Birds were taken to the laboratory and cloacal feces were immediately transferred to commercial transport medium (Eiken Chemical, Tochigi, Japan) with sterile cotton-tipped swabs. Samples were kept at cool temperature, and processed for the microbiological isolation within one day after sampling.

Isolation and identification of *Campylobacter* isolates

One ml portion of transport medium was transferred to 9 ml of Bolton broth (Oxoid, Hampshire, UK). The broth culture was incubated at 42°C under microaerobic condition (AnaeroPack-MicroAero, Mitsubishi Gas Chemical, Tokyo, Japan) for 48 hr, subsequently a loopful of culture was streaked on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (Oxoid). The plates were incubated at the same condition for 48 hr. Suspected colonies were subjected to Gram-staining, oxidase test, catalase test, Dryspot *Campylobacter* test (Oxoid). All *Campylobacter* isolates were subjected to 16S rDNA sequencing for inter species identification [162]. The reactions were performed using SYBR Premix Ex Taq Kit (Takara Bio) and amplification was carried out in a LightCycler 480 (Roche Diagnostics, Rotkreuz, Switzerland).

Detection of virulence genes

DNA extraction from *C. jejuni* isolates was performed using PrepMan Ultra Sample Preparation Reagent (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instruction. Real-time PCR was used to examine the presence of following virulence-associated genes in: *flaA*, *flaB*, *ciaB*, *cadF*, *cdtA*, *cdtB* and *cdtC*. The amplification was performed as described previously [33]. The size of the obtained amplicon was verified using a 2% gel electrophoresis and visualized with UV trans-illumination after ethidium bromide staining.

Bacterial motility

Swarming motility of *C. jejuni* isolates was investigated according to the method described by Scott *et al*. [32]. Briefly, two microliters of grown bacteria were inoculated onto Mueller Hinton II broth (BD, Franklin Lakes, NJ) plus 0.4% agar). The diameter of the resulting swarming colonies was measured following incubation for 23 hr at 42°C in microaerobic conditions. *C. jejuni* strain NCTC11168, which was a clinical isolate and widely used, was used as a control [80, 168].

Gentamicin protection assay

Invasive abilities of *C. jejuni* isolates to human colonic epithelial cell line Caco-2 cells were examined by gentamicin protection assay with slight modifications [55]. Caco-2 cells was maintained in Eagle's Minimum Essential Medium (E-MEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin-streptomycin, 100 mM L-glutamine, 100 mM sodium pyruvate and E-MEM amino acid (Sigma) at 37°C with 5% CO_2. The monolayer of Caco-2 cells (1.0×10^5 cells/well) in a 24-well culture plate was infected with *C. jejuni* at MOI of 50, and the culture plates were centrifuged at 3,000 rpm for 5 min to allow inoculated bacteria to adhere on Caco-2 cell surface. After 1 hr, non-adherent bacteria were removed by washing with 9.6 mM phosphate buffered saline (PBS), pH 7.4. The extracellular bacteria were killed by gentamicin (100 μg/ml) for 2 hr at 37°C. After washing, the

medium was replaced with E-MEM with 10% FBS then the Caco-2 cells were lysed by 500 µl of 0.1% Triton X- 100 (Wako Pure Chemical, Osaka, Japan) in PBS at 1-hr, 24-hr and 48-hr post infection. The intracellular culturable bacteria were counted on mCDDA medium.

Statistical analysis

The significance of differences between the groups was evaluated by one-way analysis of variance (ANOVA). Statistical analysis was performed with Graph Pad Prism software version 5 (Graph Pad Software Inc., La Jolla, CA). The *p*-values <0.05 were considered significant.

3. Results

Prevalence of *Campylobacter* spp. in wild birds in Obihiro

A total of 19.7% (34/173) samples from wild-living birds, including 20.9% (27/139) from the crows, 25% (6/24) from the pigeons and 10% (1/10) from the sparrows, were positive for *Campylobacter* spp. (Table 3). Of the 27 crow-originated isolates of *Campylobacter* spp., 25 (92.6%) were identified as *C. jejuni*, 1 (3.7%) as *C. coli* and 1 (3.7%) as *C. fetus*. Six samples from pigeons and one sample isolated from Eurasian tree sparrows, all *Campylobacter*-positive, were all confirmed as *C. jejuni*.

Detection of virulence genes using real-time PCR

Thirty-three isolates of *C. jejuni* from wild birds were screened for the presence of virulence genes. Real-time PCR was used to seven important *C. jejuni* virulence-related genes namely *cdtA*, *cdtB* and *cdtC*, genes related with the cytotoxin expression, *flaA*, *flaB* and *cadF*, linked with adherence and colonization; and *ciaB*, associated with invasion (Table 4). Seven *C. jejuni* isolates (six isolated from pigeons and one from a crow) harbored all the 7 virulence genes investigated. Other remaining 25 isolates, (24 from crows and 1 from a Eurasian tree sparrow), were positive for *cdtB*, *cdtC*, *flaA*, *flaB*, *cadF* and *ciaB* but *cdtA* gene (Table 4).

Motility assay on soft-agar plate

Since 7 isolates (C38, P3, P5, P6, P8, P9, and P10) were found to be positive for the 7 virulence genes, flagella-mediated motility of them was studied using a standard soft-agar assay. One isolate (C38, from crow sample) showed a high motility after 23-hr incubation, whereas no motility was observed in other isolates (Fig. 2).

Invasiveness and proliferation of *C. jejuni* isolates from wild birds

To understand the *C. jejuni* isolates invasion and proliferation ability in an epithelial cell-line, 7 isolates harboring all 7 virulence-associated genes (C38, P3, P5, P6, P8, P9, and P10) and 2 isolates lacking *cdtA* gene (C1 and C2) were used in the human intestinal Caco-2 cell-line invasion assay. As observed one hour post-infection (Fig. 3), isolates from wild birds showed a high ability to invade Caco-2 cells at a comparable level as with NCTC11168. All isolates tested adhered and invaded in to Caco-2 cells, and persisted within the cells for 48 hours. No significant difference between the isolates. After 24 hours, all the tested isolates showed 1.2 log-reduction in the internal bacteria number counts. At 48 hr, isolates from wild birds showed a slight increase (average, 0.2 Log) and underwent replication. The number of bacteria of wild bird isolates at 24 hr and 48 hr was significantly lower than those of NCTC11168 (Fig. 3).

4. Discussion

The present study yielded the following new findings. First, our study is the first report of *Campylobacter* spp. prevalence in resident wild birds of Hokkaido area. Second, we examined virulence-associated activities of wild bird *C. jejuni* isolates in comparison with those of type strain NCTC11168. To our knowledge, no reports are available that demonstrate virulence properties of *C. jejuni* isolates from wild birds.

Overall prevalence of *Campylobacter* spp. in wild birds in this study was 19.7%. The prevalence of *Campylobacter* in this study showed average level when compared with the results from other studies, which described *Campylobacter* spp. in wild birds in the range of 3-80% around the world [1]. The species identification showed that most isolates were *C. jejuni* in our study same as other domestic and overseas studies, although *C. coli* and *C. fetus* were found in one sample each. *C. jejuni* and *C. coli* account for 95% of campylobacteriosis in humans, while *C. fetus* is of veterinary importance due to its ability to colonize the intestinal mucosa and/or urogenital tract in cattle, causing abortion and sterility. *C. fetus* can also infect humans especially elderly and immunocompromized individuals [83, 148].

The isolation rates reported in previous Japanese studies on *C. jejuni* carriage in wild birds are variable. Kinjo *et al*. [98] found that 23.7% of tested pigeons (n=329) were infected by *C. jejuni* on a two-year survey. Fukuyama *et al*. [67] have reported a lower overall rate than ours. In fact, 7.9% (n=700) in all sampled birds and 13.5% (n=378) in pigeons were observed. Ito *et al*. [87] have described an overall rate of 14% (n=313) but a higher infection rate in crows, 34% (n=32) possibly due to their association with a municipal garbage dump. The infection rate varied between these studies and ours, possibly due to different sample sizes, sampling methods and investigated localities. Higher percentage of infection was found in crows and pigeons than in the Eurasian tree sparrow. The occurrence of *C. jejuni* in various wild bird species might have a link with their diet. In fact, the main food source for sparrow are seed, grain and insects while the scavenging birds such as crows and pigeons are known to feed in garbage [87]. The relationship between *Campylobacter* infection

and wild birds diet seems to be further confirmed by results reported by Ito *et al*. [87] and Kapperud and Rosef [92]. They found that herbivorous wilds birds were also infected but at a significantly lower rate than other omnivorous scavenger such as crows and gulls. These factors made the crows and pigeons to be more exposed to isolates from food animals and humans as their habitats are closely shared.

To gain insight to the pathogenicity of *C. jejuni* isolates from wild birds, we characterized the isolates for the presence of major virulence-associated genes such as *cadF*, *cdtA*, *cdtB*, *cdtC*, *ciaB*, *flaA* and *flaB*. The virulence genes screened to assess the pathogenesis of *C. jejuni* have been reviewed by Dasti *et al*. [44] and Young *et al*. [169]. The gene *cadF* encodes an outer membrane protein that interacts with host cells to bind the extracellular matrix protein fibronectin and thus is important for colonization. The cytolethal distending toxin (CDT) is encoded by *cdtA*, *cdtB* and *cdtC*, and their expression is required for their cytotoxicity effect. The tripartite CDT complex triggers the arrest of eukaryotic cells in the G2 phase of the cell cycle causing apoptosis of the concerned cell. The flagellin proteins encoded by *flaA* and *flaB* have been recognized as important factors in *C. jejuni* motility and invasion [72, 115]. Thirty-two isolates of *C. jejuni* from wild birds were screened for the presence of virulence genes, seven *C. jejuni* isolates (1 crow isolate and all 6 isolates from pigeons) were positive for all tested genes. The other 25 isolates were lacking *cdtA* in the virulence gene profile. This might be due to the complete absence of *cdtA*, or occurrence of mutations in respective gene.

Motility is critical for many of *C. jejuni* pathogenesis properties such as host colonization, secretion of virulence genes, host-cell invasion [169]. *C. jejuni* isolates from wild birds showed a reduced motility with exception of C38, which showed a high motility phenotype (Fig. 3A and 3B). On the other hand, PCR results confirmed the presence, in all isolates from wild birds, of *flaA* and *flaB*, the two major flagellin genes of *C. jejuni*. These results suggest that the defects in swarming motility are caused by lack or loss-of-function of other flagellar genes, or lack of chemotactic ability. It has been postulated that naturally, *C. jejuni* can produce non-motile deletion copies of wild type

strain that are more suitable to environments where the flagellar expression would be unnecessary otherwise [95]. These phenotypes may restore their motility, once favorable conditions are met.

Another objective of our study was to determine the invasive capabilities of *C. jejuni* isolates from wild birds to the human intestinal epithelial cell-line, Caco-2. At 1-hr post-infection, intracellular *C. jejuni* of tested isolates were comparable, followed by an identical decrease at 24-hr post infection for all isolates including the clinical isolate. However, at 48-hr post infection, isolates from wild birds showed a similar slight increase (i.e. multiplication inside Caco-2 cells) except the clinical isolate that showed a sharp significantly different increase. It has been reported that clinical isolates are hyper invasive compared to isolates from asymptomatic individuals [22, 61]. It also has been hypothesized that the virulent capacity of *C. jejuni* is attenuated by the lysosome system which prevents the bacteria to survive nor multiply inside cultured cells [109]. The tested isolates of *C. jejuni* from wild birds were invasive phenotype. Although weaker, the invasion of Caco-2 cells by the isolates of this study was observed and this highlights their potential as human pathogens.

To our knowledge, this study is the first study to investigate virulence-associated factors of *C. jejuni* isolates from wild birds. While the type strain NCTC11168 was greater in adherence and invasion capacities to Caco-2 cells than those of wild bird isolates, they invaded to the human intestinal epithelial cells, persisted and underwent replication in host cells. Since little is known about the link between wild bird origin of *Campylobacter* and human and livestock-associated strains, further studies such as hemolysis and cytotoxicity assay, *in vivo* infection studies, molecular epidemiological studies need to be performed to elucidate the pathogenicity of wild bird isolates and their impact on human and animal health.

5. Summary

The prevalence of *C. jejuni* in wild birds is a potential hazard for human and animal health. The aim of this first chapter was to establish the prevalence of *C. jejuni* in resident wild birds of the neighborhood of human house and livestock farms in Obihiro area and characterize their virulence using in vitro virulence studies such as virulence gene profiles, motility assay on soft agar, and invasion assay in Caco-2 cells.

Thirty four samples (19.7%) were positive for *Campylobacter* of which 94.1% (32/34 samples) were *C. jejuni*. Additionally, one *C. coli* and one *C. fetus* were isolated. The screening of 7 virulence genes by real-time PCR showed that 7 of the 32 *C. jejuni* isolates were fully virulent. However, most isolates from crow (with exception of one) and that from Eurasian tree sparrow were lacking *cdtA* implying possible sequence variations in this gene. The motility, an important trait of *C. jejuni* pathogenesis was assessed on soft agar for the 7 isolates harboring all the 7 virulence genes. We have found that all isolates tested but one (C38) had a reduced-motility phenotype suggesting a loss of functionality of genes involved, or lack of chemotactic ability. The cell invasion and intracellular multiplication of *C. jejuni* from wild birds compared to the reference clinical strain NCTC11168 were reduced but not cleared by Caco-2 cells immune mechanisms.

Taken together, our results showed that *C. jejuni* is prevalent in wild birds of Obihiro area. Some isolates harbored all virulence-related genes screened. Moreover, the motility and infection assay on Caco-2 cells showed that these isolates can be potentially a hazard to public health. Molecular epidemiological study of similarity between wild bird isolates and patient isolates is required to determine the potential involvement of wild bird in human infection.

Table 3 Frequency of *C. jejuni* isolation in wild-living birds in Obihiro

Birds	Total no. of samples	No. of samples positive for *Campylobacter* spp. (%)	Isolated species and proportion (%)	
Crow (*C. corone* and *C. macrorhynchos*)	139	27 (20.9)	*C. jejuni*	25 (92.6)
			C. coli	1 (3.7)
			C. fetus	1 (3.7)
Pigeon (*C. livia* and *S. orientalis*)	24	6 (25.0)	*C. jejuni*	6 (100.0)
Eurasian tree sparrow (*P. montanus*)	10	1 (10.0)	*C. jejuni*	1 (100.0)
Total	173	34 (19.7)	*C. jejuni*	32 (15.5)
			C. coli	1 (10.6)
			C. fetus	1 (10.6)

Table 4 Real-time PCR results of virulence-related genes in *C. jejuni* isolated from wild birds

Birds (no. of samples)	*cdtA*	*cdtB*	*cdtC*	*flaA*	*flaB*	*ciaB*	*cadF*
Crow (24)	-	+	+	+	+	+	+
Crow (1)	+	+	+	+	+	+	+
Pigeon (6)	+	+	+	+	+	+	+
Eurasian tree sparrow (1)	-	+	+	+	+	+	+

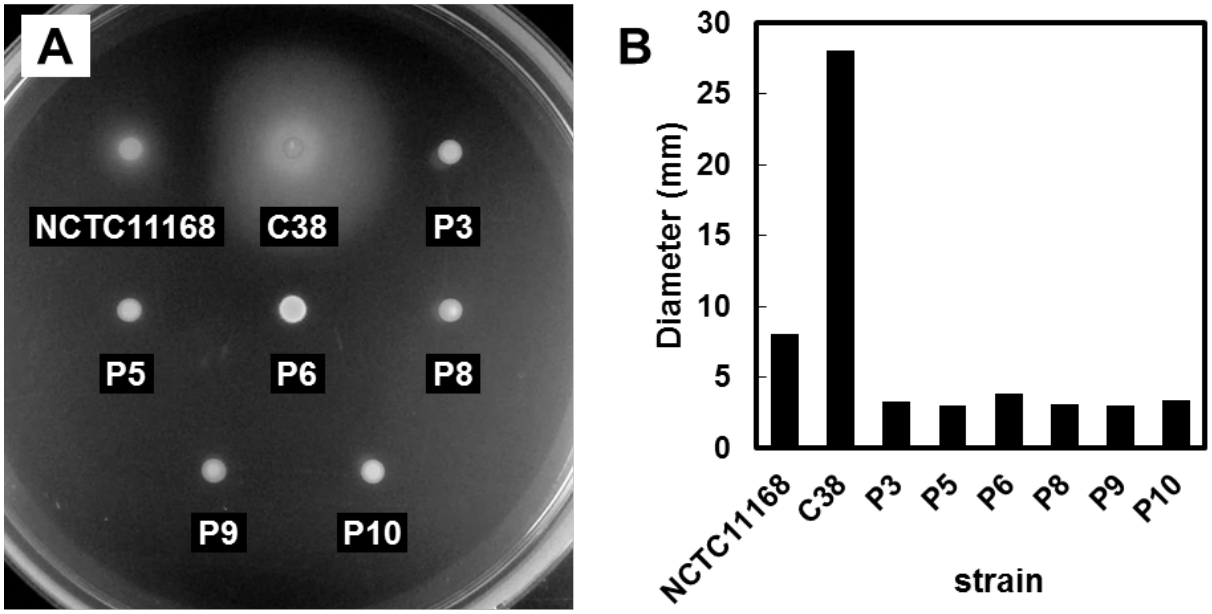


Figure 2 Flagella-mediated motility of *C. jejuni* isolates from wild-living birds. NCTC11168 was used as positive control, "C" refers to isolates from crow and "P" refers to those taken from pigeon.

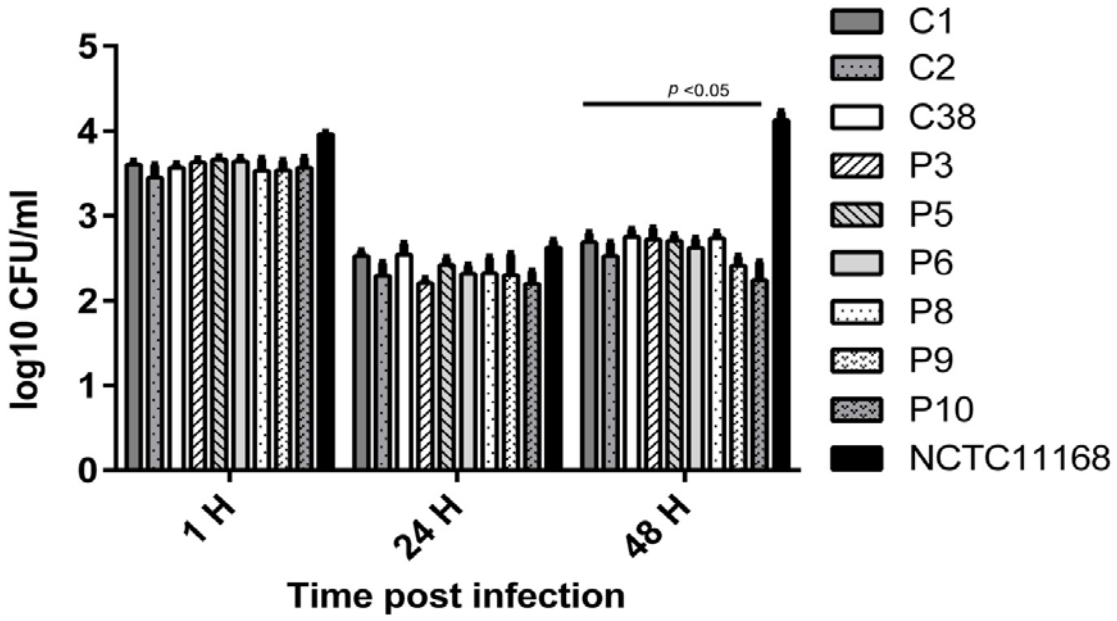


Figure 3 Flagella-mediated motility of *C. jejuni* isolates from wild-living birds. NCTC11168 was used as positive control, "C" refers to isolates from crow and "P" refers to those taken from pigeon.

Chapter II

Molecular epidemiology of *Campylobacter jejuni* isolated from wild birds

1. Introduction

C. jejuni is the leading bacterial causes of human enteritis worldwide [3, 35]. The incidence of *Campylobacter enteritis* varies in many countries, but it is believed to be a heavy burden for epidemiology and surveillance divisions of developed countries. For example, campylobacteriosis was the most frequently reported zoonosis in the European Union (EU) in 2011 [57]. Besides, the role of *C. jejuni* in causing disease in the US, EU, Canada, New Zealand and Japan, to cite only those, has been recognized [15, 57, 111, 129, 145].

Based on the available research data, it is believed that most cases of human campylobacteriosis are caused by contact or consumption of contaminated poultry, meat, milk and water [5, 30, 89, 157]. However, there are other recognized potential sources, including pets; environmental water and wild birds [2, 41, 48, 65, 105], some of which need a close monitoring in order to understand their role in the transmission of campylobacters.

The multi locus sequence typing has been validated as a tool that can be used in molecular epidemiology to infer relatedness of isolates from different hosts. To date, when applied to *C. jejuni* population for genetic analyses purposes, MLST studies have focused mostly on isolates from livestock and human campylobacteriosis. However, there has been an increasing inclusion of isolates from the environment and/or from wild birds [28, 64, 65, 71] which has a merit to turn the epidemiological survey into a more complete picture.

The isolates from food animal show a genotypic overlap with those of humans but isolates from wild birds tend to form distinct clades that suggest more marked host- specificity [28, 36, 65] as opposed to isolates from farm animals and humans where the host-specificity is less pronounced [71]. However, from previous researches and according to data available in the *C. jejuni* database (http://pubmlst.org/campylobacter), some, few admittedly, STs found in wild birds have been reported in humans, livestock and/or pets [71, 130]. Moreover, some reports have suspected human campylobacteriosis to be associated with wild bird-contaminated environments [65]. This emphasizes on the importance to keep the monitoring of *Campylobacter* spp. carriage in wild birds.

In Japan, a number of publications have established the prevalence of *C. jejuni* in wild birds. However, in spite of the usefulness of such reports, no molecular typing was done for inference of relatedness between isolates from wild birds and those susceptible to cause campylobacteriosis in human.

In our previous study (data not shown), we have isolated 32 strains of *C. jejuni* from wild birds. The results of virulence tests conducted have shown that the isolates from wild birds may be potentially hazardous to the public health, causing campylobacteriosis in human and food animals. However, the possible implication of these isolates in human campylobacteriosis could not be determined.

To address the question of possible involvement of these isolates to human and farm animal's campylobacteriosis, we have performed MLST and compared genotypes obtained with the available STs from human and livestock.

2. Material and methods

Isolates used for MLST

We have analyzed 32 *C. jejuni* isolates from wild birds isolated in Tokachi area, eastern Hokkaido, Japan. Of the 32 *C. jejuni*, 25 were isolated from crow (*C. Corone* and *C. macrorhynchos*), 6 from pigeon (*Columba livia* and *S. orientalis*) and 1 from the European Tree Sparrow (*P. montanus*). We have not included the two *Campylobacter* species, *coli* and *fetus* because they were isolated in non-representative numbers (only one isolate for each).

MLST

The MLST protocol, primers and conditions used have been described previously [51]. Briefly, the DNA fragments for seven housekeeping genes; *aspA* (aspartase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyl transferase) *pgm* (phosphoglucomutase), *tkt* (transketolase) and *uncA* (*ATP* synthase alpha subunit) were amplified and sequenced using primers shown in table 1.

Each 50-μl amplification reaction mixture comprised ~20 ng of *Campylobacter* chromosomal DNA, 1 μM each PCR primer, 1× PCR buffer (Perkin-Elmer Corp.), 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, and 1.25 U of Taq polymerase (Perkin-Elmer Corp.). The PCR reaction conditions were denaturation at 94°C for 2 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, for 35 cycles. The amplification products were purified using QIAquick PCR purification Kit. The nucleotide sequences were determined using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). DNAsis software was used to assemble and generate consensus sequences from chromatograms obtained. Allele numbers, STs and CCs were assigned by submitting DNA sequence to the *C. jejuni* MLST database website (http://pubmlst.org/campylobacter).

Assignment of allele numbers, STs and CCs

A neighbor-joining tree was constructed using concatenated MLST allele sequences of each isolate using MEGA6. The host and regional specificity were analyzed by querying additional data from the *C. jejuni* MLST database.

Phylogenetic and epidemiological analyses

A neighbor-joining tree was constructed using concatenated MLST allele sequences of each isolate using MEGA6. The host and regional specificity were analyzed by querying additional data from the *C. jejuni* MLST database.

3. Results

Diversity of *C. jejuni* MLST genotype of isolates from wild birds

Multilocus Sequence Typing (MLST) was used to type 32 *C. jejuni* isolates. The MLST analysis resulted in 19 STs including 13 novel allelic profiles and 14 (73.7%) new STs. The most frequent STs were ST-2209 and ST-448 with 5 (26.3%) isolates each, ST-5523 having 4 (21.1%), and ST-5524 and ST-5530 with 2 (10.5%) isolates each. The remaining STs have been attributed to only one isolates for each of them.

In spite of many CCs available in the *C. jejuni* database, only 8 of the 32 typed isolates could be assigned to known CCs (Table 6). In total, 5 isolates (15.6%), all from pigeon, were assigned to ST-179 CC and 3 (9.4%) isolates from crow were assigned to ST-952 CC.

The phylogenetic tree constructed from *C. jejuni* isolates from wild birds showed no clear cluster among isolates, except those from pigeon, belonging to ST-179 CC. The isolates from crow showed a more diverse genetic relationship. Also, the isolate from the Eurasian tree sparrow was not associated to any other isolate and showed higher distance from other isolates (Fig. 4)

Association between genotypes and host and geography

Firstly, in order to investigate the genetic relationship between isolates from wild birds and those from human and livestock in Japan, a maximum likelihood tree was constructed. As shown by Fig. 5A isolates from wild birds were genetically distant, with distinct clusters, which suggests a host-specificity compared to other isolates. In fact, isolates from human, cattle and poultry clustered together, showing a close genetic relationship and thus no host-specificity contrary to those from wild birds. However, when a subtree was retrieved from the maximum parsimony tree, some isolates from poultry were seen clustering with those from crow and one isolate from human lose to those of the ST-2209 (Fig. 5B), suggesting some similarity in their genotype. This finding implies two hypotheses: (i) if broader sampling was done, it may be possible to find genotypes matching those from wild birds and (ii) since the recombination is *Campylobacter* spp. is known [149], some of wild

birds isolates may have been acquired from chicken or human suggesting circulation of the bacterium in diverse hosts.

Secondly, given the lack of association between isolates from wild birds and those from humans, cattle and poultry in Japan, potential hosts of ST-952 and ST-179 complex were searched in the *C. jejuni* PubMLST database. As of January, 2015; including our data, 87 and 61 *C. jejuni* isolates belonging respectively to ST-179 and ST-952 were present in the database. The common sources for ST-179 and ST-952 complex were environmental waters (52% and 37%), wild birds (17% and 23%) and human (10% and 15%). However, other sources of less significant number, including cattle and pet were reported and deposited in the database. Moreover, several ST and CC linked to human campylobacteriosis have also been isolated from cattle and/or avian species. However, neither complex nor any of the ST found by our study were ever reported in any other host in Japan.

Lastly, to understand the genetic relationship between our isolates and those from wild birds elsewhere, we queried the MLST database and a phylogenetic tree was constructed (Fig. 6). The phylogenetic tree obtained from wild bird's isolates queried from the database showed that our isolates formed 3 specific clusters; i.e. 2 separate clusters for crow isolates and a single cluster for pigeon isolate. Moreover, as show in the previous figure, the isolate from the Eurasian Tree Sparrow was genetically distant from other isolates.

4. Discussion

Of 32 *C. jejuni* isolated and typed in this study 19 STs were determined. Out of the 19 STs observed this is the first report of 14 of them. Our data is consistent with many of previous researches showing a high genotyping diversity in *Campylobacter* isolates from various sources [50, 105, 134], including those from wild birds [71]. Within ST reported, 2 STs (2209 and 448) were detected 5 times (26.3%) each; the ST-5523, 4 times (21.1%) and two STs (5524 and 5530) twice (10.5%) each of them. ST-2209 was assigned to 5 of 6 *C. jejuni* isolates from pigeons. This ST has previously been assigned to pigeon [121]. Three novel STs (5523; 5524 and 5530) were identified more than once in the current study. These results confirmed the specificity of isolates from wild birds as it has been reported previously [71]. However, the ST-448 found in 5 different isolates was in different study isolated from different hosts including human, rabbit, environmental waters, dog and wild birds (PubMLST database). It is possible that this isolate was acquired in wild birds from livestock or human. In fact, some *C. jejuni* STs are reported to colonize various hosts [37, 50, 134]. The ST-448 was previously reported 3 times in UK and once in Switzerland from human. Our finding may have a significant implication for campylobacteriosis prevention and control as this ST is able to cause disease in human.

Five and three isolates were respectively assigned to ST-179 and ST-952 CCs. Both CCs were previously reported in samples from wild birds and environmental waters [101, 121]. The CC-179 previously reported as a pigeon-associated CC [121] was isolated in UK from beach sands and wilds birds (PubMLST database), but was also found in clinical isolate in children from Lithuania [130]. Data from our study allowed assigning this CC to 5 of the 6 isolates from pigeon confirming its host-specificity. It has been suggested that isolates from pigeon show a lower diversity than other birds [121]. The CC-952 assigned to 3 of our isolates has been previously allocated to isolates from rabbit, environmental waters and wild birds [101]. *C. jejuni* is reported to undergo phase variation in passage through hosts, this allows the pathogen to adapt and colonize a range of hosts [97, 155]. It is noteworthy mentioning that the CC-952 was once reported in a human infection case (PubMLST

database) involving the ST-2111 also isolated in one of our samples. This CC has a broad host range-target making it a good candidate for human campylobacteriosis [71].

Our study aimed at establishing the relatedness of isolates of this study and those from human, poultry and cattle in Japan to determine a possible overlap between genotypes which would imply a circulation of same genotypes between those hosts and human. Our finding conformed with previous reports on the high degree of host-specificity seen in wild-birds [28, 36, 38, 39] as no overlap was observed between *C. jejuni* isolates and those from human, poultry and cattle. Also, the 2 CCs assigned to isolates of this study; as well as STs were not yet reported in Japan.

Our study is the first one to perform a molecular typing using MLST on *C. jejuni* isolates from wild birds in Japan which, in addition to the host-specificity discussed above, justify the lack of previous reports on the 2 CCs. Geographic-specificity for *C. jejuni* has been suggested previously [65], indeed our results are in tandem. Nineteen STs assigned, 14 were reported for the first time, i.e. are only found in Japan. However, the 2 CCs assigned to some isolates of this study have been reported elsewhere suggesting that if broader sampling is performed; shared STs among isolates might be found.

5. Summary

In Japan, campylobacteriosis is typically conveyed to human through consumption of contaminated animal products, mainly poultry. However, thus far no study has yet evaluated the potential contribution of environmental sources such as wild birds to this infection. Wild birds live in close contact with human and livestock even though they are known carriers of *Campylobacter* spp. The aim of the current study was to establish MLST profiles of *C. jejuni* isolated from wild birds and compare the obtained genotypes to those from humans, poultry and cattle in Japan.

Thirty two strains of *C. jejuni* isolated from resident wild birds in Tokachi area, Hokkaido, Japan were analyzed using MLST method. Of the 32 isolates, 19 STs were determined, of which 13 were novel and reported for the first time by this study. As previously reported, our results confirmed the low clonality of genotypes of *C. jejuni* isolated from wild birds. In fact, only two CCs were found in all the 32 isolates: ST-179 complex attributed to 5 isolates were all from pigeons and ST-952 complex assigned to three isolates were from crows. This is by far the first study to report the 19 STs in Japan, suggesting that wild birds are not a significant route of transmission of campylobacteriosis to human and food animals. The two CCs found are mostly associated with wild birds and environmental samples as documented by other studies across the world. However, the two CCs and some genotypes found in this study were sometimes linked to human, livestock or pets cases, suggesting that they may trigger campylobacteriosis.

Taken together, the current study showed that the genotypes of *C. jejuni* isolates from wild birds are host-specific and not genetically close to genotypes affecting human or food animal in Japan so far. However, some genotypes of isolates found in wild birds have been reported in humans or other human-contaminating pathways in certain countries. In conclusion, the current study hypothesizes that the isolates from wild birds are a potential threat to human and animal health in regard to the transmission of campylobacteriosis.

Table 5 PCR and sequencing primers used for MLST.

Gene	Reaction	Primer sequence	Amplicon size (bp)
asp	PCR	5′-AGTACTAATGATGCTTATCC-3′	899
		5′-ATTTCATCAATTTGTTCTTTGC-3′	
	Sequencing	5′-CCAACTGCAAGATGCTGTACC-3′	
		5′-TTAATTTGCGGTAATACCATC-3′	
gln	PCR	5′-TAGGAACTTGGCATCATATTACC-3′	1,262
		5′-TTGGACGAGCTTCTACTGGC-3′	
	Sequencing	5′-CATGCAATCAATGAAGAAAC-3′	
		5′-TTCCATAAGCTCATATGAAC-3′	
glt	PCR	5′-GGGCTTGACTTCTACAGCTACTTG-3′	1,012
		5′-CCAAATAAAGTTGTCTTGGACGG-3′	
	Sequencing	5′-GTGGCTATCCTATAGAGTGGC-3′	
		5′-CCAAAGCGCACCAATACCTG-3′	
gly	PCR	5′-GAGTTAGAGCGTCAATGTGAAGG-3′	816
		5′-AAACCTCTGGCAGTAAGGGC-3′	
	Sequencing	5′-AGCTAATCAAGGTGTTTATGC GG-3′	
		5′-AGGTGATTATCCGTTCCATCGC-3′	
pgm	PCR	5′-TACTAATAATATCTTAGTAGG-3′	1,150
		5′-CACAACATTTTTCATTTCTTTTTC-3′	
	Sequencing	5-GGTTTTAGATGTGGCTCATG-3′	
		3′-TCCAGAATAGCGAAATAAGG-3′	
tkt	PCR	5′-GCAAACTCAGGACACCCAGG-3′	1,102
		5′-AAAGCATTGTTAATGGCTGC-3′	
	Sequencing	5′-GCTTAGCAGATATTTTAAGTG-3′	
		5′-ACTTCTTCACCCAAAGGTGCG-3′	
unc	PCR	5′-ATGGACTTAAGAATATTATGG C-3′	1,120
		5′-GCTAAGCGGAGAATAAGGTGG-3′	
	Sequencing	5′-TGTTGCAATTGGTCAAAAGC-3′	
		5′-TGCCTCATCTAAATCACTAGC-3′	

Table 6 Diversity of *C. jejuni* isolates from wild birds typed by MLST

CC	ST	Number of Isolates	Isolates
ST-179 Complex	2209	5	P3, P5, P8, P9, P10
ST-952 Complex	5527	1	C19
	2761	1	C21
Unassigned	448	4	C10, C24, C25, P6
	2111	1	C56
	3322	1	C74
	5523	4	C1, C7, C41, C78
	5524	2	C2, C12
	5525	1	C8
	5526	1	C9
	5528	1	C14
	5529	1	C33
	5530	2	C38, C50
	5531	1	C47
	5532	1	C63
	5533	1	C65
	5534	1	C67
	5535	1	C68
	5536	1	S1

Table 7 Prevalent sources for *C. jejuni* ST-179 and ST-952 complex

CC	No. of ST	Most frequent ST (No/%)	No. of isolates	Source (No./ %)
ST-179	32	3889 (24/28)	87	Environmental waters (45/52)
				Wild birds (15/17)
				Sand bathing (9/10)
				Human (9/10)
				Chicken (3/3)
				Cattle (2/2)
				Dog (1/1)
				Unknown (3/3)
ST-952	49	4871 (3/6)	61	Environmental waters (22/37)
				Wild birds (14/23)
				Human (9/15)
				Chicken (7/12)
				Cattle (2/3)
				Non-specified animals (2/3)
				Farm environment (1/2)
				Dog (1/2)
				Rabbit (1/2)
				Unknown (1/2)

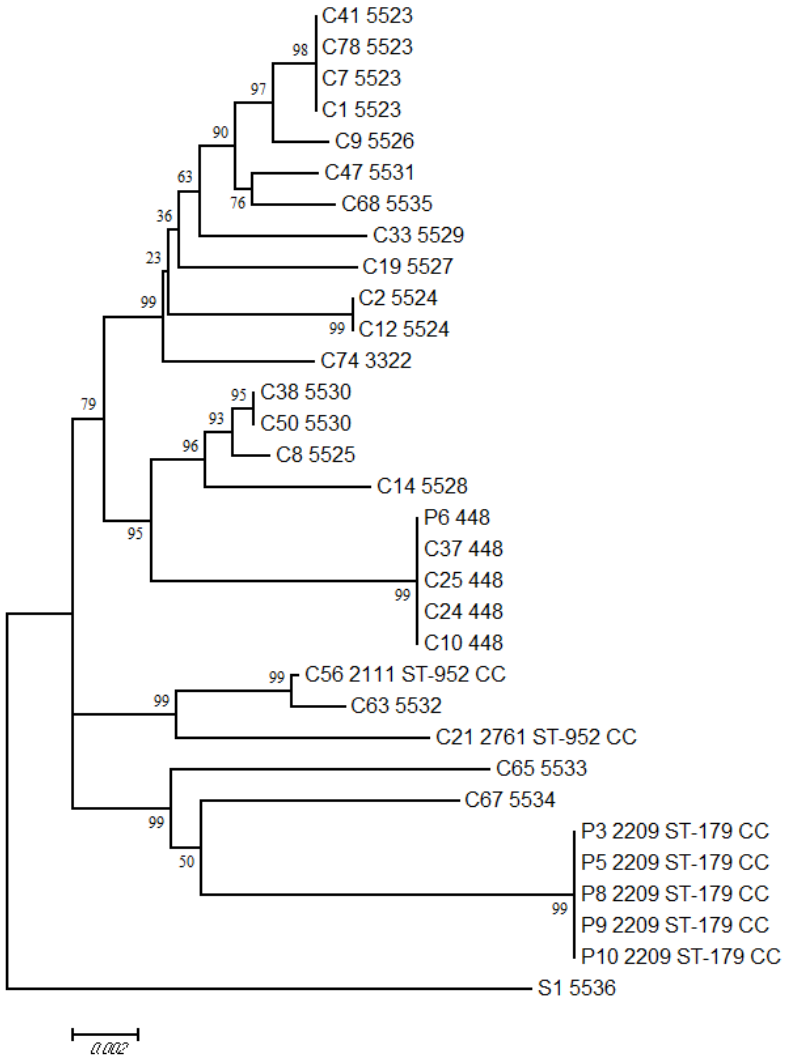
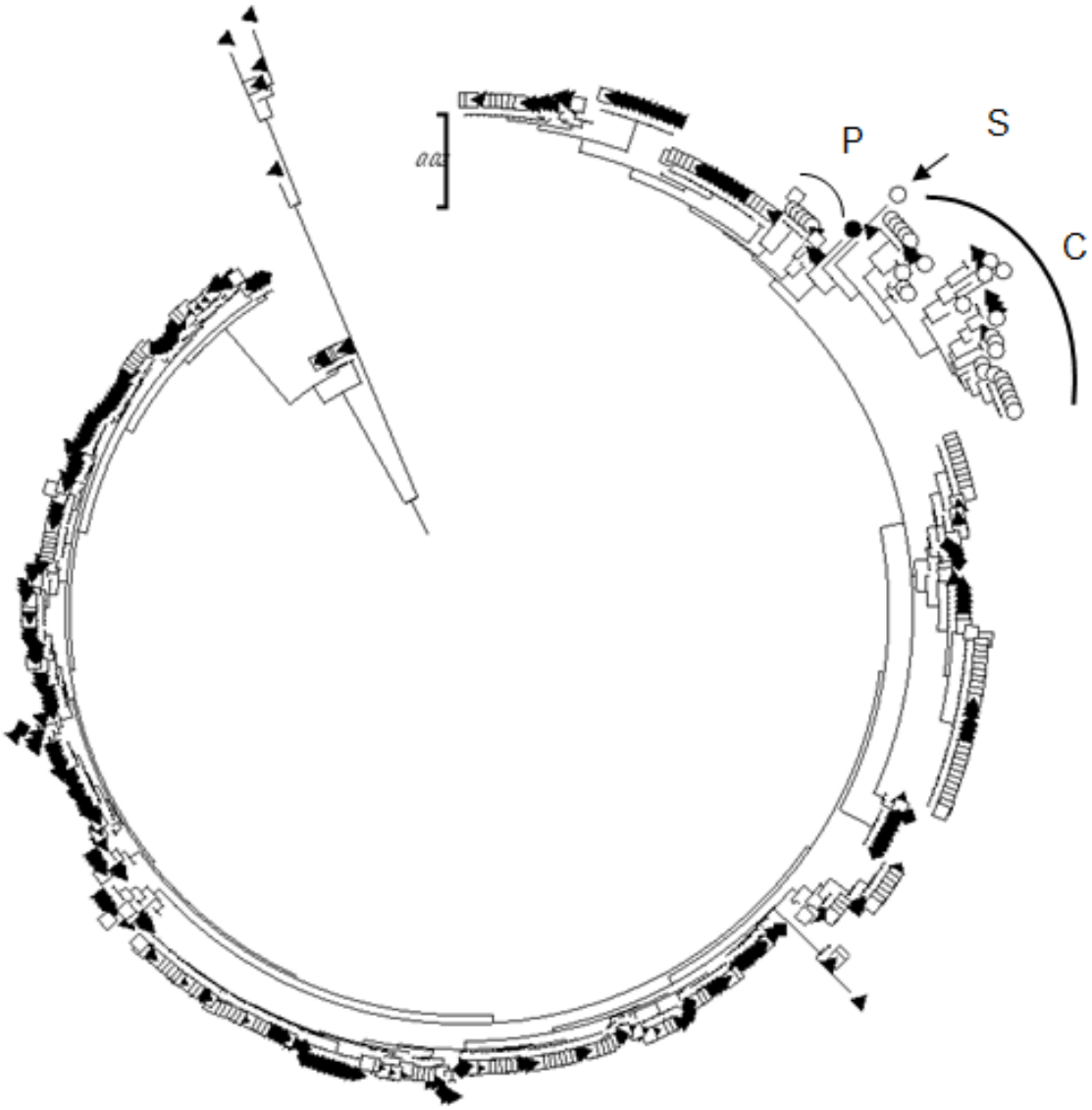


Figure 4 Phylogenetic tree of *C. jejuni* isolates from wild birds typed using MLST was constructed by MEGA software [151] using neighbor-Joining method [139]. Sequence data for the analysis are from the MLST allele for each isolate. The sequences were aligned using Muscle [54]. The number shown on nodes are bootstrap values (multiplied by 100) based on 10000 replicates [53, 136]. The first letter and number represent the origin ("C" for crow, "P" for pigeon and "S" for isolates from the Eurasian tree sparrow) and numerical order for each isolate, the second number show the ST assigned to isolate after MLST analysis and lastly, the CC is shown when applicable.

A



B

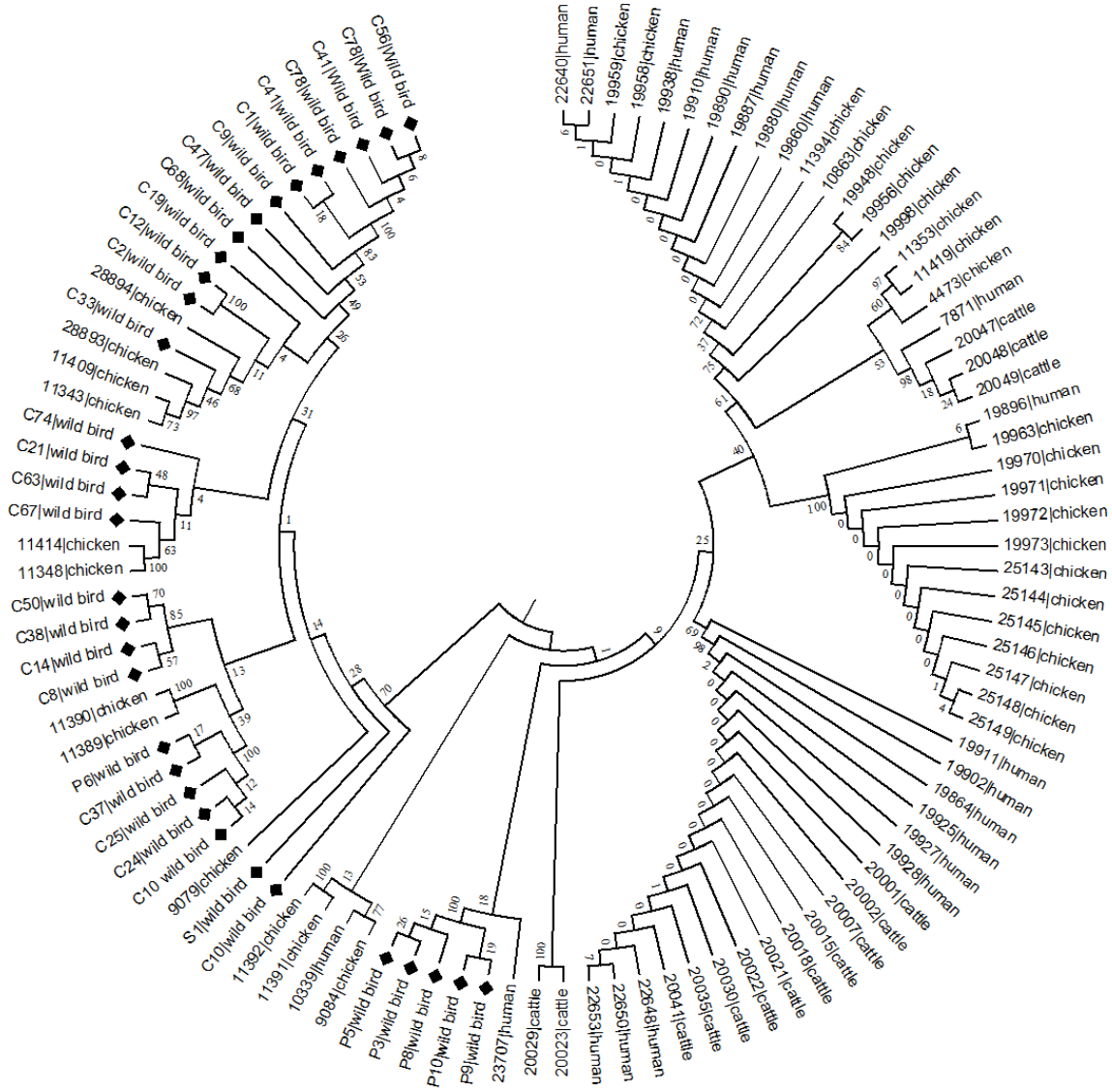


Figure 5 Maximum likelihood tree constructed using MLST sequences of isolates from humans, cattle, poultry and wild birds of Japan queried on pubmlst database website. Sequences were concatenated and aligned using Muscle [54] and tree constructed using MEGA 6 [151]. [A] Isolates from poultry (closed triangle), those from cattle (closed diamond), isolates from human (empty rectangles) and from wild birds (closed circles) are shown [B] A subtree was generated from the main tree, and zoom on the clade made by isolates from wild birds.

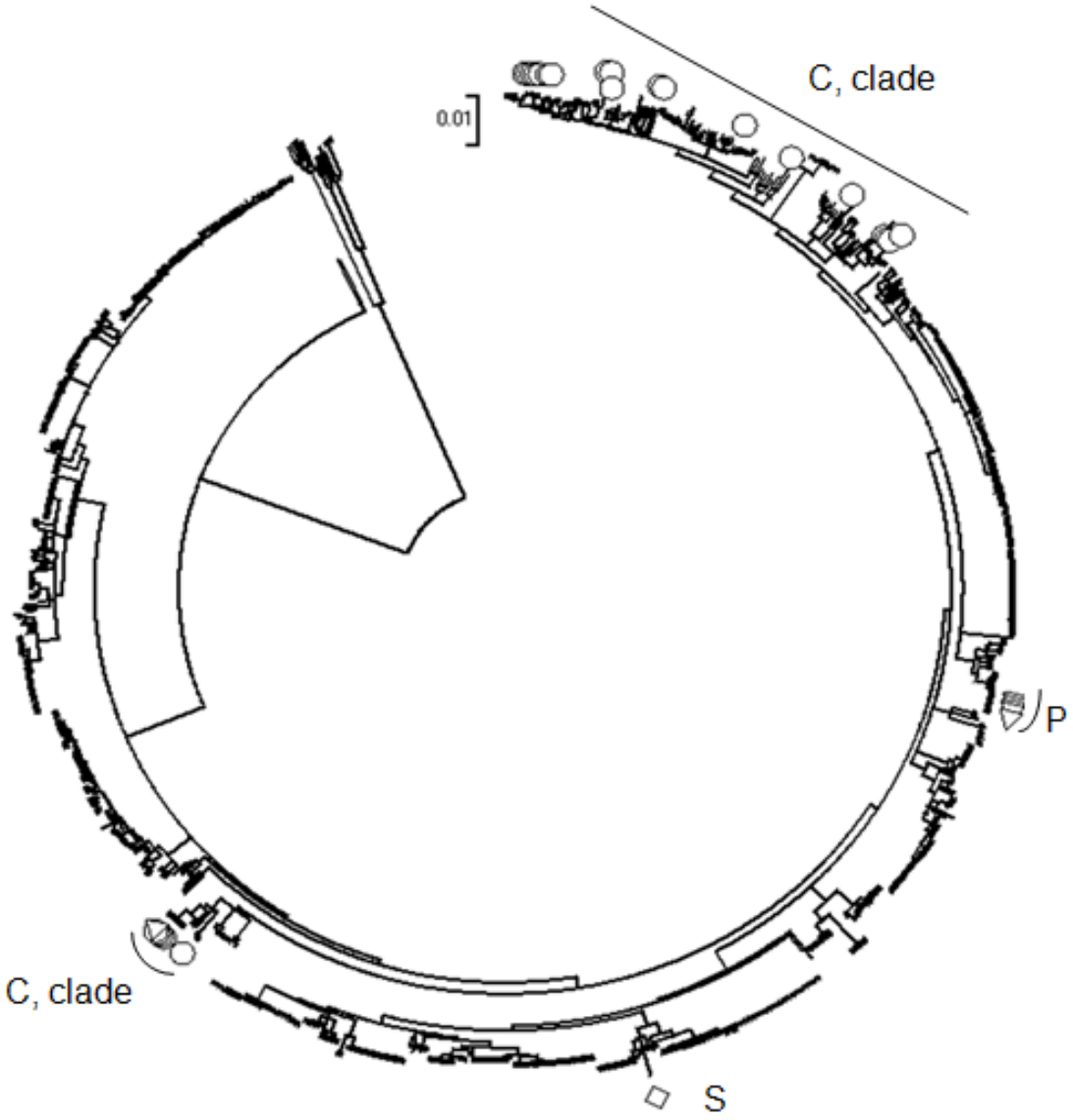


Figure 6 An unrooted phylogenetic tree of *C. jejuni* wild birds isolates queried at the *C. jejuni* MLST database website (http://pubmlst.org/campylobacter) against isolates of our study. The nucleotide sequences of the 7 MLST alleles were concatenated and aligned using MUSCLE algorithm [54] and the evolutionary history was inferred using the Neighbor-Joining method [139] and distances computed using the Jukes-Cantor method. The analysis involved 841 nucleotide sequences. Evolutionary analyses were conducted in MEGA6 [151]. Open circle shape show the position of isolates of our study.

Chapter III

Comparative genome analysis

of *Campylobacter jejuni* isolates from wild birds

1. Introduction

C. jejuni are fastidious bacteria, spiral-shaped, gram-negative bacilli harboring a flagellum at either one or both ends which enable them to be motile. *C. jejuni* has the ability to colonize multiple animal hosts; domestic and wild animals, and can be found in environment sources. Yet, this bacterium is a leading bacterial cause of foodborne poisoning worldwide and some patients develop post-infection complications such as GBS.

The primary route of campylobacteriosis transmission to human is undeniably the consumption and handling of contaminated animal food products and water, poultry being the most common source.

In contrast, environmental pathways of human campylobacteriosis continue to be poorly understood. However, few risk factors have been identified for sporadic campylobacteriosis and they include drinking contaminated water during swimming [66], which in turn can be contaminated by dropping of wild animals [127].

The MLST has contributed greatly to understanding the predilection of some clones amongst *C. jejuni* isolates from various sources. It has been shown that some MLST CCs such as the ST-21 complex are widely distributed in various hosts whereas others such as ST-179 complex seem to be more host-specific.

The occurrence of *C. jejuni* in wild birds has been extensively reported and these hosts are considered a potential hazard in the transmission of campylobacteriosis to human. In a study in the UK, the ST-179 complex was found in pigeons and was described as pigeon-associated. This CC has also been found in human [130] and sand from UK bathing beaches (PubMLST database). The ST-448 was previously reported (PubMLST database) in human three times in UK and one time in Switzerland. This genotype was also found in other sources including dog, wild birds and environmental waters making it broad-range target. Lastly, the ST-5523 and ST-5524 were first reported by our study and are only isolated from wild birds so far.

Whereas *C. jejuni* is able to colonize multiple animal species, this pathogen mostly expresses its virulence in humans. The factors influencing the ability of *C. jejuni* to colonize specific hosts are not well elucidated.

To understand the genetic acquisition and virulence mechanisms in various hosts, it is important to have the genomes from these different hosts. However, there are no genomes available yet from wild birds.

In our previous study, 32 strains of *C. jejuni* were isolated from various wild birds residing in Tokachi area, Obihiro-Hokkaido, Japan. The virulence tests in vitro and typing using MLST showed that some isolates are fully virulent and that some genotypes were reported to cause campylobacteriosis in human, food animals or pets. However, most of isolates were assigned to genotypes that are clearly distinct from those found in humans or in human-contaminating pathways. Moreover, for most of them, the virulence phenotype was either lacking one of the virulence genes tested or having an impaired motility on soft agar, suggesting that some variation in the gene sequences may be responsible.

In this chapter, whole genome sequencing of 8 isolates from wild birds was performed. We chose the ST-448 and ST-2209 of the ST-179 complex, because they are adapted to wild birds but might be transmitted to human or other animals. In contrast, ST-5523 and ST-5524 were chosen to represent new genotypes that are restricted to wild birds.

2. Material and methods

Isolates used

In total, eight isolates of *C. jejuni* were analyzed. All these were isolated from cloacal swabs of wild birds in the period from May to December 2010. Of the eight samples, 4 (C1; C2; C10 and C12) were from crow (*C. corone* and *C. macrorhynchos*) and other 4 (P3; P5; P9 and P10) from pigeon (*S. orientalis* and *Columba livia*). In a previous experiment, the used isolates were typed using Multilocus Sequence Typing (MLST) method. Thus, they were assigned to ST-5523 and ST-448 for C1 and C10 respectively, and ST-5524 for C2 and C12. All isolates from pigeons were assigned to ST-2209. The isolates will therefore be referred to as, C1-5523, C2-5524, C10-448, and C12-5524 for those from crow, and P3-2209, P5-2209, P9-2209 and P10-2209 for pigeon's. All isolates were sequenced from fleshly cultivated bacteria from frozen stocks.

Genomic DNA preparation and quantification

To prepare the genomic DNA, each of 8 samples was cultured from frozen stock on modified charcoal cefoperazone agar (mCDDA) and incubated in microaerophilic environment at 42 ºC for 48 h. Next, a well-isolated characteristic colony was inoculated into 10 ml of Bolton broth (OXOID) supplemented with horse defibrinated-blood and incubated in same condition as above. From the second culture, 2 ml were used to collect bacteria pellet to be used for genomic DNA extraction using Dneasy Blood & Tissue kit (250) (Qiagen, Hilden, Germany) following manufacturer's instructions, except that the final genomic DNA was eluted in double-distilled water and quantified with Qubit 2.0 fluorometer before processing for preparation of sequencing library.

Library preparation and genomic DNA sequencing

Genomic DNA sequencing library was prepared using Nextera XT sample preparation Kit (illumina, Inc. San Diego, Ca, USA) following the manufacturer's instructions. The library preparation was done using 5 µl of genomic DNA (final concentration, 1 ng per reaction) according

to the Nextera XT protocol (Version October, 2012). Briefly, the DNA was tagged and fragmented (tagmented) in 5 µl of Amplicon Tagment Mix and 10 µl of Tagment DNA buffer (illumina, Inc. San Diego, Ca, USA).

Tagmentation reactions were performed by incubation at 55ºC for 5 min, followed by a neutralization step using 5 µl of Neutralize Tagment Buffer for 5 min. Tagmented DNA (25 µl) was used as template in a 50 µl limited-cycle PCR (12 cycles) along with carefully-selected dual indicies and processed as outlined in the Nextera XT protocol.

Amplified DNA was purified using 25 µl of AMPure XP beads (Beckman Coulter, Fullerton, CA, USA), washed in a freshly-prepared 80% Ethanol and resuspended in Resuspension buffer (Illumina, San Diego, CA, USA).

The fragment size distribution of this library was analyzed using a 2100 Bioanalyzer with a High Sensitivity DNA assay kit (Agilent Technologies, Santa Clara, CA, USA).

The purified DNA was normalized with mixed Library normalization beads/additives and 0.2 N NaOH (final concentration, 0.1 N NaOH). In preparation for cluster generation and sequencing, equal volumes of normalized library from each sample were combined, diluted in hybridization buffer and heat-denatured before pooling to Miseq cartridge.

Libraries were sequenced using the Miseq Sequencer (illumina Inc., San Diego, CA, USA) running a Miseq control software version 2.1.13.0, and the machine was run to generate, after demultiplexing step, FASTQ-only data, convenient for further analysis using a third-part software.

Sequencing analysis, assembly and mapping

Reads obtained from Miseq run, were trimmed and assembled "*de novo*" using CLC Genomic Workbench 6.5.2 (CLC bio, Qiagen, Hilden, Germany). The trimming was carried out using the "trim sequences" tool. Trimming settings were set to: "No ambiguous nucleotides allowed, low quality limit of 0.03 and removal of 20 nucleotides from the 3' which frequently have a lower quality". Furthermore, Nextera XT adapter sequences were removed using the adapter trimming option and the Nextera XT transposases sequences.

After trimming, reads were used to generate the quality control report and then were "*de novo*" assembled using "*De novo* assembly tool" with default settings and mapping, automatic bubble size determination and 64-word size. The scaffold was performed with auto-detect paired distances.

Furthermore, trimmed reads were mapped to various *C. jejuni* genomes, to *C. coli* and to 5 known *C. jejuni* plasmids using "map reads to reference" tool with default settings.

Contigs obtained from *de novo* assembly, were ordered automatically with Mauve [42] and finally aligned to the genome of *C. jejuni* NCTC11168 strain using progressive Mauve [43].

Lastly, for quality assurance purposes, obtained whole genome of *C. jejuni* strains from wild birds were submitted for MLST profiles prediction using online service of the center for genomic epidemiology located at https://cge.cbs.dtu.dk/services/MLST/ in order to verify is same allele, ST and CC are assigned as in our previous experiment.

Genome annotation

Functional annotation was performed with the Rapid Annotation Subsystem Technology (RAST) online pipeline [13], using Glimmer [47] for gene calling and allowing frameshift correction, backfilling of gaps and automatic fixing of errors. Assigned functions were checked using BLAST. Phage sequences were detected using PhAge Search Tool web server available at http://phast.wishartlab.com [173].

Comparative genomic analysis

To order contigs and compare the genomes of our eight sequenced isolates with that of the strain NCTC11168, Mauve was used [42]. For the generation of visual circular comparisons between our sequenced genomes and the references, the Blast Ring Image Generator (BRIG) was used [4] and ACT-Artemis [31] were used. For alignment and genealogy construction, the CLC genomic workbench 6.5.2 and MEGA 6 were used.

3. Results

General features of the sequenced 8 *C. jejuni* isolates from wild birds

An illumina Miseq system was used to produce short read sequences of 150 bp from genomic DNA of the sequenced 8 *C.jejuni* isolates from wild birds. In total; 1435488, 3540622, 3839026, 2968922, 2338804, 2275220, 1659742 and 2773330 sequences in pair were obtained from C1-5523, C2-5524, C10448, C12-5524, P3-2209, P5-2209, P9-2209 and P10-2209 with an overall yield of 3,4 Gb for the eight library pooled-together in a Miseq cartridge. The trimming procedure discarded 21766 (0.10%) sequences that did not meet settings used.

Obtained trimmed sequences were assembled on a CLC Genomic Workbench using NCTC11168 as a closest complete reference genome for mapping (scaffolding) and assuming that all short reads map back to it. Thus, assembly generated 126, 78, 84, 81 contigs for respectively C1-5523 to C12-5524 isolates from crow and 74, 52, 70, 64 contigs for isolates from pigeon P3-2209 to P10-2209 with an average coverage of 79, 242, 231 and 207-fold for crow-originated isolates C1-5523 to C12-5524 respectively and an average depth coverage of 147, 191, 133 and 183-fold for isolates P3-2209 to P10-2209 respectively.

Moreover, the trimmed short reads were mapped to the 6 reference strains and 5 plasmids using CLC Genomic Workbench. The results are displayed in table 8 to 11. The estimated chromosome size of the eight isolates, the % GC content, the predicted protein-coding genes and RNA-coding genes are displayed in table 12. Lastly, the submission to the MLST prediction website has conformed our previous finding on isolates ST and CC.

The whole genome alignment was performed between our isolates and reference. In spite of large syntenic regions shown by genomes of *C. jejuni* isolates from wild birds compared to the reference NCTC11168 (fig. 7), it is striking that the genome size of isolates from crow are bigger by nearly 100Mb than those from pigeon and *C. jejuni* NCTC11168 taken as a reference. Accordingly, the number of gene calls vary according to the size of sequenced isolates. Thus, the number of genes from crow isolates was higher than that of isolates from pigeon and the reference NCTC11168.

The main difference between the isolates from wild birds and the human-originated NCTC11168 were associated to the insertion of prophages and known hypervariable regions of *C. jejuni*, which are the lipooligosaccharide, the flagellar modification locus and the surface polysaccharide (Fig. 8).

Phylogenetic analyses

Phylogeny of *C. jejuni* isolates from wild birds was inferred using sequences from 16s rRNA gene (Fig. 9). As expected, isolates from wild birds showed distinct clusters distantly located in the phylogenetic tree compared to isolates from human (Cjj 81116, Cjj 11168 and Cjj 81-176) and that obtained from chicken (Cjj RM1221) (Fig. 9). Moreover, isolates from pigeon belonging to the same ST 2209 (as assigned by MLST), and two isolates from crow (C2-5524 and C12-5524, belonging to the same ST 5524) are clustered together respectively.

Hypothetical virulence-associated genes and features in isolates from wild birds

C. jejuni in the manner of other members of the epsilonproteobacteria has its flagellum located at either one or both cellular poles. The importance of *C. jejuni* motility in colonizing animals, invading cultured cells and causing disease in susceptible animals, has been demonstrated. According to our results, the flagellar filament proteins (*flaA* and *flaB*) were found extremely truncated and thus very short (Fig. 10). We therefore questioned whether other genes involved in flagellar biosynthesis, modification and assembly were present.

Our results showed that major genes were present with a good nucleotide identity (Fig. 12A), including regulators of flagellar biosynthesis. However, *fliD* (encoding for flagellar cap) in all isolates and *fliK* (associated with the flagellar-hook-length control) only in isolates from pigeon, showed low sequence similarity compared to *C. jejuni* strains used as references (Fig. 10). From our results, major genes involved in iron-uptake were compared. The ferric enterobactin uptake receptor (*cfrA*) was identified in isolates from crow but is shorter of ~20 bp compared to that from NCTC11168 or RM1221. Moreover, it has a low sequence identity compared to references (Fig.

11A and 11B). Analogous to the very pathogenic *C. jejuni* subsp. *jejuni* strain 81-176, *cfrA* is missing in isolates from pigeon. However, the strain 81-176 encodes for a substitute ferric enterobactin receptor (*cfrB*) [172] which was not found neither in crow isolates nor in pigeon isolates that miss the principal ferric enterobactin receptor *cfrA*.

Furthermore, *tonB1*, *tonB3*, *exbB1*, *exbD1* and the iron transport protein (cj0177 in NCTC11168) showed a high level of variability in isolates from wild birds and 81-176 compared to NCTC11168 and RM1221(Fig. 11B).

The gene encoding for the tetracycline resistance protein tetO was found in C1-5523, C2-5524 and C12-5524 and not in other isolates including NCTC11168.

The alignment of *tetO* sequences (Fig. 12) between isolates from wild birds and sequences from *C. jejuni* strains 81-176 and HB93-13 and *C. coli* strain RM2228 showed that (i) the six sequences have same length (639 aa) and (ii) that they are very well conserved between bacteria compared.

The FHA protein functions as an adhesin in *Bordetella pertussis*. This protein is known to enhance the binding of possessing bacteria to epithelial cell surfaces due to its high affinity to carbohydrates [128].

FHA protein was identified in pigeon and not in crow isolates. Asakura et al. [9] reported a high affinity of FHA-positive *C.jejuni* in comparison to isolates possessing truncated FHA proteins. Moreover, the author observed that a prior incubation with recombinant protein reduced adhesion of FHA-positive bacteria to Caco-2 cells.

The Cytolethal distending toxin operon was found in all the eight sequenced isolates. However, while the length of the Cytolethal distending toxin (Cdt) operon was identical between isolates from pigeon and reference genomes used, cdt operon in isolates from crow was longer by 6 bp than other *C. jejuni* compared with (fig.13).

The type VI secretion system (T6SS) was found in C10_ST448 from crow sample. Various reported genes of this T6SS were annotated and blast searched for their homologues in *Vibrio cholerae* and in *Helicobacter hepaticus* (Table 13).

4. Discussion

The present study performed a comparative genomic analysis, by using whole-genome sequencing technology to examine the polymorphism of *C. jejuni* strains isolated from wild birds in Obihiro, Japan.

The whole genome sequence alignment using progressive Mauve [43] showed that our strains and *C. jejuni* NCTC11168 used as reference, have extended syntenic regions as shown by large locally collinear blocks which indicate regions that do not contain rearrangements of homologous sequence. However, the main differences were associated with insertion of prophage-like elements that were seen in isolates from crow. CJIE2-like elements were identified in all 4 isolates from crow. The CJIE4-like elements were identified in only C2-5524, C10-448 and C12-5524 (Fig. 8B). As expected, the hypervariable regions of *C, jejuni* also associated to the modification of cell surface structures showed a great degree of variability between compared strains (Fig. 8A).

The genomes of isolates from crow were strikingly significantly larger that their homologues from pigeon and NCTC11168 as a consequence of insertion of prophage sequences. Moreover, *tetO* gene and a type VI secretion system in one isolate from crow were identified. The impact of these findings is yet to be established, for example the involvement of these finding in the virulence of the possessing isolates.

C. jejuni, in the manner of many other bacteria, needs an iron source for an effective colonization of mammalian cells. Since it does not possess any endogenous siderophore of its own, iron acquisition relies on iron hunting using outer-membrane transport system specific for iron source [110]. Analogous to the highly invasive *C. jejuni* strain 81-176 [80], the isolates from pigeon were missing the ferric enterobactin uptake receptor (*cfrA*). Moreover, this gene showed low level of identity in isolates from crow. The adjacent gene *tonB3* and the *tonB1* transport system (exbB1/*exbD1*/ *tonB1*) showed also a very low level of homology compared to query genes from *C. jejuni* strains NCTC11168 and RM1221. The sequence homologies were rather comparable to those

from strain 81-176. However, the later is known to encode for an alternative ferric enterobactin receptor *cfrB* which is a pseudogene in NCTC11168. This gene was found neither in isolates from crow or in those from pigeon. *chuA* has been reported to be required for hemin and hemoglobin utilization in *C. jejuni* [132]. *chuA*, annotated as hemin uptake system outer-membrane receptor by RAST was found in all isolates from wild birds. This different system of iron uptake and transport, suggest an adaptation to different colonization.

In all *C. jejuni* isolates from wild birds, the Cytolethal distending toxin operon was identified. However, this operon showed some distinctive features compared to NCTC11168. In fact, *cdtA* gene in isolates from crow is shorter by 9 bp whereas, *cdtB* and *cdtC* genes are longer by 9 and 3 bp respectively. This modification can explain our previous negative results from PCR screening of *cdtA* genes in isolates from crow. Cytolethal distending toxin (CDT) causes cell distention followed by its death due to the blocking of cell division. It has been suggested that *cdt* genes are universally found in *C. jejuni* and *C. coli* [126] and their distinct species divergence was reported [59]. However, our results have found a high degree of sequence divergence for isolates from crow, challenging the assumption that species of *C. jejuni* can be identified based solely on amplification of *cdt* gene [10]. This however, remains to be demonstrated with a more diverse and larger isolate collection.

Of interest, we have identified a *tetO* gene in C1-5523, C2-5524 and C12-5524 isolates. This gene is involved in tetracycline resistance in *Campylobacter* spp. and seems plasmid-mediated [154]. The fact that this gene was found in only genotype showing a host-specificity restricted to wild birds should raise further analysis.

A T6SS was identified in one isolate from wild birds, C10-448. Some research findings associate the T6SS in bacterial pathogenicity as a virulence factor [24]. Yet, T6SS are also found in non-pathogenic bacteria suggesting roles other than interactions with the host [103].

The most hypervariable regions found in our isolates compared to the NCTC 11168, were associated with the clusters of genes responsible for LOS biosynthesis, Extracellular polysaccharide biosynthesis and flagellar modification (Fig.8).

Lipooligosaccharide are localized on the surface of many mucosal pathogens, and in *C. jejuni*, LOS have been shown to play a significant role in adhesion to human intestinal cells, invasion, and evasion from complement-mediated killing [75]. Moreover, the LOS are involved in mimicry reactions of human antigens [76], which reactions are implicated in GBS and Miller Fisher syndrome [8]. Studies have focused on the diversity in gene content of the LOS [69]. The comparative sequence analyses of the LOS have allowed characterizing gene clusters and assigning them to distinct LOS classes. The analysis of cluster genes of the LOS for our isolates showed that they possesses both a putative acetyltransferase and ß-1,4-N-acetylgalactosaminyltransferase, which genes are associated to the *C. jejuni* LOS class B-type. it has been postulated that the genes unique to class A and B loci (both genes named above), and genes that are involved in sialic acid biosynthesis or transfer, may be crucial for the induction of neuropathogenic cross-reactive antibodies and thus be taken as GBS indicator genes [69]. However, isolates C2-5524 and C12-5524 lack any genes involved in sialic acid transfer and thus cannot be associated to this class.

The flagellar modification locus is another hypervariable region of *C. jejuni* [124]. Our isolates showed two patterns in this locus. First, isolates from crow showed a flagellar modification locus that is more close to that of the strain 81-176 than to NCTC11168. Conversely, isolates from pigeons were seen to be more identical to NCTC11168 than to the strain 81-176. Moreover, flagellin proteins FlaA and flaB were seen to be very truncated. It has been reported that these two major flagellin proteins, FlaA and flaB display antigen variation [74] and are post translationally modified by glycosylation [52]. Another well identified mechanism is the presence of short homopolymeric set of nucleotides commonly found in genes encoding the biosynthesis or modification of surface structures mainly. The genome of *C. jejuni* shows an abundance of hypervariable homopolymeric tracts of G/C residues and are clustered in three loci involved in biosynthesis of the surface-located structures capsular polysaccharide and lipooligosaccharide, as well as the flagellin glycosylation locus [124]. These homopolymeric tracts are the basis of the well-known phase variation, that can be understood as a random and reversible switching on/off of gene expression [79].

5. Summary

The ubiquity of *C. jejuni* in the environment and most importantly in wild birds, make the risk associated to human infection more complicated. In fact, apart from well-established pathways, such as the consumption of contaminated animal products and water, other possibilities are being discovered. They include contamination of environment by domestic and wild animal feces that trigger human infection via recreational water use or direct contamination in playgrounds. *C. jejuni* has a small genome, and live mainly as commensal organisms in the gastrointestinal tract of various mammalian and avian hosts.

Nevertheless, this commensal organism is the major cause of food-borne bacterial gastroenteritis in humans worldwide. However, *C. jejuni* has been isolated from diverse animal, human and environmental sources including wild birds. Factors prompting the ability of some *C. jejuni* strains to colonize particular hosts or persist in specific environmental niches are not well understood. Moreover, reasons behind its virulence in humans and not in the majority of other hosts are unclear.

Using comparative genomic sequencing on 8 isolates from wild birds, we have identified modifications in the hypervariable regions that may be responsible for surface exposure change allowing adaptation to their niche. Moreover, prophage-like elements were identified in isolates from crow, contributing to the accessory genomes of those strains. Lastly, the iron-uptake system seems to be impaired, as some genes were found missing or with low homology to others identified in other strains.

We have also identified a number of tools acquired by isolates from wild birds, including a T6SS in one isolate from crow, tetracycline resistance-encoding gene in 3 isolates from crow and a FHA domain protein in pigeon samples.

All together, we suggest that genomic divergence observed constitutes evidence of adaptation necessary for wild bird colonization.

Table 8 Characteristics of chosen 6 genomes of *C. jejuni* and *C. coli* and results of mapping the short reads from *C. jejuni* isolates from crow samples ("C").

			C1-5523		C2-5524		C10-448		C12-5524	
Genome Project	Genome Length (Mb)	No. of genes	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped
NCTC 11168	1.642	1634	976,595	68.11	2,996,610	84.75	3,323,289	86.66	2,479,334	83.66
subsp. *jejuni* RM1221	1.778	1838	983,786	68.61	3,079,764	87.1	3,409,979	88.93	2,554,678	86.2
subsp. *Jejuni* S3	1.681	1761	964,272	67.25	3,012,345	85.2	3,332,975	86.92	2,494,082	84.16
subsp. *jejuni* 81116	1.628	1681	973,106	67.86	2,977,556	84.21	3,350,453	87.37	2,460,979	83.04
subsp. *doylei* 269.97	1.845	1731	949,066	66.19	2,929,367	82.85	3,238,878	84.46	2,422,313	81.73
C. coli CVM N29710	1.673	1323	775,927	54.11	2,352,649	66.54	2,575,734	67.17	1,912,213	64.52

Table 9 Characteristics of the 5 plasmids of *C. jejuni* and results of mapping the short reads from *C. jejuni* isolates from crow samples ("C").

			C1-5523		C2-5524		C10-448		C12-5524	
Genome Project	Genome length (Mb)	No. of genes	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped
81-176 plasmid pTet	0.045	44	5,281	0.37	15,538	0.44	23,795	0.62	11,977	0.4
plasmid pCJ419	0.004	4	19	0	221	0.01	280	0.01	215	0.01
81-176 plasmid pVir	0.037	52	591	0.04	848	0.02	1,209	0.03	803	0.03
plasmid pCJ1170	0.004	3	78	0.01	247	0.01	122	0	231	0.01
plasmid pCJ01	0.003	4	23	0	185	0.01	251	0.01	191	0.01

Table 10 Characteristics of chosen 6 genomes of *C. jejuni* and *C. coli* and results of mapping the short reads from *C. jejuni* isolates from pigeon samples ("P").

			P3-2209		P5-2209		P9-2209		P10-2209	
Genome Project	Genome Length (Mb)	No. of genes	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped
NCTC 11168	1.642	1,634	1,864,841	79.79	1,928,988	84.91	1,303,655	78.65	2,303,872	83.18
subsp. *jejuni* RM1221	1.778	1,838	1,848,275	79.08	1,914,119	84.25	1,292,021	77.95	2,282,939	82.42
subsp. *jejuni* S3	1.681	1,761	1,843,042	78.86	1,908,959	84.02	1,288,203	77.71	2,276,382	82.19
subsp. *jejuni* 81116	1.628	1,681	1,862,512	79.69	1,931,218	85	1,302,036	78.55	2,300,279	83.05
subsp. *doylei* 269.97	1.845	1,731	1,765,592	75.55	1,829,267	80.52	1,233,380	74.41	2,179,869	78.7
C. coli CVM N29710	1.673	1,323	1,441,810	61.69	1,490,033	65.59	1,002,691	60.49	1,765,716	63.75

Table 11 Characteristics of the 5 plasmids of *C. jejuni* and results of mapping the short reads from *C. jejuni* isolates from pigeon samples ("P").

			P3-2209		P5-2209		P9-2209		P10-2209	
Genome Project	Genome Length (Mb)	No of genes	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped	Mapped reads	% reads mapped
81-176 plasmid pTet	0.045	44	200	0.01	342	0.02	204	0.01	317	0.01
plasmid pCJ419	0.004	4	46,605	1.99	38,468	1.69	38,199	2.3	54,834	1.98
81-176 plasmid pVir	0.037	52	7,010	0.3	5,672	0.25	5,726	0.35	8,551	0.31
plasmid pCJ1170	0.004	3	5,851	0.25	4,939	0.22	4,636	0.28	7,214	0.26
plasmid pCJ01	0.003	4	24,775	1.06	20,627	0.91	20,194	1.22	29,421	1.06

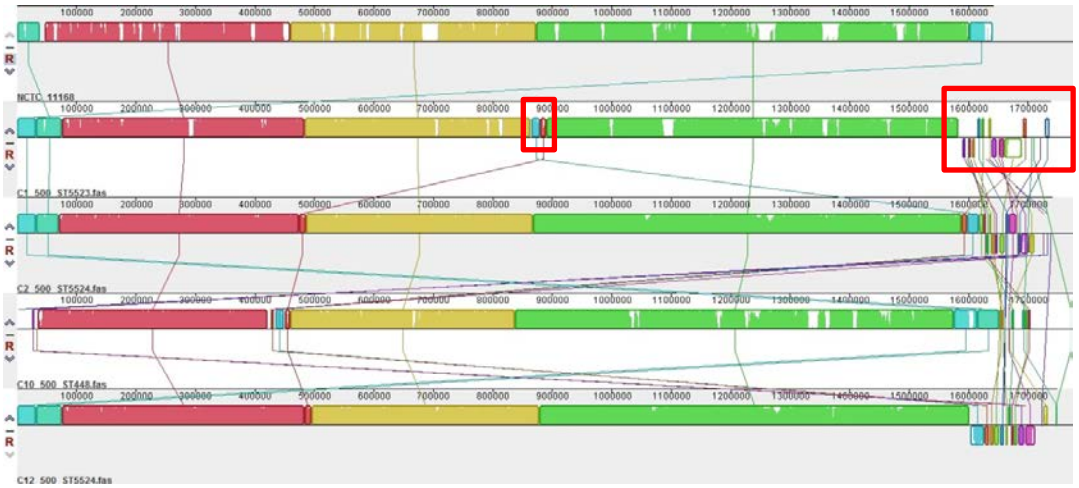
Table 12 General characteristics for the draft genome of eight *C. jejuni* isolates from wild birds

Genome characteristics	C1-5523	C2-5524	C10-448	C12-5524	P3-2209	P5-2209	P9-2209	P10-2209
No. Contigs	126	78	84	81	74	52	70	64
Total Contig size (Mbp)	1.727	1.74	1.772	1.747	1.67	1.658	1.656	1.655
Average coverage	79	242	231	207	147	191	133	183
N50 (bp) after scaffolding	37086	87990	107221	71617	153531	147170	69932	96955
Largest Contig size (bp)	146205	183591	186605	191218	331256	225387	198306	200111
GC Content	30.7	30.3	30.1	30.3	30.9	30.7	30.7	30.7
rRNA	3	3	3	4	4	3	3	3
tRNA	39	36	35	38	36	40	38	38
No. CDS calls	1732	1787	1842	1801	1676	1671	1667	1670
Accession number	JYDX00000000	JXTR00000000	JYDY00000000	JYDZ00000000	JYEA00000000	JYEB00000000	JXTS00000000	JYEC00000000

Table 13 Annotated T6SS proteins in C10-448 and their orthologs in other species

C10-448		*Vibrio cholerae* O1 biovar eltor		*H. hepaticus* 51449	
Name	Size (aa)	Ortholog (accession number)	Size (aa)	Ortholog (accession number)	Size (aa)
IcmF-related protein	1176	VC_A0120 (NP_232521.1)	1181	HH0252 (NP_859783.1)	1154
Outer membrane protein ImpK/VasF, OmpA/motB	258	VC_A0115 (NP_232516.1)	257	HH0251 (NP_859782.1)	262
Protein ImpG/VasA	574	VC_A0110 (NP_232511.1)	589	HH0245 (NP_859776.1)	571
Type VI secretion lipoprotein /VasD	149	VC_A0113	158	-	-
Uncharacterized protein ImpA	416	VC_A0119	469	-	-
Uncharacterized protein ImpB	162	VC_A0107 (NP_232508.1)	168	Conserved Hypothetical protein (AAP_76845.1)	165
Uncharacterized protein ImpC	485	VC_A0108 (NP_232508.1)	492	Conserved Hypothetical protein (AAP_76844.1)	490
Uncharacterized protein ImpH/VasB	301	VC_A0111 (NP_232512.1)	338	HH0244 (NP_859775.1)	331
Uncharacterized protein ImpJ/VasE	466	VC_A0114 (NP_232515.1)	444	HH0250 (NP_85978.1)	459
Uncharacterized protein similar to VCA 0109	64	VC_A0109 (NP_232510.1)	145	HH0246 (NP_859777.1)	129
VgrG protein	318	VC_A0123 (NP_232524.1)	1017	HH0291 (NP_859822.1)	469
ClpB protein	858	ClpB (NP_230360.1)	857	Chaperone protein ClpB (Q7VJY3.1)	859

A



B

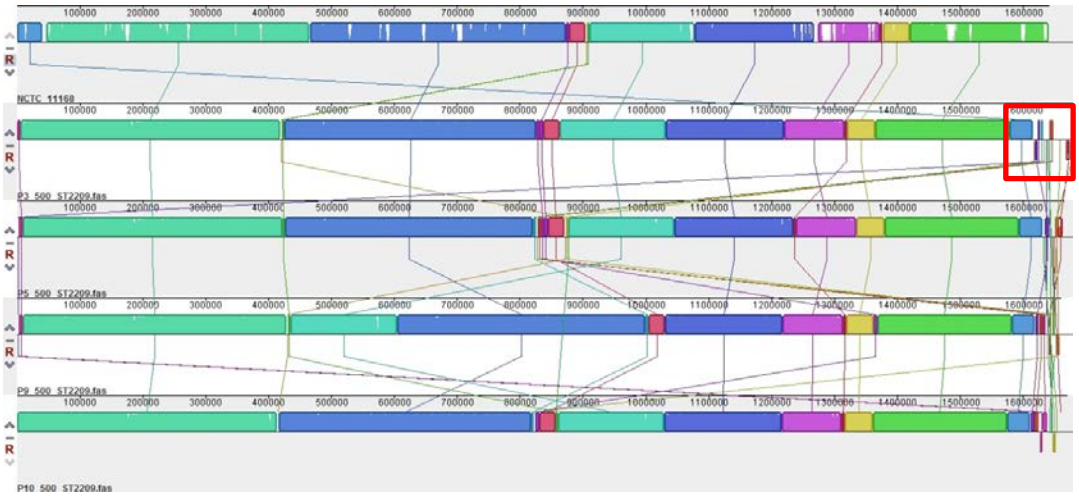
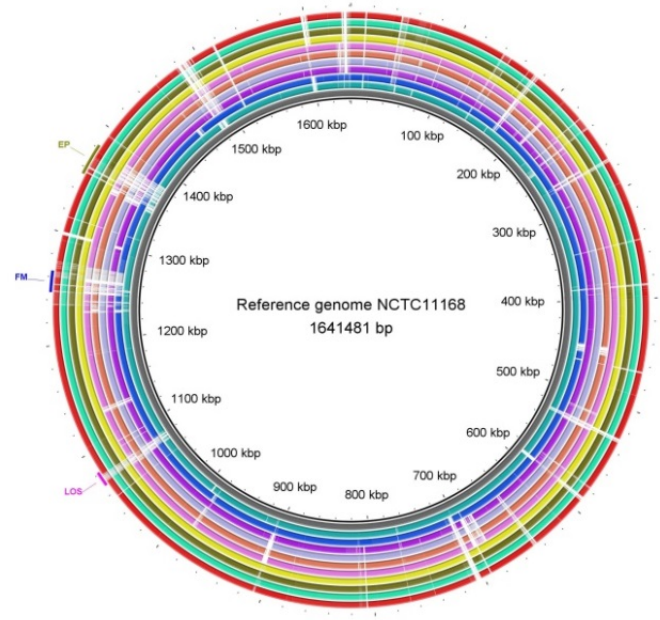


Figure 7 Alignment of whole genome sequences of (A) isolates from crow and (B) those from pigeons using progressive Mauve. Genome rearrangements-free, colored conserved segments found in *C. jejuni* NCTC11168 are connected by lines to similar colored blocks that are homologous in isolates from wild birds. Inverted regions in *C. jejuni* from wild birds are presented as blocks below center lines of the genomes. The surrounded area, are unique features found in *C. jejuni* isolates of our study and not in the reference.

A



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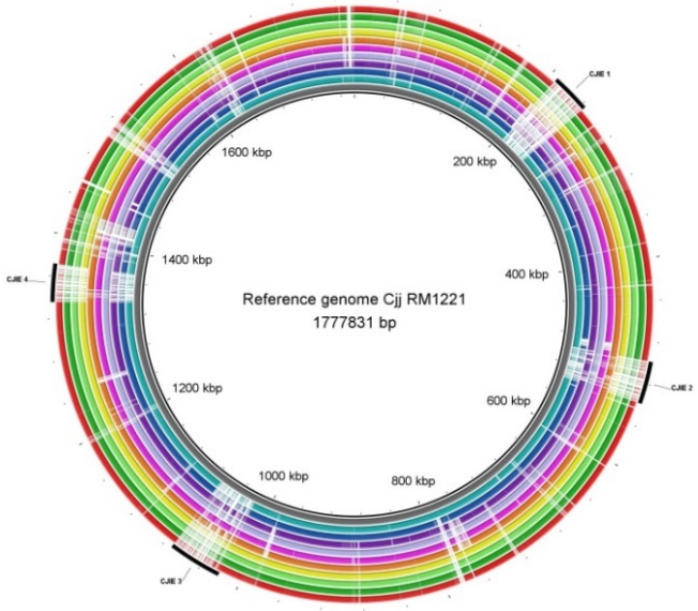


Figure 8 BRIG image highlighting sequence differences in (A) extracellular polysaccharide (upper, fuschia color) flagellar modification (center, blue) and LOS (down, pink) using NCTC11168 as reference and in (B) CJIEs-like elements using strain RM1221 as a reference. Upper and lower identity thresholds of 90% (dark color) and 70% (light color) were utilized. From inner to outermost rings are NCTC11168 (in A) or RM1221 (in B), RM1221 (in A) or NCTC11168 (in B), Cjj 81-176, C1, C2, C10, C12, P3, P5, P9 and P10.

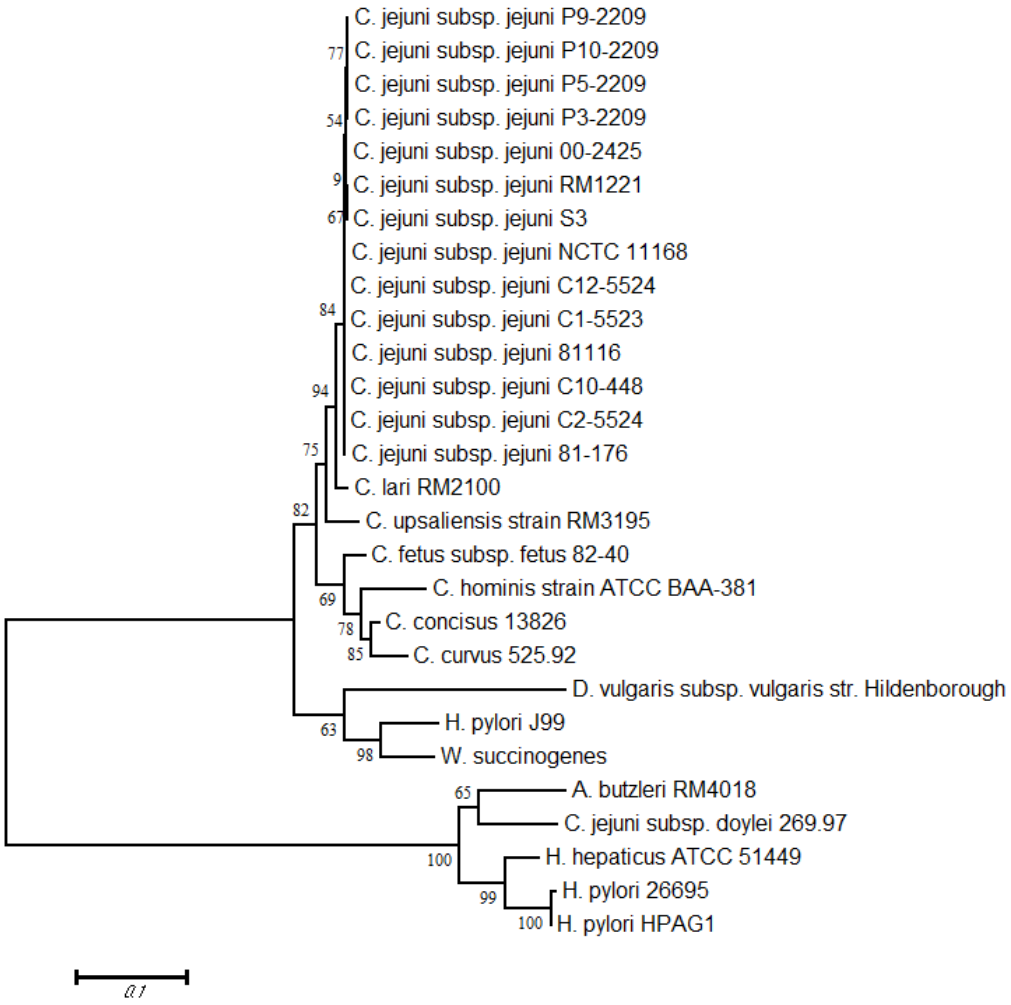
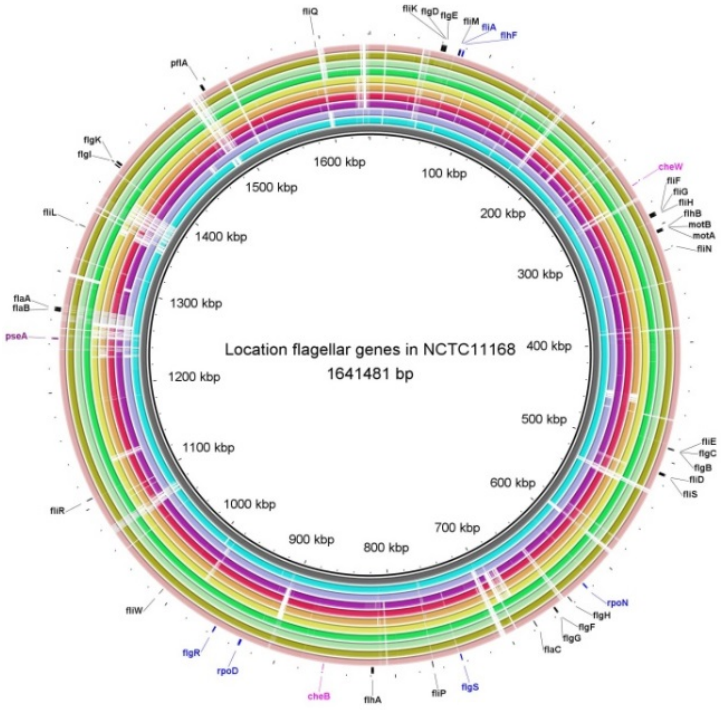


Figure 9 Phylogenetic analysis of *C. jejuni* isolates from wild birds. A radial un-rooted, Neighbor-Joining tree of the concatenated sequences of conserved proteins16s rRNA of isolates from wild birds and reference genomes. The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the isolates analyzed. The bootstrap values are shown next to the branches.

A



B

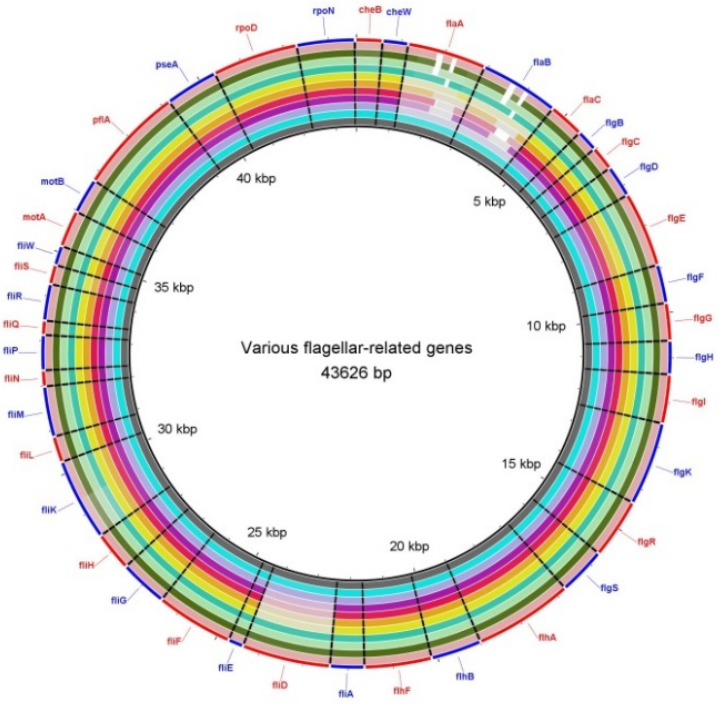
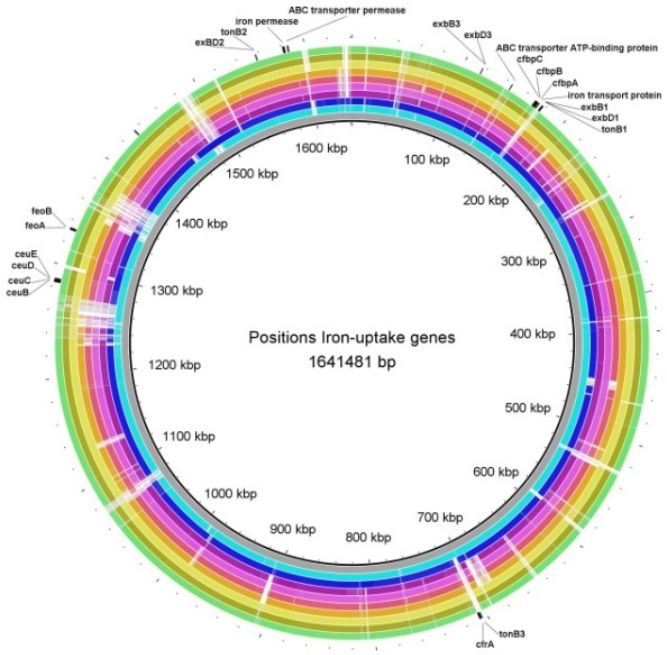


Figure 10 Sequence conservation in flagellar-associated genes visualized using BRIG. (A) Chromosomal location of genes involved in flagellar assembly (in black), modification (in purple), regulators of biosynthesis (in blue) and chemotaxis (in fuschia) using NCTC11168 as reference. (B) Multi-Fasta sequence analysis of flagellar genes variations between the reference and isolates from

wild birds. Upper and lower identity thresholds of 90% (dark color) and 70% (light color) were used for visualizing the difference between sequences. White areas correspond to sequences with less than 70% similarity (BLASTN) against the reference. From the innermost ring to the outermost are, NCTC11168, RM1221, Cjj 81-176, isolates C1, C2, C10, C12, P3, P5, P9 and P10 respectively.

A



B

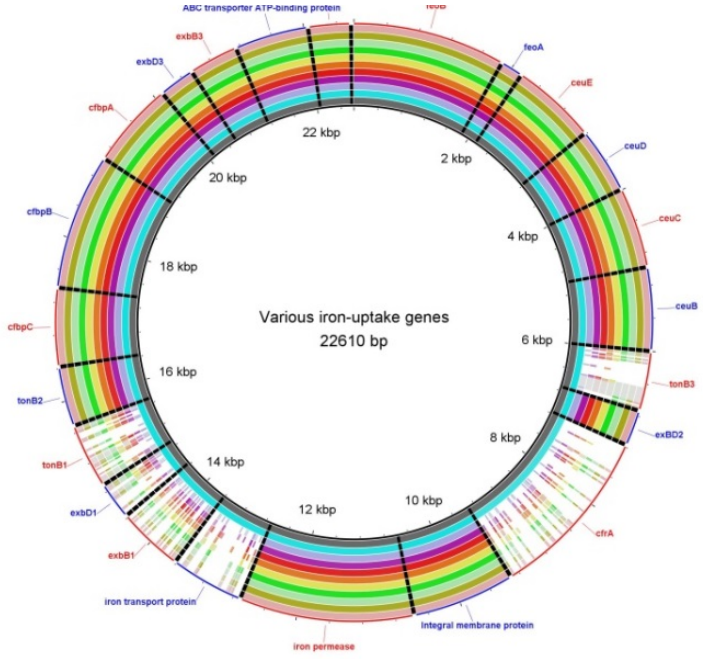


Figure 11 BRIG image picturing sequence conservation in flagellar-associated genes. (A) Chromosomal location of genes selected involved in flagellar assembly (in black), flagellar modification (in purple), regulators for flagellar biosynthesis (in blue) and chemotaxis (in fuschia) in *C. jejuni* using NCTC11168 as a reference. (B) Detailed analysis of genes sequence variation

compared to references. Upper and lower identity thresholds of 90% (dark color) and 70% (light color) were used for visualizing the difference between sequences. White areas correspond to sequences with less than 70% similarity (BLASTN) against the reference. From the most inner ring to the outermost are, NCTC11168, RM1221, Cjj 81-176, isolates C1, C2, C10, C12, P3, P5, P9 and P10 respectively.

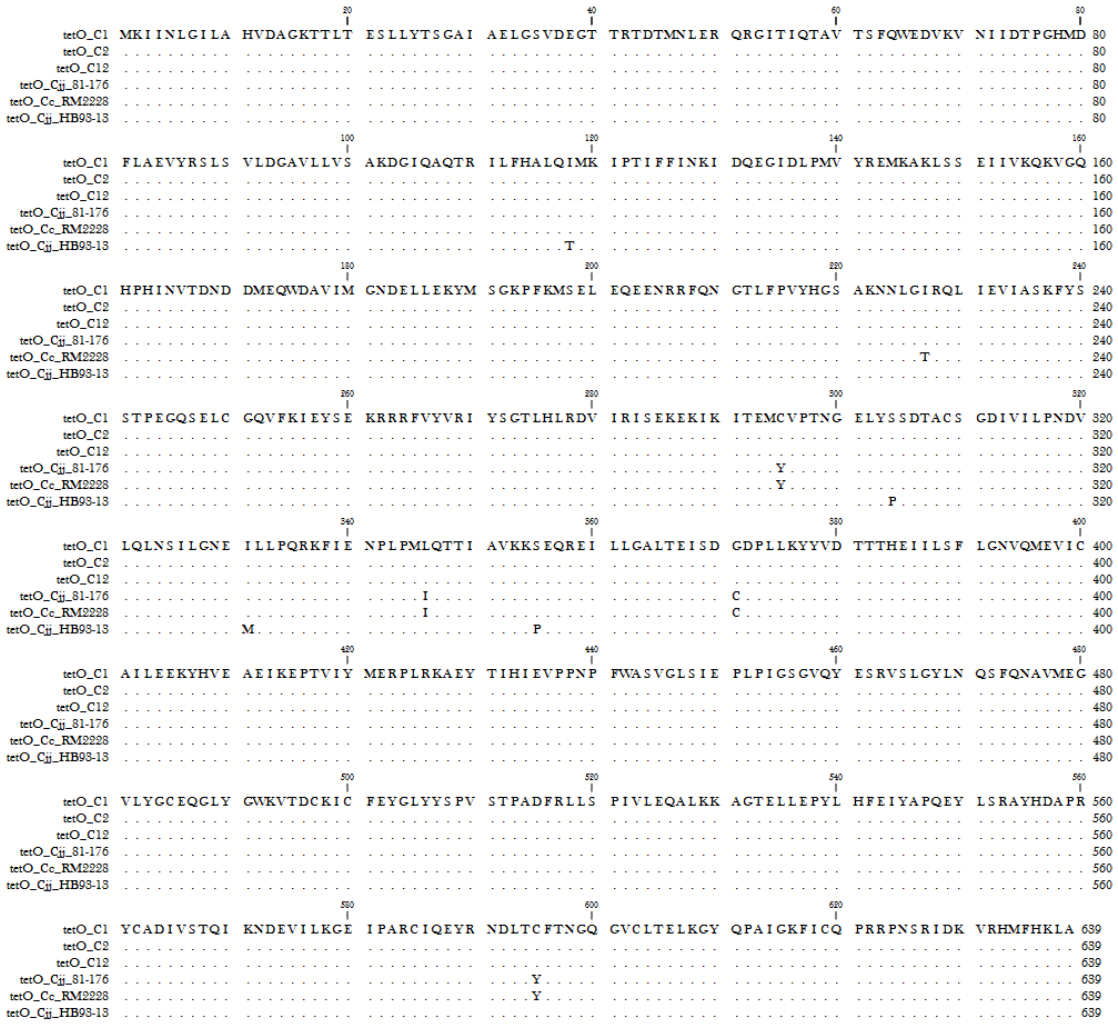


Figure 12 TetO protein alignment between isolates from wild birds (C1, C2 and C12) and *C. jejuni* subsp. *jejuni* strain 81-176 and strain HB93-13 and *C. coli* strain RM2223.

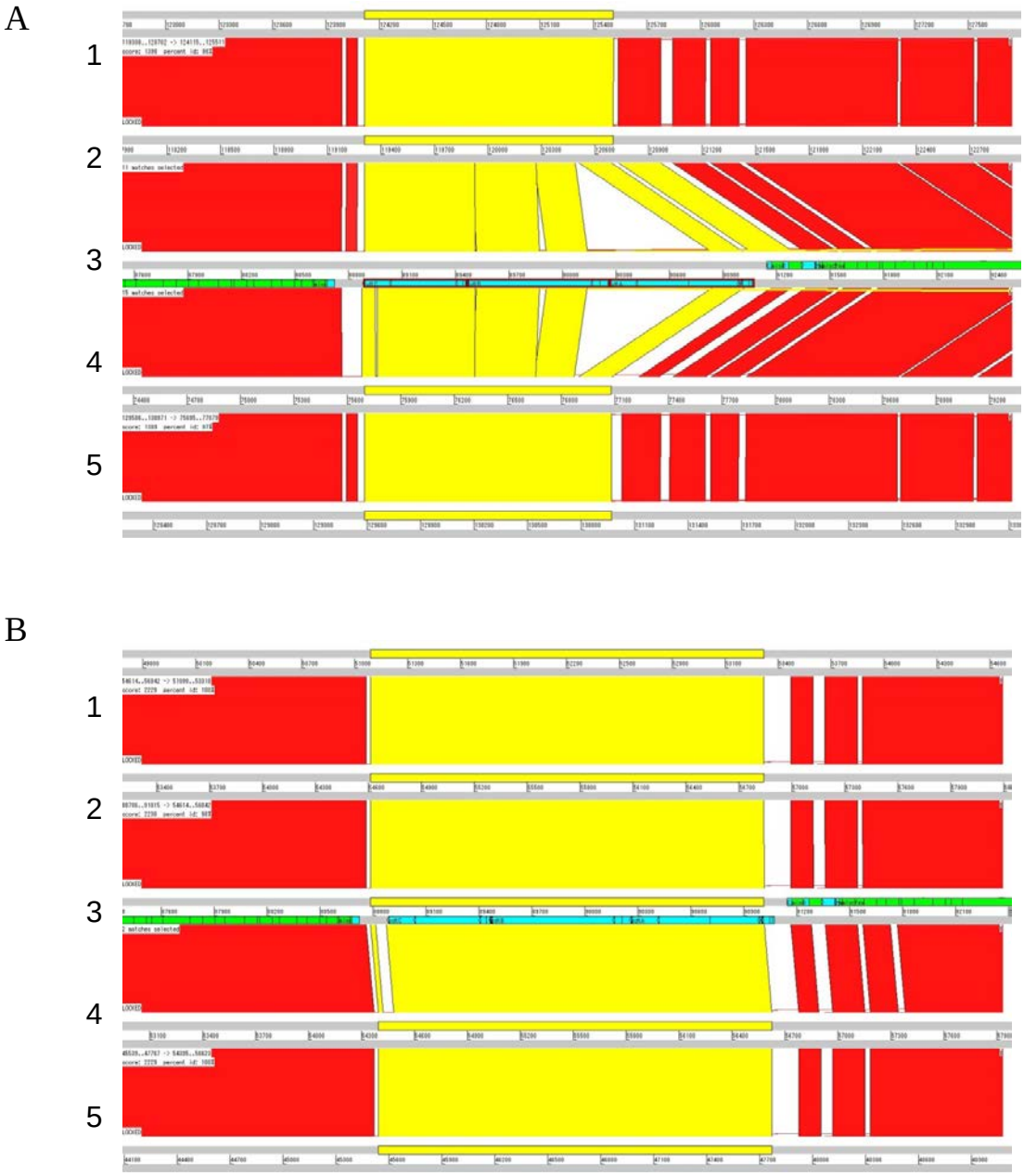


Figure 13 Variation of cdt operon sequences in isolates (A) from crow, where 1, 2, 3, 4, and 5 refers to C1-5523, C2-5524, NCTC11168, C10-448 and C12-5524 respectively, and (B) from pigeon compared to NCTC11168 with P3-2209, P5-2209, NCTC11168, P9-2209 and P10-2209.

General Discussion

Campylobacter spp. are a leading bacterial cause of zoonotic disease worldwide, touching around 55.49 per 100,000 population in 2012 [57] and infecting as much as 13 of every 100,000 persons in the US yearly [145]. In Japan, *Campylobacter* is also the most frequently diagnosed bacterial causes of human gastroenteritis [111]. This bacteria is also prevalent and of hygienic importance in developing countries as it is the most commonly isolated bacterial pathogen from under 2 years-old children [35].

Campylobacter spp. have got the ability to colonize and survive in a broad range of animal species, and environment which make them exceptionally difficult to control [29, 65, 89, 117, 165]. The main objective of the current study was the evaluation of the threat caused by wild birds in the transmission of *Campylobacter* to human, through isolation from wild birds, molecular typing and genomic characterization of obtained isolates. Indeed, the current trend in *Campylobacter* research is focusing on evaluating (i) the role of other possible pathways for the transmission of *C. jejuni* [71] and (ii) on the genomic characterization of isolates from environment and wild life animals in an effort to understand more on *Campylobacter* pathogenesis and host colonization [78].

Thirty two isolates of *C. jejuni* and one isolate of *C. coli*, the two main causes of gastroenteritis were isolated from wild birds inhabiting the surroundings of livestock farms in Tokachi area, eastern Hokkaido, Japan. Moreover, one isolate of *C. fetus* was found in wild birds. The occurrence of *Campylobacter* spp. in resident wild birds is of public health importance as the wild birds sampled inhabit the areas shared by humans and livestock. Moreover, the high rate found in crow implies that this scavenging bird can shed infection in the neighborhood of the study area. Of interest, this is the first report on occurrence of *C. jejuni* in wild birds in Hokkaido, northern Japan.

The virulence of wild bird isolates was examined *in vitro*. We found that the isolates from wild birds have a reduced motility as shown by their swarming ability tested on soft agar suggesting impairment in motility-related genes or in chemotaxis ability. For the virulence genes tested by real-

time PCR, results were negative to *cdtA*, a subunit of the CDT toxin, for most of isolates from crow and that from Eurasian tree sparrow. Nonetheless, some isolates harbored all the 7 virulence genes tested, including one isolate with high swarming ability. This shows that wild birds shed *C. jejuni*, some of which may be fully virulent.

Interestingly, independent of their origin, isolates from wild birds were able to attach and translocate into Caco-2 cells as shown by Gentamicin-killing assay. Their potential to invade and colonize epithelial human cell line, Caco-2 cell, was lower relative to the ability shown by the NCTC11168 used as positive control. This suggests that some of the mechanisms important for invasion and colonization were impaired or at least not fully expressed for virulence. However, at 48 hr post-infection, a slight increase in the number of intracellular bacteria was observed, showing that the immune mechanisms of Caco-2 cells were not enough to clear all invading *Campylobacter* cells. This suggests that the isolates from wild birds can be infective under particular conditions, thereby weakening the host immune mechanisms.

To address the question on the probable involvement of wild birds in the transmission of *Campylobacter* to human, MLST method was performed to characterize the 32 *C. jejuni* isolates. Our results have shown that most of isolates typed (19/32) are of novel STs, thus unique to wild birds niche. Moreover, 5 of 6 isolates from pigeon were of ST-2209 already assigned to pigeon in UK [121]. Interestingly, other remaining isolates (8/32) were assigned to STs that have already been isolated from various sources including human stool, environmental water, livestock, pet and wild birds (http://pubmlst.org/campylobacter). Although we could not find matching STs in human or livestock isolates from Japan, the fact that they have been reported in other countries are important. It is possible that if investigated, *C. jejuni* isolates of the same types as those isolates in wild birds can be found. To confirm this hypothesis, the isolation of *C. jejuni* from human and livestock should be performed.

From the previous experiments, some of the STs of isolates in this study were already reported in humans, livestock and pet animals (http://pubmlst.org/campylobacter). Moreover, we have demonstrated that these isolates may show, admittedly reduced, a virulence phenotype.

Therefore, we went on to characterize isolates from wild birds using whole genome sequencing strategy on eight isolates from both crow and pigeon. We have found that isolates from wild birds show almost complete synteny to the human-originated NCTC11168 strain. However, we have noted that isolates from pigeon seem more close to NCTC11168 as no element insertion was seen in any of them. On the contrary, isolates from crow showed insertion of CJIE-like sequences similar to those reported in *C. jejuni* strain RM1221 [63]. Moreover, three of four isolates acquired a tetracycline resistance gene and one isolate had a type VI secretion system, probably allowing them to adapt to its versatile host range [103]. These insertions materials are the reason of a seen bigger genome size of isolates from crow.

The flagellin proteins of isolates from wild bird were found truncated and the hypervariable regions of *C. jejuni* were very diverse due to the presence of homopolymeric tracts in genes located in those regions. Although the draft genomes provide valuable information on the genomic structure and content of isolates from wild birds, to conclude on the presence or absence of a given gene will require completing these genomes [106]. However, these quality draft genome open up a way toward genetic study for example in an attempt to determine a particular gene function or expression.

General Summary

Campylobacter spp. are the leading bacterial causes of gastroenteritis worldwide. Moreover, the disease caused by this bacterium, campylobacteriosis, can in some cases trigger deadly complications such as the GBS and the reactive arthritis. Campylobacteriosis is mainly associated with consumption or contact with meat, poultry mainly, milk and water. However, sporadic cases are associated with other pathways including environmental sources and wild animals. For an effective control of human gastroenteritis caused by *Campylobacter*, it is crucial to evaluate the entire potential sources and their contributions to the *Campylobacter* spp. occurring in nature. Hence, *Campylobacter* prevalence in wild birds must be explored thoroughly, especially in countries with high human *C. jejuni* infection. In fact, wild birds come into contact with humans, livestock, pet animals and their environment including water. Since *Campylobacter* spp. have a broad range targets, it is believed that wild birds can act as a vehicle transmitting the bacterium from one host to another by means of their droppings.

The aim of the current study was firstly to establish the prevalence of *Campylobacter* spp. in wild birds in Tokachi area, eastern Hokkaido, Japan and evaluate the virulence of obtained isolates. Secondly, to determine the genotypes of *C. jejuni* from wild birds and establish the probable implication of wild birds in the transmission of campylobacteriosis in Japan using MLST method. Thirdly, to provide whole genomes of *C. jejuni* from wild birds and initiate a comparative genomics that evaluates virulence and unique features of these isolates.

Chapter one describes an investigation on the carriage of *Campylobacter* spp. in wild birds in Hokkaido, Japan and the virulence of the obtained isolates using *in vitro* tests. We have found that overall, 34 of 173 birds were positive, representing 19.7% of the samples. The species identification showed that most of the isolates were *C. jejuni* (32/34) but also one isolate each for *C. coli* and *C. fetus* was seen. This supports the finding of public health importance of *C. jejuni* and *C. coli* as the main bacterial causes of gastroenteritis in humans. Moreover, *C. fetus* is of veterinary medicine importance, but may also infect humans. The virulence of isolates from wild birds using a human

epithelial cell line, Caco-2, showed that isolates possess ability to invade and multiply inside cells in vitro, although the full expression of their virulence was hindered by unknown mechanisms. Supported by PCR results, we have found that 7 out of 32 tested isolates harbored all the virulence-related genes investigated. Lastly, the swarming motility on soft agar was reduced for all tested isolates, except for one isolate from crow. This shows that some of the *C. jejuni* introduced in the environment by wild birds may be fully virulent.

In chapter two, we used multilocus sequence typing, MLST, as an ideal epidemiological tool to infer similarity between isolates from our study and those from human, poultry and cattle in Japan. Our study found 19 STs, 13 of which are newly reported from the results of our study. In addition, some STs could be assigned to two known CC; ST-179 and ST-952 complex, commonly reported by various studies in wild birds and environmental samples, implying host-specificity of wild birds isolates. We have not found close relationship between genotypes of isolates from wild birds and those from human, poultry and cattle in Japan, signifying that wild birds are less likely involved in the transmission of campylobacteriosis in Japan. Yet, given that some STs and both CC were found in other countries in host other than wild birds, we suggest that these isolates need a continuous monitoring as potential source of *Campylobacter* infection.

Chapter three describes the sequencing of eight whole genome sequences of *C. jejuni* from wild birds were generated using Next-generation sequencing technology. An endeavor was made to use generated genomes in the comparative genomics. We have provided first whole genomes of *C. jejuni* isolates from wild birds and deposited them at Genbank. Moreover, the obtained genomes were automatically annotated using RAST server and a comparative genomics done. The current study has shown that some virulence genes such as CDT operon, flagella-associated genes and iron-acquisition genes, have undergone important alterations. We have reported acquisition by some of these genomes, of insertion elements similar to known *C. jejuni* prophages, *tetO* gene and a type-VI secretion system. The observed plasticity of these genomes from wild birds may be related to evolution required for a safe colonization of wild birds. However, additional research experiments

should be carried out to isolate *Campylobacter* from human and livestock and to fully conclude on the potential hazard caused by the prevalence of this pathogen in wild birds.

In conclusion, our study showed a relatively high occurrence of *Campylobacter* spp. in resident wild bird in Tokachi area, Hokkaido, Japan. The species identified, showed that the campylobacters obtained were of public importance as *C. jejuni*, *C. coli* and *C. fetus* were found in sampled wild birds. Moreover, some of the isolates of *C. jejuni* were seen fully virulent using *in vitro* tests. No similarity was found between isolates of the current study and those previously reported in humans, poultry and cattle in Japan, using MLST method. Wild birds should however, be taken as a potential source of campylobacteriosis since some genotypes of our study was reported overseas in clinical outbreaks. Lastly, we have sequenced and deposited whole genomes of *C. jejuni* and they will contribute to the understanding of the diversity of this pathogen.

This current study on the prevalence, molecular typing and genomic characterization of *C. jejuni* isolates from wild birds will serve as a benchmark for more research to improve the understanding of the impacts and importance of the prevalence of *C. jejuni* in wild animals. Moreover, it will help provide valuable genomic data on this pathogen, whose pathogenesis is still poorly understood.

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Japanese Summary

ヒトのカンピロバクター感染症はカンピロバクター（*Campylobacter*）属菌の感染によって引き起こされ、下痢を主徴とする急性胃腸炎である。日本をはじめとする先進国では発生数が増加しており、細菌性食中毒の上位に位置しており、途上国においても急性細菌性胃腸炎の主要原因菌である。本属菌はグラム陰性、微好気性のらせん状の桿菌で、2015 年現在で 17 菌種 6 亜種が知られているが、ヒト患者から分離される起因菌の約 9 割以上を *C. jejuni* が占め、残りは *C. coli* となっている。また、まれではあるが *C. lari* や C. *upsaliensis* も患者糞便から分離されている。カンピロバクター属菌は家畜、野鳥、環境中に広く分布し、ヒトには汚染食品の摂取や保菌動物との接触により感染する。感染源として特に鳥類が問題で、鳥類の腸内環境は *C. jejuni* を含む比較的高温を好むカンピロバクター属菌の増殖に適しており、家禽は高率に本菌を保有している。これまでに我々は十勝地方においてヒトと生活圏を共有する身近な野鳥を対象にカンピロバクター属菌の疫学調査を行い、カラス、ハトおよびスズメから *C. jejuni*、*C. coli*、*C. fetus* を分離した。また、国内外の様々な野鳥においても *C. jejuni* をはじめとする *Campylobacter* 属菌の分離が報告されている。しかし、野鳥由来 *C. jujuni* の病原性については報告がなく、また、これらの野鳥から他の動物への病原菌の伝搬の有無や、ヒトのカンピロバクター症への関与についても不明な点が多い。そこで、本研究では、野鳥由来 *Campylobacter* 属菌の公衆衛生上の問題について明らかにするため、野鳥由来株の病原性および分子疫学解析を行った。

第一章においては、十勝地方の野鳥から分離された *C. jejuni* 株の病原性を検討するため、主要病原遺伝子の保有、軟寒天培地における運動性、ヒト結腸癌由来上皮細胞株の Caco-2 細胞への感染実験により検討した。これらの検討の比較対照として、ヒト臨床株で世界中の研究室で使用されている Type Strain の NCTC11168 を用いた。軟寒天培地を用いて運動性を比較したところ、カラス由来の 1 株は NCTC11168 よりも高い運動性が観察された。

一方でそれ以外の株では運動性は観察されなかった。Caco-2 感染実験では、感染 1、24、48 時間後の細胞内生菌数を測定、比較したところ、NCTC11168 では、感染 1 時間後の菌数は 24 時間後にはいったん減少したが、48 時間後には増加しており、宿主細胞内での増殖が観察された。一方、野鳥株でも Caco-2 への侵入性が見られたが、24 時間後には細胞内生菌数はいずれの株も大きく減少した。24 時間後と 48 時間後の細胞内生菌数を比較すると、野鳥株では臨床株ほど増殖せず、菌数は維持されていた。48 時間後の細胞内生菌数を比較すると、NCTC11168 と野鳥株の間には有意な差が認められた。これらの検討により、野鳥株はヒトに対して病原性を示す可能性が示された一方で、ヒト臨床株と比較してその病原性は低いと考えられた。

第二章においては、野鳥株と家畜や家禽由来株、ヒト臨床株との遺伝的関連性を明らかにするため、Multilocus sequence typing (MLST) による遺伝的多型解析による分子疫学的解析を行った。定法に従い、*aspA, ginA, gltA, glyA, pgm, tkt, uncA* の 7 遺伝子の特定領域を PCR で増幅後、塩基配列を決定した。各遺伝子配列のアレル型を *Campylobacter* PubMLST データベース上の登録された塩基配列と照合し、7 遺伝子のアレルプロファイルにより Sequence type (ST) を決定した。同データベース上に未登録の配列については、新規アレル型として登録した。カラス分離株では解析した 27 株中、74.1%にあたる 20 株が新規 ST であり、うち 10 株が新規アレル型を含んでいた。その他、ST-448 が 3 株、ST-2761 が 1 株、ST-3322 が 1 株認められた。ハト分離株は全て既知の ST で、1 株 (ST-448) を除き全て ST-2209 であった。またスズメ分離株では 7 遺伝子のうち 5 つが新規アレル型であった。カラス分離株は 2 つのクラスターに分かれ、それぞれが clonal complex を構成する可能性が考えられた。ハト分離株、スズメ分離株は検体数が少ないが、カラス分離株とは異なるクラスターにあり、遺伝子型別と宿主の種別には相関性があることが示唆された。ST-2761 は牛から、ST-448 はヒト臨床例からの分離が報告されているが、今回得られた分離株の大部分は野鳥に特異的なものが多く、ヒトや牛由来株との関連性が一部で認められたのみで食

鳥由来株との関連性は認められなかった。また、分離株の多くが新規アレル型を持つことから、宿主特異性および地域特異性の存在が示唆された。

第一章および二章の解析から、野鳥由来 *C. jejuni* 株は宿主特異性を保ちながら進化、適応してきたと推測された。また、病原性についてもヒト臨床株と比べ異なることが明らかとなった。カンピロバクター属菌の鳥類消化管での定着機構や感受性動物におけるカンピロバクター症の発症機序については依然として不明な点が多い。そこで、第三章では、これらの分子メカニズムについて既知遺伝子以外の情報を得るため、*Campylobacter* の野鳥分離株のゲノムシークエンスを行い、既に全ゲノム配列が明らかな NCTC11168 と比較解析を行った。野鳥由来 *C. jejuni* 株 32 株のうち、カラス由来の 4 株、ハト由来の 4 株についてドラフト塩基配列解析を行った。野鳥株では CDT オペロン、べん毛関連遺伝子、鉄代謝関連遺伝子の幾つかの遺伝子に重要な違いが見られた。またカラス由来株の一部で、Type 6 secretion system、*C. jejuni* プロファージおよびテトラサイクリン耐性遺伝子をコードすると思われる遺伝子が存在していた。また、ハト由来株には線維状赤血球凝集素（FHA）をコードする遺伝子が存在していた。

本研究の成果から、野鳥由来 *C. jejuni* 株は Caco-2 への侵入性など *in vitro* での検討で病原性を示した。ヒトに感染した場合の病原性については不明だが、NCTC11168 との比較から、野鳥株はヒト細胞に対して病原性を示す可能性が示された一方で、ヒト臨床株と比較してその病原性は低いと考えられた。また、日本の野鳥由来の *C. jejuni* の MLST により新規アレル、新規 ST を含むデータを登録した。今回得られた野鳥由来株は海外の野鳥由来株と比較して地域特異性の存在を示唆し、さらに現段階では国内のヒト臨床由来株や鶏由来株との関連性は認められず野鳥に特徴的であったが、鶏や環境などを介した野鳥由来株のヒトへの感染経路についての可能性も否定できない。今後、野鳥や他の宿主のサンプリングを広範に継続して行い、遺伝的多様性および分子疫学情報としての知見を蓄積していく必要がある。