Population genetics and characterization of *Campylobacter jejuni* isolates in western jackdaws and game birds in Finland

Sara Kovanen[1], Mirko Rossi[1], Mari Pohja-Mykrä[2], Timo Nieminen[2a], Mirja Raunio-Saarnisto[3], Mikaela Sauvala[1], Maria Fredriksson-Ahomaa[1], Marja-Liisa Hänninen[1], and Rauni Kivistö[1*]

[1]Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, FI-00014, Finland

[2]Ruralia Institute, Faculty of Agriculture and Forestry, University of Helsinki, Seinäjoki, FI-60320, Finland

[3]EVIRA, Finnish Food Safety Authority, Seinäjoki, FI-60101, Finland

[a]Current address: Tukes, Finnish Safety and Chemical Agency, Helsinki, FI-00520, Finland

[*]Corresponding author: rauni.kivisto@helsinki.fi.

ABSTRACT

Poultry is considered a major reservoir and source of human campylobacteriosis but the roles of environmental reservoirs, including wild birds, have not been assessed in depth. In this study, we isolated and characterized *Campylobacter jejuni* from western jackdaws (n=91, 43%), mallard ducks (n=82, 76%) and pheasants (n=9, 9%). Most of the western jackdaw and mallard duck *C. jejuni* isolates represented MLST sequence types (STs) that diverged from those previously isolated from human patients and various animal species, whereas all pheasant isolates represented ST-19, a common ST among human patients and other hosts worldwide. Whole-genome MLST revealed that mallard duck ST-2314 and pheasant ST-19 isolates represented bacterial clones that were genetically highly similar to human isolates detected previously. Further analyses revealed that in addition to divergent ClonalFrame genealogy certain genomic

characteristics, e.g. novel *cdtABC* gene cluster and T6SS of the western jackdaw *C. jejuni* isolates may affect their host-specificity and virulence. Game birds may thus pose a risk for acquiring campylobacteriosis, and therefore, hygienic measures during slaughter and meat handling warrant special attention.

IMPORTANCE

The roles of environmental reservoirs, including wild birds, in the molecular epidemiology of *Campylobacter jejuni* have not been assessed in depth. Our results showed that game birds may pose a risk for acquiring campylobacteriosis, because they had *C. jejuni* genomotypes highly similar to human isolates detected previously. Therefore, hygienic measures during slaughter and meat handling warrant special attention. On the contrary a unique phylogeny was revealed for the western jackdaw isolates and certain genomic characteristics identified among these isolates are hypothesized to affect their host-specificity and virulence. Comparative genomics within STs, using wgMLST, and phylogenomics are efficient methods to analyze the genomic relationships of *C. jejuni* isolates.

Introduction

With more than 200,000 annually reported cases, *Campylobacter jejuni* continues to be the most common cause of human bacterial gastroenteritis in the European Union (EU), including Finland (1). Poultry has been recognized to be a major reservoir and source of human campylobacteriosis (1). However, in Finland, the prevalence of *Campylobacter*-positive broiler flocks has remained low since 2004, when systematic reporting started (2), while simultaneously the incidence in the human population has shown an increasing trend (incidence 69/100,000 in 2004 and 85/100,000 in 2016) (3). Besides poultry, wild birds have often been shown to carry *C. jejuni*, and hence, are considered to act as wildlife reservoirs, causing fecal contamination of the environment of food production farms and possibly transmitting *Campylobacter* to domestic animals as well as to humans (4). *C. jejuni* has been found in various wild bird species (4-7), however, relatively little is known about its occurrence in western jackdaws and game birds.

Western jackdaw (*Corvus monedula*) belongs to the family of crows and is one of the most common bird species in some urban and agricultural surroundings in Finland, thus being also a concern from the public health perspective as a potential reservoir of zoonotic pathogens, including *Campylobacter jejuni*. The Finnish jackdaw population has increased rapidly during the last thirty years (8). Jackdaws form large flocks producing noise and considerable fecal loads that contaminate the environment, including the surroundings of food production farms and city parks. The western jackdaw is an omnivore, feeding on plants and invertebrates as well as food waste. Jackdaw flocks also feed in farmland and may cause marked agricultural damage (8). Jackdaws are partial migratory birds; adults overwinter near their nests, whereas juveniles migrate to the Southern parts of the Baltic Sea.

Besides natural wild game bird populations that are hunted during hunting season, several bird species, including waterfowl, pheasants, and pigeons, are bred and raised on specific farms and released for hunting purposes. Mallard duck (*Anas platyrhynchos*) is the most common game bird in Finland, with 210,000 to 300,000 birds hunted annually (www.riista.fi/en/). Mallard ducks live close to the water systems and feed on plants and invertebrates. Most of the Finnish natural mallard duck population migrates in late autumn to Central Europe, but some flocks stay in Finland year-round. The hunting season lasts approximately four months (August to December), and meat is mostly consumed by individual hunters (Ministry of Agriculture and Forestry, www.mmm.fi).

Pheasants (*Phasianus colchicus*) belong to the same family as partridges and are a common domesticated game bird in Europe and in the USA (9). In the UK and Ireland alone, up to 30 million pheasants are hunted annually (10). These birds live typically in open spaces and fields and feed on plants, grain, seeds, insects, and invertebrates. In Finland, 15,000 to 75,000 pheasants are hunted annually (www.riista.fi/en/), and the meat is mostly consumed by individual hunters, but also sold to restaurants (Ministry of Agriculture and Forestry, www.mmm.fi).

Multilocus sequence typing (MLST) has been an invaluable molecular typing method, providing essential knowledge about *C. jejuni* types occurring in various hosts and sources worldwide. MLST is, however, limited to the characterization and discrimination of isolates to sequence type (ST) (11-13), and thus, more accurate methods, such as whole-genome (wg) MLST, are increasingly being used (11, 14) to compare genetically related isolates in more detail and to be able to identify clones potentially originating from the same source. In previous studies, most of the STs found among wild birds, including mallard ducks (5), barnacle geese (7), starlings (15),

and several other bird species (16), have been considered to represent mainly host-associated STs, differing from those STs reported in human patients or domestic animals. Thus, wild birds are commonly considered to have a minor role in human campylobacteriosis. However, certain STs and generalist lineages that are common in human patients (e.g. ST-45 CC) have been detected in several wild bird species as well (5-7, 16, 17), indicating that wild birds are potential reservoirs for certain common STs also infecting humans. For example, ST-45, ST-677, and ST-267, which have been common STs in human infections also in Finland (18, 19), have been found among both blackbird and chicken isolates from Sweden (6). However, to our knowledge there have been no comprehensive wgMLST level studies, and only one study including comparative genomic analyses on wild bird isolates (American crows) has been published to date (17).

The aim of this study was to assess the occurrence, population genetics, and diversity of *Campylobacter* spp. in western jackdaws and game birds (i.e. mallard duck and pheasant) in Finland using whole genome sequencing. We further explored the presence of genomic features associated with virulence and antimicrobial resistance among the isolates to evaluate their importance from a public health perspective.

Results

Occurrence and Multi-Locus Sequence Typing (MLST)

Campylobacter spp. was isolated from 91 western jackdaw (43%), 79 farmed game mallard duck (79%), 9 pheasant (9%), and 3 wild mallard duck (38%) samples. All isolates were identified as *C. jejuni*.

Whole-genome sequencing (WGS) was successful for 87 (of 91) western jackdaw *C. jejuni* isolates. MLST showed great diversity, as 62 different sequence types (STs) were found, 46 of which were novel to the PubMLST database (pubMLST.org/campylobacter) (Supplementary Table S1). ST-1282 and ST-6460 were the most frequently found STs and also the most widely distributed both temporally and geographically. Among mallard ducks, 36 of 38 selected representative isolates (35 farmed, chosen based on PFGE screening, and 3 wild mallard duck isolates) were successfully sequenced. Altogether 16 STs were identified, 4 of which were novel to the PubMLST database. Taking into account the combined results of PFGE and MLST, the most common STs in mallard ducks were ST-2314 (n=26), ST-1299 (n=16), ST-991 (n=7), ST-2839 (n=7), and ST-995 (n=5). All of the nine pheasant isolates represented ST-19 (ST-21 CC).

Phylogenomics

A phylogenomic tree based on the core genome alignment of altogether 1261 *C. jejuni* strains (Supplementary Dataset S1.), including the ones sequenced in this study and those collected from public databases, is shown in Fig. S1. The western jackdaw isolates clustered together in a diffuse branch including also American crow and few chicken isolates and a very limited number of genomes representing sandhill crane, mallard duck and other mammals. All the other isolates even inside the branch were clearly distinct from the western jackdaw isolates which also were highly diverse. The mallard duck isolates also showed great diversity, however the majority clustered together with *C. jejuni* strains isolated from few chicken and barnacle goose. The pheasant isolates formed a more homogenous cluster and shared the same branch with a higher proportion of agricultural *C. jejuni* isolates from chicken, some bovine and other mammals.

ClonalFrame genealogy

ClonalFrame genealogy representing the phylogeny of the STs is shown in Fig. 1. ClonalFrame revealed that most of the STs occurring in western jackdaws were in their own diffuse cluster and differed from those that have been previously detected from other hosts in Finland, including other wild bird species. Exceptions were ST-4565 and ST-7841, which were clearly distinct from other western jackdaw STs and located in the same branch with the STs detected from humans, poultry, and bovines (ST-677 CC and related STs), and also ST-6460, which was a common ST among the western jackdaw isolates, but has previously been found also in poultry in Finland.

STs detected from mallard ducks occurred mostly in the same branches as STs detected previously from barnacle geese. Exceptions were ST-6228 and ST-7843, which were in the same cluster with western jackdaw STs, ST-995, which has previously been isolated also from poultry, and ST-6788, which was closely related to ST-1332, previously isolated from human patients and poultry. The most commonly found ST among farmed mallard ducks, ST-2314, was not closely related to any other ST previously reported in Finland.

ST-19 (ST-21 CC), represented by the pheasant isolates, has previously been isolated from two human patients in Finland, and it has been common in several other countries in various sources, including humans, poultry, and bovine (www.pubMLST.org/campylobacter).

Whole-genome (wg) MLST

WgMLST analysis of ST-1282, the most common ST among western jackdaw isolates, obtained from two different locations and on five separate occasions, revealed high numbers of allelic differences (62 to 439) between the isolates (Fig. 2A). The two isolates with the fewest allelic differences (n=62), CB346 and CB384, were isolated from the same town, but temporally five

146 months apart, while two isolates (e.g. CB299 and CB288) were collected from the same
147 sampling site on successive sampling days, but they had 313 allelic differences. WgMLST of
148 ST-6460, representing the second most common ST, revealed 87-89 allelic differences between
149 the western jackdaw isolates and a chicken isolate obtained in 2012 from the Finnish
150 *Campylobacter* monitoring program (Fig. 2B). Western jackdaw ST-6460 isolates were more
151 closely related to each other, as only 8 to 16 allelic differences occurred among the isolates,
152 which had been collected from two different locations and on three separate occasions (Fig. 2B).
153 *C. jejuni* pairs (CB294/CB286) and (CB329/CB358) in panel (B) were isolated on the same days
154 and from the same sampling points, however originating from different towns.

155 WgMLST analysis of ST-2314 isolates from Finnish mallard ducks and human patients from the
156 UK (isolated in 2010, 2011, and 2013) is shown in Fig. 2C. The isolates formed three clusters,
157 differing by 195 to 303 alleles. Between the mallard duck ST-2314 isolates, only 2 to 7 allelic
158 differences were detected, and 17 to 21 allelic differences (approximately 1% of the 1614 shared
159 loci) were found between the five mallard duck isolates and a clinical isolate from the UK
160 (OXC8492) in 2013 (Fig. 2C).

161 WgMLST of ST-19, describing the allelic differences between the *C. jejuni* isolates from
162 pheasants and human patients, collected in the UK in 2010-2014
163 (www.pubMLST.org/campylobacter) and in Finland in 2012 (19), is shown in Fig. 2D. The
164 isolates formed four distinct (from 600 to 945 allelic differences) clusters. One of the clusters
165 that consisted of all of the genetically highly related pheasant isolates (differing by 3 to 4 alleles)
166 and one clinical isolate obtained from the UK in 2014 (OXC9006), which diverged from each
167 other by 33 to 37 allelic differences, accounted for 2% of the 1581 shared loci (Fig. 2D).

Description of genetic features

Integrated elements (CJIEs)

Integrated elements CJIE1, 2, 3, and 4 were present in 20% (17/87), 86% (77/87), 85% (74/87) and 14% (12/87) of the western jackdaw isolates, respectively. However, there were large deletions and other major differences in the genomic structure of CJIE2 in the majority of the isolates compared with reference strain RM1221 (20). Among mallard duck isolates, integrated elements CJIE1, 2, 3, and 4 were present in 3% (1/36), 39% (14/36), 81% (29/36), and 3% (1/36) of the isolates, respectively. The only integrated element present in pheasant isolates was CJIE4, which was present in 100% of the isolates.

Type VI secretion system

A total of 43 of 87 (49%) of the western jackdaw isolates carried a type VI secretion system (T6SS), similar in gene synteny and sequence (96% query coverage and 96% identity in BLASTN) to the T6SS of strain 108 (21). Interestingly, the majority (72%) of the mallard duck isolates also carried the T6SS, whereas none of the pheasant isolates carried either the CJIE3 or T6SS.

Cytolethal distending toxin

Fig. 3 shows an alignment of the cytolethal distending toxin (*cdtABC*) gene clusters of western jackdaw isolate CB287 against the reference strain NCTC 11168. The *cdtABC* operon, located in the same genomic site as in NCTC 11168, was complete in only two of the western jackdaw isolates, missing completely in three isolates, and degenerated and thus likely dysfunctional in most (82 of 87) of the western jackdaw isolates (e.g. CB287, Fig. 3). However, another homologous *cdtABC* gene cluster (69% identity, 84% query coverage against other *C. jejuni*

sequences in nr using BLASTN) was present in another location of the genome in 59% (51/87) of the western jackdaw isolates (e.g. CB287, Fig. 3). For further analysis, a phylogenetic neighbor-joining tree was constructed based on the conserved amino acid sequences of the *cdtB* gene. The novel jackdaw CdtB sequence was relatively distinct from those of other *C. jejuni* strains and even from other *Campylobacter* spp., but highly similar to those of crow *C. jejuni* isolates from California, USA (Fig. 4). The two western jackdaw isolates that had an intact *ctdABC* operon, located in a similar position as in strain NCTC 11168, represented ST-4565 and ST-7841, which were clearly distinct from the majority of the western jackdaw STs also in the ClonalFrame genealogy and located in the generalist clade (Fig. 1). Similarly, the CdtB phylogenetic tree (Fig. 4) showed that the sequences of these two isolates were more closely related to other *C. jejuni* strains, including the pheasant isolates from our study and generalist *C. jejuni* isolates from crows in a previous study (17).

The *cdtABC* operon was degenerated also in all mallard duck isolates, except one isolate that carried the novel *cdtABC* gene cluster, homologous to most of the western jackdaw isolates. This mallard duck isolate represented ST-6228, which was located in the same clade with western jackdaw STs in the ClonalFrame genealogy (Fig. 1) and had identical or closely related CdtB amino acid sequence to the western jackdaw and crow isolates (Fig. 4). In contrast to western jackdaw and mallard duck isolates, the *cdtABC* operon (similar to strain NCTC 11168) was intact and most likely functional among all pheasant isolates (ST-19).

Antimicrobial resistance-associated genetic markers

The *tetO* gene encoding for tetracycline resistance ribosomal protection protein (TetO) was found to be present in 14% (12/87) of the jackdaw isolates, but only in one of the 36 mallard duck isolates (3%) and none of the pheasant isolates. The 23S rRNA gene in positions 2074 and

2075 was wildtype, suggesting susceptibility to macrolides. Gene *AadE*, conferring streptomycin resistance, was absent among all bird isolates. No mutations associated with ciprofloxacin resistance in the *gyrA* gene were detected among mallard ducks, but one T86I substitution in *gyrA* was present in a western jackdaw isolate. Similarly, the T86I mutation in *gyrA* was found among all pheasant isolates.

The neighbor-joining tree representing the alignment of family class D beta-lactamase (*bla-OXA*) gene sequences is shown in Supplementary Figure S2. All pheasant isolates carried *bla-OXA 61* family class D beta-lactamase gene with sequences identical to *C. jejuni* type strain NCTC 11168. In contrast, *bla-OXA 184* family class D beta-lactamase was found to be present in 69% (60/87) of the western jackdaw isolates and in all mallard duck isolates. Furthermore, based on the beta-lactamase gene sequences among the western jackdaws and mallard ducks, they formed separate branches in the neighbor-joining tree (Fig. S2), with the exception of the sequences of two mallard duck isolates: SS6R (ST-6228) and SO-68 (ST-7843), located in the same cluster as the majority of the western jackdaw STs in the ClonalFrame analysis (Fig. 1).

Discussion

Birds are well-recognized natural reservoirs of *C. jejuni* and different wild bird species have been found to carry *C. jejuni*, among other *Campylobacter* spp., with variable frequencies. Thus, wild birds may pose a risk for public health either indirectly by transmitting *C. jejuni* to other animals, food production farms, and environmental waters, and through these, also to humans, or directly, when bird meat is consumed as game (4). This study is the first to characterize *C. jejuni* isolates from western jackdaws and game birds using MLST and comparative genomics in order to elucidate their potential public health significance. Western jackdaws have not been

235 previously investigated extensively. In this study, we found 43% (n=212) of our western jackdaw
236 isolates to carry *C. jejuni*. In addition to western jackdaws, 76% (n=108) of the mallard ducks
237 carried *C. jejuni*. An earlier study from New Zealand reported 23% (n=702) of wild mallard
238 ducks to be positive for *C. jejuni* (5), and in a study from the UK, farmed mallard ducks carried
239 high frequencies (93.3-100%) of *Campylobacter*, emphasizing their role also as a risk for human
240 health (22). The relatively high frequency and diversity of *C. jejuni* among mallard ducks in this
241 study may be at least partly a consequence of mixing with natural *C. jejuni* populations from
242 other waterfowl since the farmed-mallard ducks were raised in natural ponds, which were co-
243 habited by wild birds. In contrast to western jackdaws and mallard ducks, a low prevalence of *C.*
244 *jejuni* was found among farmed pheasants (9%). In a previous study from the Czech Republic,
245 70% of pheasants that were farmed with intensive production were positive for *Campylobacter*
246 sp., 41% being *C. jejuni* (23). However, in the same study, a lower frequency (25.7%) was
247 detected in wild pheasants; only 16% were *C. jejuni*-positive (23). Colonization dynamics of *C.*
248 *jejuni* in wild birds is complicated and may depend on, for instance, host ecology, age of the
249 bird, social behavior, and season (24).

250 Only limited epidemiological data exist on cases where western jackdaws, mallard ducks, or
251 pheasants have been suspected as a source of human infections. However, from the 1980s to
252 1990s in the UK, several milkborne outbreaks were associated with western jackdaws and
253 magpies that were pecking milk bottles located outside the front doors of houses (25, 26). Also,
254 in a Finnish epidemiological study in the 1990s we found two patients suspected of having
255 acquired campylobacteriosis after contact with pheasants (27).

256 The high occurrence of novel or rarely detected STs, which have only infrequently previously
257 been associated with human disease, suggests that while western jackdaws are not a major, they

are a potential source of campylobacteriosis in humans. The most commonly detected STs were ST-1282 and ST-6460, the former being previously isolated from a wild bird in Sweden and environmental water in Luxembourg (www.pubMLST.org). ST-6460, in turn, has been infrequently detected in a chicken slaughter batch in Finland in 2012 (2) and in a human patient in Sweden (www.pubMLST.org). Other STs found among western jackdaws isolated in human stools, included ST-1539 (UK and Netherlands), ST-6589 (Sweden), and ST-6590 (Sweden) (www.pubMLST.org/campylobacter). However, overall STs previously reported in human disease accounted for only 9 of 87 isolates (10%), and those reported previously in chickens (ST-6460 and ST-5543) accounted for 7 of 87 isolates (8%), supporting the result that jackdaws are not a major source of *C. jejuni* in human infections. Chicken farming is located in the same area in western Finland where the sampling of western jackdaws took place, suggesting that western jackdaw flocks living close to chicken farms may pose a risk of contamination.

Despite the high frequency (76%) of *C. jejuni* among Finnish mallard ducks, all STs differed from those previously found among Finnish human patients with infections acquired from domestic sources (18, 19, 28, 29). However, the five most common STs (ST-2314, ST-1299, ST-2839, ST-991, and ST-995) have previously been reported in the pubMLST database in different geographical areas from human stools (except ST-2839, unknown source) and from other wild birds (except ST-2314 and ST-2839), suggesting that these STs may have a wider host range and the capability of also causing human infections. Furthermore, the ST-2314 mallard duck isolates, representing one-third of all of the mallard duck isolates based on PFGE types (data not shown), differed in wgMLST analysis from each other by only 2 to 7 alleles, indicating that these isolates represent the same clone, which was also found to be genetically highly similar to a human isolate previously isolated in the UK. In addition, both the farmed and wild mallard ducks in this

study carried ST-995 (UA), which has been detected among mallard ducks also in Sweden (16) and New Zealand (5) (Fig. S3), suggesting a strong host association of this ST with mallard ducks.

All *C. jejuni* isolates from pheasants represented ST-19, which has previously been frequently isolated from human patients with gastroenteritis as well as from a wide variety of different hosts worldwide (22, 30-32). Since all the pheasants carried the same clone of *C. jejuni* (3 to 4 allelic differences in wgMLST analysis), but were hunted on three separate occasions and from two different locations, the birds had most likely been colonized already in the breeding farm at a very early stage, before they were moved to the separate hunting farms.

The phylogenomic tree based on the core genomes clearly indicated that although the western jackdaw isolates form a very distinct set of strains from the rest of the *C. jejuni* population, they do not form a cohesive single population. ClonalFrame genealogy further confirmed that the majority of the *C. jejuni* STs from western jackdaws clustered separately from the other STs previously detected in human patients, poultry, bovine, and other wild birds in Finland (2, 7, 18, 19, 28, 29), confirming the suggestion that these STs have evolved in western jackdaws and are not frequently transferred between different animal host species. Only two STs (ST-7841 and ST-4565) were located in the same branch, which comprised also STs isolated from human patients or poultry. ClonalFrame also showed that most of the mallard duck STs formed two branches, containing also STs detected previously from Finnish barnacle geese (7), which may indicate that these STs have adapted more widely to waterfowl. This was supported by the phylogenomic analysis. In conclusion, ClonalFrame genealogy of our study showed concordance with earlier studies (6, 7, 16), revealing that wild birds are mostly colonized by host-adapted

303 STs, but also have a minor set of STs shared with other hosts, including domestic animals and
304 human patients.

305 Comparative genomics using wgMLST was done to explore genomic diversity within the most
306 frequently detected STs. In conclusion, wgMLST using GeP (14) to detect closely related *C.*
307 *jejuni* isolates revealed that birds living in flocks in close contact are colonized by both
308 genetically highly related (e.g. ST-6460, ST-2314, and ST-19) and diverse STs (e.g. ST-1282).
309 Each strain and ST will most likely have their own capability to evolve, as shown earlier for ST-
310 45 CC (12), and for ST-2314 and ST-19 in this study, but more spatial and temporal data from
311 each genetic lineage are needed to make robust conclusions. Our results add to the evidence that
312 comparative genomics within STs, using wgMLST, is a suitable method to analyse the genomic
313 relationships of *C. jejuni* isolates. These results further confirm the results of our previous
314 studies, where we found that isolates originating from the same outbreak usually have only a few
315 allelic differences, whereas isolates from unassociated sources more likely have tens to several
316 hundreds of allelic differences (33-35).

317 Even though several putative virulence factors of *C. jejuni* have been suggested to be associated
318 with the pathogenesis of human gastroenteritis (20, 21, 36, 37), their functions are not well-
319 known and it remains unclear whether all strains have the capability to cause human infections
320 (38). Most of the STs detected among western jackdaw and mallard duck isolates were novel,
321 thus they have not been associated previously with human infections or detected in other
322 animals. Therefore, we studied genetic characteristics of these isolates to screen for virulence-
323 associated genes in their genomes. One of the most recently described putative virulence-
324 associated systems in *C. jejuni* (39) and in *C. coli* (40) is T6SS. In our study, a complete T6SS
325 gene cluster was found to be common among western jackdaw (46%) and mallard duck isolates

(72%). The high frequency of T6SS in our study is an interesting result because T6SS has been recognized to contribute to bacterial pathogenesis by toxic effects on host cells or competing bacterial species (39). The *C. jejuni* T6SS has been found to have pleiotropic effects, ranging from adaptation to host cell adherence, *in vivo* colonization, invasion, and cytotoxicity towards human erythrocytes (39, 41, 42). In addition, patients harboring a bacterial strain with T6SS often had bacteremia (39), suggesting more severe disease. The T6SS gene cluster has been found with variable frequencies in different studies, most probably depending on the origin of the strain. Commonly, approximately 10% of studied strains harbor T6SS, containing 13 ORFs similar to those described in *C. jejuni* strain 108 (39). T6SS was not detected among common STs and clonal lineages occurring in human patients and animal sources in the UK (e.g. ST-45 CC, ST-21 CC), but it was more often detected among human patient isolates from Vietnam and Pakistan, representing rare STs not commonly detected in Europe (42). This finding corroborates our results because most of the western jackdaw and mallard duck isolates had novel STs, which were not detected previously in human patients or other animal hosts. More studies on patients infected with uncommon STs containing T6SS are warranted to strengthen the evidence of an association between bacteremia and certain genetic lineages of *C. jejuni*.

Another virulence-associated system known to be common in *C. jejuni* is the cytolethal distending toxin (CDT), encoded by a three component operon *cdtABC* (37). CdtB is a DNase that causes DNA double-strand breaks in the nucleus, resulting in cell cycle arrest at the G2/M stage and apoptosis (43). Previous PCR-based studies directed at the CDT operon or a single gene of the operon have shown more than 95% of *C. jejuni* strains to be positive (36). In the present study, all, except two, of our western jackdaw isolates carried a *cdtABC* operon, but surprisingly it was degenerated in most (94%) of the isolates. The two isolates with an intact

copy of the *cdtABC* clustered more closely with the generalist clade in the ClonalFrame genealogy and also in the CdtB phylogeny. In addition, the gene cluster was totally missing from three of the jackdaw isolates. Interestingly, almost all isolates with a degenerated *cdtABC* locus also had another intact homologous *cdtABC* gene cluster. The newly identified *cdtABC* gene cluster was consistently present only in the western jackdaw clade in the ClonalFrame genealogy. This is the first time, to our knowledge, that another complete *cdtABC* gene cluster has been reported to be present in *C. jejuni*. Further analysis of the CdtB sequences of the newly identified gene cluster revealed that the sequences of *C. jejuni* isolates from western jackdaws were identical or closely related to those of crow isolates collected in California, USA (17). Comparison of the CdtB sequences also revealed that the jackdaw and crow sequences were more closely related to *C. lari* than other *C. jejuni* isolates, including the pheasant isolates from this study. This is an interesting finding and warrants further studies. All mallard duck isolates carried only the degenerated *cdtABC* locus. Whether the degeneration of the *cdtABC* locus is connected only to wild bird-associated genetic lineages remains unanswered and awaits future research.

The *tetO* gene, predicting resistance against tetracycline (TET), was found to be present among 14% of the western jackdaw isolates. Among mallard ducks, the *tetO* gene was present in only one isolate, and in none of the pheasant isolates, suggesting low resistance against TET. The T86I mutation in *gyrA*, previously linked to high fluoroquinolone-resistance in *Campylobacter* strains (44, 45), was found in only one jackdaw isolate. Among mallard ducks, *gyrA* was found to be wildtype, however, among pheasants, the T86I substitution in *gyrA* was found in all isolates, predicting resistance against ciprofloxacin. The MIC value of ciprofloxacin was confirmed to be $\geq$ 16 µg/ml and 64 µg/ml for nalidixic acid using VetMIC Camp EU (SVA,

372 Sverige). In a previous study, no resistance against ciprofloxacin (CIP) was found in wild bird
373 isolates in Finland (46), indicating that the results of the present study are mostly in line with the
374 low occurrence of fluoroquinolone resistance among wild birds since the pheasant isolates most
375 likely represented a single clone. It is intriguing why this single clone was so successful and no
376 other types were found among the pheasant isolates. The farm raising the pheasants for hunting
377 did not use any antibiotics and this may be excluded as a reason for the clonal expansion of this
378 resistant type. Contact with wildlife is likely in this production system from very early stages on,
379 however, only after the birds are released to the surroundings as 3-4 week old. Interestingly, a
380 small proportion of the hatched pheasant eggs are imported from France on a yearly basis, and
381 this may be a possible source of resistance since the birds carried the same clone on separate
382 farms even though they had been separated as one-day-old chicks. Furthermore, ST-19 is a
383 common generalist type known to colonize a variety of hosts, including wild bird in Canada and
384 ducks in the UK (https://pubmlst.org/campylobacter/), and carry the CIP-NAL resistance
385 phenotype (47, 48). According to EFSA and ECDC, resistance against ciprofloxacin is generally
386 high among human, chicken and turkey *C. jejuni* isolates in the EU, and showing a rising trend
387 (49). Further studies will reveal if fluoroquinolone resistance is on the rise also among *C. jejuni*
388 in wild bird populations. A previous study (50) reported that certain strains with this mutation in
389 *gyrA* showed no biological cost and fluoroquinolone-resistant strains could even outcompete
390 susceptible ones *in vivo* in chickens in the absence of selective pressure, which could also
391 explain the clonal expansion of successful clones. Genomic markers, conferring streptomycin
392 *(AadE*) and macrolide (23S rRNA gene mutation in positions 2074 and 2075) resistance, were
393 absent among all bird isolates. Overall, antimicrobial resistance has remained low in Finland

among the *C. jejuni* isolates of domestic origin relative to several other countries (46) and seems to be low also in birds migrating to Finland.

β-lactams (including penicillin, amoxicillin, and ampicillin) have been widely used in veterinary and clinical medicine, however, emerging resistance has compromised their use. High-level β-lactam resistance and β-lactamase activity have been shown to be linked to an upregulated *bla-OXA 61* gene in *C. jejuni* (51). Interestingly, among our *C. jejuni* collection the *bla-OXA 184* family (instead of *bla-OXA 61*) class D beta-lactamase was present in 69% of the western jackdaw isolates, and it was also present in all mallard duck isolates. In contrast, all pheasant isolates carried the OXA-61 family class D beta-lactamase. Further phylogenetic analysis revealed that the *bla-OXA 184* sequences between mallard duck and western jackdaw isolates differed markedly, suggesting an evolutionary link with the host. Previously, the OXA 184 family beta-lactamases have been found to be associated also with crow isolates compared with OXA 61, which has been associated with strains isolated from either domestic animals or human patients (17). These results reveal that OXA 184 family class D beta-lactamase is for an unknown reason associated with wild bird isolates, but future studies will extend our knowledge of their evolution and functionality.

In conclusion, phylogenetic studies performed on MLST data, CdtB and beta-lactamase gene sequences support a unique evolutionary history of *C. jejuni* in western jackdaws. Due to a high frequency of novel STs and STs only rarely detected among human patients and broiler batches, western jackdaws are, however, considered to be an infrequent source of campylobacteriosis in humans and contamination source of poultry production. Although the STs among western jackdaws and mallard ducks seem to be clearly host-associated, they may have the capability to transfer into domestic animals as well as occasionally cause human infections. Game birds

417 especially may pose a higher risk for acquiring campylobacteriosis, and both the carcasses and
418 meat should be handled accordingly. More evidence is anticipated to accumulate when MLST,
419 particularly wgMLST, is applied more extensively on different sources worldwide.

420 Materials and methods

421 Bacterial isolates

422 *Campylobacter* spp. was isolated from western jackdaws from two small towns, located in
423 Southern Finland (Lahti) and Western Finland (Seinäjoki), during a five-month period from
424 September 2014 to February 2015. Fecal droppings (n=212) were collected by placing a plastic
425 carpet under the known roosting trees of jackdaws. When jackdaws had settled in their roosting
426 trees, the flock was scared away to cause an automatic defecation of the birds. Individual
427 droppings were immediately swabbed into transport tubes (TS0001, Oxoid, Thermo Fisher
428 Scientific, Vantaa, Finland) and delivered to the laboratory chilled (+4ºC). Contamination of the
429 collected fecal material is unlikely as we checked that no other bird species were present in the
430 same tree before scaring the jackdaw flocks away. Of note, especially hooded crows tend to roost
431 with jackdaws.

432 Samples from game birds that were farmed for hunting were collected during the hunting season
433 in Southern Finland. The birds were shot by licensed hunters and no animals were killed for the
434 purpose of this study. Mallard ducks (n=100) were hunted from September to November in 2014
435 and pheasants (n=100) from November to December in 2013. The birds were plucked (mallard
436 ducks) or skinned (pheasants) and eviscerated immediately after hunting. Intestinal samples were
437 collected during evisceration, and transported to the laboratory chilled (+4ºC). The mallard ducks
438 were hunted on the surroundings of a single farm. The birds were hatched on another breeding

439 farm and three- to four-week-old birds were transferred and released to the natural ponds of the
440 hunting farm where they were raised by regular feeding. Pheasants were hatched on the same
441 farm as mallard ducks were raised on. Six batches stayed on the same farm for hunting and three
442 batches of one-day-old chicks were transferred to yet another hunting farm where they were
443 raised. In both hunting farms, the four-week-old birds were released into the surroundings where
444 their feeding was continued.

445 In addition to farmed game mallard ducks, fresh fecal samples (n=8) from the wild mallard duck
446 population, living in a lake of an urban park in Southern Finland, were collected from individual
447 droppings into transport tubes (TS0001, Oxoid, Thermo Fisher Scientific, Vantaa, Finland) in
448 November 2014.

449 Fecal samples were cultivated on mCCDA plates (Oxoid Ltd., Hampshire, UK). Typical colonies
450 were Gram-stained and genomic DNA was extracted using PureLink™ Genomic DNA minikit
451 (Invitrogen, Waltham, MA, USA). *Campylobacter* species was identified by species-specific
452 PCR (52). DNA of all *C. jejuni* isolates from western jackdaws, pheasants, and wild mallard
453 ducks was subjected to WGS.

454 For *C. jejuni*-positive game mallard duck isolates, pulsed-field gel electrophoresis (PFGE), using
455 KpnI restriction enzyme, was first used to screen the diversity of PFGE types among the isolates.
456 PFGE was performed as described previously (53). Thirty-five isolates, representing all of the
457 different PFGE profiles (some in duplicate or triplicate), were selected and their DNA was
458 subjected to WGS.

Whole-genome sequencing

Whole-genome sequencing (WGS) was performed using Illumina HiSeq Technology at the Institute for Molecular Medicine Finland (FIMM). Raw data were assembled into contigs using INNUca pipeline (https://github.com/B-UMMI/INNUca) and genome sequences (n=6) that did not pass the quality threshold were excluded from further analyses.

Phylogenetic analyses

A phylogenomic tree was constructed based on the core genomes of 1261 *C. jejuni* strains selected from the INNUENDO *Campylobacter jejuni* dataset (54) and including the genomes sequenced in this study (Supplementary Dataset S1). The genomes were annotated using Prokka 1.12 (55). A pan genome analysis was performed using Roary 3.7.0 (56) with 95% protein identity as cutoff for defining gene clusters and the core genes. FastTree 2.1 (57) was used with the Jukes-Cantor model of nucleotide evolution for building the approximation of a maximum-likelihood (ML) phylogenetic tree based on the core genome alignment (99% shared loci in Roary analysis, including 942 core genes). When necessary the jobs were run in parallel using GNU Parallel (58). iTOL (59) v4.2.3 was used for visualization. The tree was rooted at midpoint for better visualization.

ClonalFrame genealogy was used to describe the phylogeny of the *C. jejuni* MLST allele sequences collected in this study and including also MLST sequence data of *C. jejuni* isolates from our previous studies originating from several sources (poultry, bovine, barnacle geese and human) in Finland (2, 7, 18, 19, 28, 29). ClonalFrame was run using default parameters (60), and the consensus tree was displayed in MEGA6 and labelled using CorelDRAW X8.

GoeBURST full Minimum spanning tree (MST) was constructed using PHYLOViZ (61). Besides our wild bird isolates, MLST data for mallard ducks from Sweden (16) and New Zealand (5) were obtained from the PubMLST database (www.pubMLST.org/campylobacter).

Comparative genomics

Genome profiler (GeP) (14) was used in wgMLST to further compare the draft genomes. In addition to our isolates, WGS data from clinical isolates from the UK were obtained from the PubMLST database (pubMLST.org/campylobacter) for reference. The clinical ST-19 isolates from the UK were chosen to represent all available subtypes as defined by using coregenome (cg) MLST for 700 loci (https://github.com/mickaelsilva/chewBBACA_deprecated/wiki), and different years. Allelic differences between strains of each ST, obtained using GeP, were visualized using SplitsTree4 (62) and edited in CorelDRAW X8.

RAST (Rapid Annotation using Subsystem Technology) server (63) was used for preliminary annotation and genomic characterization of the isolates. Sequence-based comparisons, genome browsing, and alignments for visualization of synteny of homologous genomic regions were performed using the SEED viewer (64, 65) with default parameters. Novel open reading frames, genes associated with cytolethal distending toxin (*cdtABC*) operon, integrated elements (CJIE1, 2, 3, and 4), type VI secretion system (T6SS), family class D beta-lactamases, and antimicrobial resistance were further analyzed using the InterProScan (66) and by BLASTN and/or BLASTP searches against the non-redundant nucleotide/protein sequences database (nr) in GenBank. Phylogenetic analyses of genes of interest were performed by aligning the amino acid sequences of the *cdtB* gene and nucleotide sequences of family class D beta-lactamases using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) and MEGA6 (67) was used to construct a

phylogenetic neighbor-joining tree based on the alignments. The resulting phylogenetic trees were text edited in CorelDRAW 2017.

Sequence deposition

Whole-genome sequences of those bird isolates that represented novel STs were deposited to the pubMLST database (www.pubMLST.org/campylobacter) to assign new sequence types and allelic profiles.

The whole-genome sequences of all of the isolates sequenced in this study have been deposited in GenBank under BioProject PRJNA430314. The relevant BioSample and sequence accession numbers are listed in Supplementary Table S1. NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) was used to annotate the genomes.

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References

1. **European Food Safety Authority, EFSA, European Centre for Disease Prevention and Control, ECDC.** 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA Journal doi:10.2903/j.efsa.2016.4634.

2. **Llarena AK, Huneau A, Hakkinen M, Hänninen M-L.** 2015. Predominant *Campylobacter jejuni* sequence types persist in Finnish chicken production. PLoS One **10:**e0116585. doi:10.1371/journal.pone.0116585.

3. **Jaakola S, Lyytikäinen O, Rimhanen-Finne R, Salmenlinna S, Savolainen-Kopra C, Liitsola K, Jalava J, Toropainen M, Nohynek H, Virtanen M, Löflund J-E, Kuusi M, Salminen M.** 2017. Tartuntataudit Suomessa 2016. THL - Raportti 5/2017 http://urn.fi/URN:ISBN:978-952-302-890-6.

4. **Waldenström J, Griekspoor P.** 2014. Ecology and Host Associations of *Campylobacter* in Wild Birds, p. 265-284. *In* Sheppard S (ed), *Campylobacter* Ecology and Evolution, 1st ed, Caister Academic Press, Norfolk, UK.

5. **Mohan V, Stevenson M, Marshall J, Fearnhead P, Holland BR, Hotter G, French NP.** 2013. *Campylobacter jejuni* colonization and population structure in urban populations of ducks and starlings in New Zealand. MicrobiologyOpen **2:**659-673. doi:10.1002/mbo3.102.

6. **Griekspoor P, Hansbro PM, Waldenström J, Olsen B.** 2015. *Campylobacter jejuni* sequence types show remarkable spatial and temporal stability in Blackbirds. Infect Ecol Epidemiol **5:**28383. doi:10.3402/iee.v5.28383.

7. **Llarena AK, Skarp-de Haan CP, Rossi M, Hänninen M-L.** 2015. Characterization of the *Campylobacter jejuni* population in the barnacle geese reservoir. Zoonoses Public Health **62:**209-221. doi:10.1111/zph.12141.

8. **Pohja-Mykrä M (ed), Koskinen S, Mykrä S, Nieminen T, Sillanpää H.** 2016. Naakka ja ihminen - Naakkojen aiheuttamien haittojen hallinta, Suomen Ympäristö. Suomen Ympäristö **2:**110-p.110.

9. **Sokos CK, Birtsas PK, Tsachalidis EP.** 2008. The aims of galliforms release and choice of techniques. Wildlife Biology **14:**412-422.

10. **Matheson SM, Donbavand J, Sandilands V, Pennycott T, Turner SP.** 2015. An ethological approach to determining housing requirements of gamebirds in raised laying units. Appl Anim Behav Sci **165:**17. https://doi.org/10.1016/j.applanim.2015.02.001.

11. **Cody AJ, McCarthy ND, Jansen van Rensburg M, Isinkaye T, Bentley SD, Parkhill J, Dingle KE, Bowler IC, Jolley KA, Maiden MC.** 2013. Real-time genomic epidemiological evaluation of human *Campylobacter* isolates by use of whole-genome multilocus sequence typing. J Clin Microbiol **51:**2526-2534. doi:10.1128/JCM.00066-13.

12. **Llarena AK, Zhang J, Vehkala M, Välimäki N, Hakkinen M, Hänninen M-L, Roasto M, Mäesaar M, Taboada E, Barker D, Garofolo G, Cammà C, Di Giannatale E, Corander J, Rossi M.** 2016. Monomorphic genotypes within a generalist lineage of *Campylobacter jejuni* show signs of global dispersion. Microb Genom **2:**e000088. doi:10.1099/mgen.0.000088.

562 13. **Cody AJ, Bray JE, Jolley KA, McCarthy ND, Maiden MCJ.** 2017. Core genome
563 multilocus sequence typing scheme for stable, comparative analyses of *Campylobacter jejuni* and
564 *C. coli* human disease isolates. J Clin Microbiol **55:**2086-2097. doi:10.1128/JCM.00080-17.

565 14. **Zhang J, Halkilahti J, Hänninen M-L, Rossi M.** 2015. Refinement of whole-genome
566 multilocus sequence typing analysis by addressing gene paralogy. J Clin Microbiol **53:**1765-
567 1767. doi:10.1128/JCM.00051-15.

568 15. **Colles FM, McCarthy ND, Howe JC, Devereux CL, Gosler AG, Maiden MC.** 2009.
569 Dynamics of *Campylobacter* colonization of a natural host, *Sturnus vulgaris* (European starling).
570 Environ Microbiol **11:**258-267. doi:10.1111/j.1462-2920.2008.01773.x.

571 16. **Griekspoor P, Colles FM, McCarthy ND, Hansbro PM, Ashhurst-Smith C, Olsen B,**
572 **Hasselquist D, Maiden MC, Waldenstrom J.** 2013. Marked host specificity and lack of
573 phylogeographic population structure of *Campylobacter jejuni* in wild birds. Mol Ecol **22:**1463-
574 1472. doi:10.1111/mec.12144.

575 17. **Weis AM, Storey DB, Taff CC, Townsend AK, Huang BC, Kong NT, Clothier KA,**
576 **Spinner A, Byrne BA, Weimer BC.** 2016. Genomic comparison of *Campylobacter* spp. and
577 their potential for zoonotic transmission between birds, primates, and livestock. Appl Environ
578 Microbiol **82:**7165-7175.

579 18. **de Haan CP, Kivistö R, Hakkinen M, Rautelin H, Hänninen M-L.** 2010. Decreasing
580 trend of overlapping multilocus sequence types between human and chicken *Campylobacter*
581 *jejuni* isolates over a decade in Finland. Appl Environ Microbiol **76:**5228-5236.
582 doi:10.1128/AEM.00581-10.

19. **Kovanen SM, Kivistö RI, Rossi M, Schott T, Kärkkäinen UM, Tuuminen T, Uksila J, Rautelin H, Hänninen M-L.** 2014. Multilocus sequence typing (MLST) and whole-genome MLST of *Campylobacter jejuni* isolates from human infections in three districts during a seasonal peak in Finland. J Clin Microbiol **52:**4147-4154. doi:10.1128/JCM.01959-14.

20. **Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Parker CT, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, Sullivan SA, Shetty JU, Ayodeji MA, Shvartsbeyn A, Schatz MC, Badger JH, Fraser CM, Nelson KE.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol doi:10.1371/journal.pbio.0030015.

21. **Ugarte-Ruiz M, Stabler RA, Dominguez L, Porrero MC, Wren BW, Dorrell N, Gundogdu O.** 2015. Prevalence of Type VI Secretion System in Spanish *Campylobacter jejuni* Isolates. Zoonoses Public Health **62:**497-500. doi:10.1111/zph.12176.

22. **Colles FM, Ali JS, Sheppard SK, McCarthy ND, Maiden MC.** 2011. *Campylobacter* populations in wild and domesticated Mallard ducks (*Anas platyrhynchos*). Environ Microbiol Rep **3:**574-580. doi:10.1111/j.1758-2229.2011.00265.x.

23. **Nebola M, Borilova G, Steinhauserova I.** 2007. Prevalence of *Campylobacter* subtypes in pheasants (*Phasianus colchicus* spp. *torquatus*) in the Czech Republic. Veterinarni medicina **52:**496-501.

24. **Taff CC, Weis AM, Wheeler S, Hinton MG, Weimer BC, Barker CM, Jones M, Logsdon R, Smith WA, Boyce WM, Townsend AK.** 2016. Influence of Host Ecology and Behavior on *Campylobacter jejuni* Prevalence and Environmental Contamination Risk in a

604 Synanthropic Wild Bird Species. Appl Environ Microbiol **82:**4811-4820.
605 doi:10.1128/AEM.01456-16.

606 25. **Southern JP, Smith RM, Palmer SR.** 1990. Bird attack on milk bottles: possible mode of
607 transmission of *Campylobacter jejuni* to man. Lancet **336:**1425-1427.

608 26. **Hudson SJ, Lightfoot NF, Coulson JC, Russell K, Sisson PR, Sobo AO.** 1991. Jackdaws
609 and magpies as vectors of milkborne human *Campylobacter* infection. Epidemiol Infect
610 **107:**363-372.

611 27. **Hänninen M-L, Pajarre S, Klossner ML, Rautelin H.** 1998. Typing of human
612 *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. J Clin Microbiol
613 **36:**1787-1789.

614 28. **Kärenlampi R, Rautelin H, Schönberg-Norio D, Paulin L, Hänninen M-L.** 2007.
615 Longitudinal study of Finnish *Campylobacter jejuni* and *C. coli* isolates from humans, using
616 multilocus sequence typing, including comparison with epidemiological data and isolates from
617 poultry and cattle. Appl Environ Microbiol **73:**148-155. doi:10.1128/AEM.01488-06.

618 29. **de Haan CP, Kivistö RI, Hakkinen M, Corander J, Hänninen M-L.** 2010. Multilocus
619 sequence types of Finnish bovine *Campylobacter jejuni* isolates and their attribution to human
620 infections. BMC Microbiol **10:**200-2180-10-200. doi:10.1186/1471-2180-10-200.

621 30. **Rotariu O, Dallas JF, Ogden ID, Macrae M, Sheppard SK, Maiden M, Gormley FJ,**
622 **Forbes KJ, Strachan NJ.** 2009. Spatiotemporal homogeneity of *Campylobacter* subtypes from

623 cattle and sheep across NE and SW Scotland. Appl Environ Microbiol .
624 doi:10.1128/AEM.00499-09.

625 31. **Cody AJ, McCarthy NM, Wimalarathna HL, Colles FM, Clark L, Bowler IC, Maiden**
626 **MC, Dingle KE.** 2012. A longitudinal 6-year study of the molecular epidemiology of clinical
627 *Campylobacter* isolates in Oxfordshire, United kingdom. J Clin Microbiol **50:**3193-3201.
628 doi:10.1128/JCM.01086-12.

629 32. **Mossong J, Mughini-Gras L, Penny C, Devaux A, Olinger C, Losch S, Cauchie HM,**
630 **van Pelt W, Ragimbeau C.** 2016. Human campylobacteriosis in Luxembourg, 2010-2013: A
631 case-control study combined with multilocus sequence typing for source attribution and risk
632 factor analysis. Sci Rep **6:**20939. doi:10.1038/srep20939.

633 33. **Revez J, Llarena AK, Schott T, Kuusi M, Hakkinen M, Kivistö R, Hänninen M-L, Rossi**
634 **M.** 2014. Genome analysis of *Campylobacter jejuni* strains isolated from a waterborne outbreak.
635 BMC Genomics **15:**768-2164-15-768. doi:10.1186/1471-2164-15-768.

636 34. **Revez J, Zhang J, Schott T, Kivistö R, Rossi M, Hänninen M-L.** 2014. Genomic variation
637 between *Campylobacter jejuni* isolates associated with milk-borne-disease outbreaks. J Clin
638 Microbiol **52:**2782-2786. doi:10.1128/JCM.00931-14.

639 35. **Kovanen S, Kivistö R, Llarena AK, Zhang J, Kärkkäinen UM, Tuuminen T, Uksila J,**
640 **Hakkinen M, Rossi M, Hänninen M-L.** 2016. Tracing isolates from domestic human
641 *Campylobacter jejuni* infections to chicken slaughter batches and swimming water using whole-
642 genome multilocus sequence typing. Int J Food Microbiol **226:**53-60.
643 doi:10.1016/j.ijfoodmicro.2016.03.009.

644 36. **Mortensen NP, Schiellerup P, Boisen N, Klein BM, Locht H, Abuoun M, Newell D,**
645 **Krogfelt KA.** 2011. The role of *Campylobacter jejuni* cytolethal distending toxin in
646 gastroenteritis: toxin detection, antibody production, and clinical outcome. APMIS **119:**626-634.
647 doi:10.1111/j.1600-0463.2011.02781.x.

648 37. **Lai CK, Chen YA, Lin CJ, Lin HJ, Kao MC, Huang MZ, Lin YH, Chiang-Ni C, Chen**
649 **CJ, Lo UG, Lin LC, Lin H, Hsieh JT, Lai CH.** 2016. Molecular Mechanisms and Potential
650 Clinical Applications of *Campylobacter jejuni* Cytolethal Distending Toxin. Front Cell Infect
651 Microbiol **6:**9. doi:10.3389/fcimb.2016.00009.

652 38. **Bolton DJ.** 2015. *Campylobacter* virulence and survival factors. Food Microbiol **48:**99-108.
653 doi:10.1016/j.fm.2014.11.017.

654 39. **Bleumink-Pluym NM, van Alphen LB, Bouwman LI, Wosten MM, van Putten JP.** 2013.
655 Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring
656 capsule polysaccharide sensitive cytotoxicity. PLoS Pathog **9:**e1003393.
657 doi:10.1371/journal.ppat.1003393.

658 40. **Ghatak S, He Y, Reed S, Strobaugh T,Jr, Irwin P.** 2017. Whole genome sequencing and
659 analysis of *Campylobacter coli* YH502 from retail chicken reveals a plasmid-borne type VI
660 secretion system. Genom Data **11:**128-131. doi:10.1016/j.gdata.2017.02.005.

661 41. **Lertpiriyapong K, Gamazon ER, Feng Y, Park DS, Pang J, Botka G, Graffam ME, Ge**
662 **Z, Fox JG.** 2012. *Campylobacter jejuni* type VI secretion system: roles in adaptation to
663 deoxycholic acid, host cell adherence, invasion, and in vivo colonization. PLoS One **7:**e42842.
664 doi:10.1371/journal.pone.0042842.

42. **Harrison JW, Dung TT, Siddiqui F, Korbrisate S, Bukhari H, Tra MP, Hoang NV, Carrique-Mas J, Bryant J, Campbell JI, Studholme DJ, Wren BW, Baker S, Titball RW, Champion OL.** 2014. Identification of possible virulence marker from *Campylobacter jejuni* isolates. Emerg Infect Dis **20:**1026-1029. doi:10.3201/eid2006.130635.

43. **Pickett CL, Pesci EC, Cottle DL, Russell G, Erdem AN, Zeytin H.** 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* gene. Infect Immun **64:**2070-2078.

44. **Wang Y, Huang WM, Taylor DE.** 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob Agents Chemother **37:**457-463.

45. **Han J, Wang Y, Sahin O, Shen Z, Guo B, Shen J, Zhang Q.** 2012. A fluoroquinolone resistance associated mutation in *gyrA* affects DNA supercoiling in *Campylobacter jejuni*. Front Cell Infect Microbiol **2:**21. doi:10.3389/fcimb.2012.00021.

46. **Olkkola S, Nykasenoja S, Raulo S, Llarena AK, Kovanen S, Kivistö R, Myllyniemi AL, Hänninen M-L.** 2016. Antimicrobial Resistance and Multilocus Sequence Types of Finnish *Campylobacter jejuni* Isolates from Multiple Sources. Zoonoses Public Health **63:**10-19. doi:10.1111/zph.12198.

47. **Kovac J, Cadez N, Stessl B, Stingl K, Gruntar I, Ocepek M, Trkov M, Wagner M, Smole Mozina S.** 2015. High genetic similarity of ciprofloxacin-resistant *Campylobacter jejuni* in central Europe. Front Microbiol **6:**1169. doi:10.3389/fmicb.2015.01169.

685 48. **Habib I, Miller WG, Uyttendaele M, Houf K, De Zutter L.** 2009. Clonal population
686 structure and antimicrobial resistance of *Campylobacter jejuni* in chicken meat from Belgium.
687 Appl Environ Microbiol **75:**4264-4272. doi:10.1128/AEM.00168-09.

688 49. **EFSA (European Food Safety Authority), ECDC (European Centre for Disease**
689 **Prevention and Control).** 2018. The European Union summary report on antimicrobial
690 resistance inzoonotic and indicator bacteria from humans, animals and food in 2016. EFSA
691 Journal **16(2):5182:**270 pp.

692 50. **Luo N, Pereira S, Sahin O, Lin J, Huang S, Michel L, Zhang Q.** 2005. Enhanced *in vivo*
693 fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection
694 pressure. Proc Natl Acad Sci U S A **102:**541-546.

695 51. **Zeng X, Brown S, Gillespie B, Lin J.** 2014. A single nucleotide in the promoter region
696 modulates the expression of the beta-lactamase OXA-61 in *Campylobacter jejuni*. J Antimicrob
697 Chemother **69:**1215-1223. doi:10.1093/jac/dkt515.

698 52. **Denis M, Soumet C, Rivoal K, Ermel G, Blivet D, Salvat G, Colin P.** 1999. Development
699 of a m-PCR assay for simultaneous identification of Campylobacter jejuni and C. coli. Lett Appl
700 Microbiol **29:**406-410.

701 53. **Kärenlampi R, Rautelin H, Hakkinen M, Hänninen M-L.** 2003. Temporal and
702 geographical distribution and overlap of Penner heat-stable serotypes and pulsed-field gel
703 electrophoresis genotypes of *Campylobacter jejuni* isolates collected from humans and chickens
704 in Finland during a seasonal peak. J Clin Microbiol **41:**4870-4872.

54. **Rossi M, Silva MSD, Ribeiro-Gonçalves BF, Silva DN, Machado MP, Oleastro M, Borges V, Isidro J, Viera L, Barker DO, Llarena A, Halkilahti J, Jaakkonen A, Kivistö R, Kovanen S, Nieminen T, Hänninen M-L, Salmenlinna S, Hakkinen M, Garaizar J, Bikandi J, Hilbert F, Taboada EN, Carriço JA.** 2018. INNUENDO whole genome and core genome MLST schemas and datasets for Campylobacter jejuni. doi:10.5281/zenodo.1322564.

55. **Seemann T.** 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics **30:**2068-2069. doi:10.1093/bioinformatics/btu153.

56. **Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush D, Keane JA, Parkhill J.** 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics **31:**3691-3693. doi:10.1093/bioinformatics/btv421.

57. **Price MN, Dehal PS, Arkin AP.** 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One **5:**e9490. doi:10.1371/journal.pone.0009490.

58. **Tange O.** 2011. GNU Parallel - The Command-Line Power Tool. ;login: The USENIX Magazine **36:**42-47.

59. **Letunic I, Bork P.** 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res **44:**W242-5. doi:10.1093/nar/gkw290.

60. **Didelot X, Falush D.** 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics **175:**1251-1266. doi:10.1534/genetics.106.063305.

61. **Francisco AP, Bugalho M, Ramirez M, Carrico JA.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics **10:**152-2105-10-152. doi:10.1186/1471-2105-10-152.

62. **Huson DH, Bryant D.** 2006. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol **23:**254-267. doi:10.1093/molbev/msj030.

63. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9:**75-2164-9-75. doi:10.1186/1471-2164-9-75.

64. **Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, de Crecy-Lagard V, Diaz N, Disz T, Edwards R, Fonstein M, Frank ED, Gerdes S, Glass EM, Goesmann A, Hanson A, Iwata-Reuyl D, Jensen R, Jamshidi N, Krause L, Kubal M, Larsen N, Linke B, McHardy AC, Meyer F, Neuweger H, Olsen G, Olson R, Osterman A, Portnoy V, Pusch GD, Rodionov DA, Ruckert C, Steiner J, Stevens R, Thiele I, Vassieva O, Ye Y, Zagnitko O, Vonstein V.** 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res **33:**5691-5702. doi:10.1093/nar/gki866.

65. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the

744 Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids
745 Res **42:**D206-14. doi:10.1093/nar/gkt1226.

746 66. **Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R.** 2005.
747 InterProScan: protein domains identifier. Nucleic Acids Res **33:**W116-20.
748 doi:10.1093/nar/gki442.

749 67. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular
750 Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725-2729.
751 doi:10.1093/molbev/mst197.

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Figure legends

Figure 1. ClonalFrame genealogy of MLST allele sequences in different hosts detected in this study (western jackdaws and mallard ducks) and previously in Finland (2, 7, 18, 19, 28, 29). Different hosts and combinations of hosts are indicated with different colors, and those STs that were further analyzed in wgMLST are indicated with arrows. A dashed line separates the STs detected from human patients, poultry, bovine, and wild birds (generalist clade) from the STs mainly detected from western jackdaws (western jackdaw clade).

Figure 2. WgMLST describing the allelic differences among shared loci of ST-1282 (A), ST-6460 (B), ST-2314 (C) and ST-19 (D) isolates using GeP (14). Numbers between strains indicate allelic differences. Jackdaw isolates are indicated in dark blue, chicken isolate in light blue, mallard duck isolates in orange, pheasant isolates in red, clinical isolates from the UK (www.pubMLST.org/campylobacter) in black and Finland in green.

Figure 3. Seed Viewer homology-based sequence alignment of the two *cdtABC* gene clusters (top and bottom rows) of western jackdaw strain CB287. The chromosomal region of the focus gene (top) is compared with four similar organisms (*C.* = *Campylobacter*, *H.* = *Helicobacter*). The graphic is centered on the focus gene (*cdtC*), which is red and numbered 1. Sets of genes with similar sequence are grouped with matching number and color code. Genes that are not conserved remain unnumbered and are shown in gray.

Figure 4. Neighbor-joining tree representing the alignment of CdtB amino acid sequences. Accession number, species, and strain number are indicated for each sequence. Strains from this study are indicated in boldface and with CB for western jackdaw, SO or SS for mallard duck, and FA for pheasant in the strain name.