

# Genetic Diversity of *Campylobacter jejuni* Isolates from Farm Animals and the Farm Environment

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**The genetic diversity of *Campylobacter jejuni* isolates from farm animals and their environment was investigated by multilocus sequence typing (MLST). A total of 30 genotypes, defined by allelic profiles (assigned to sequence types [STs]), were found in 112 *C. jejuni* isolates originating in poultry, cattle, sheep, starlings, and slurry. All but two of these genotypes belonged to one of nine *C. jejuni* clonal complexes previously identified in isolates from human disease and retail food samples and one clonal complex previously associated with an environmental source. There was some evidence for the association of certain clonal complexes with particular farm animals: isolates belonging to the ST-45 complex predominated among poultry isolates but were absent among sheep isolates, while isolates belonging to the ST-61 and ST-42 complexes were predominant among sheep isolates but were absent from the poultry isolates. In contrast, ST-21 complex isolates were distributed among the different isolation sources. Comparison with MLST data from 91 human disease isolates showed small but significant genetic differentiation between the farm and human isolates; however, representatives of six clonal complexes were found in both samples. These data demonstrate that MLST and the clonal complex model can be used to identify and compare the genotypes of *C. jejuni* isolates from farm animals and the environment with those from retail food and human disease.**

*Campylobacter jejuni* continues to be the most common etiological agent of bacterial gastroenteritis in the developed world, with an estimated 2.5 million cases occurring per year in the United States (22) and over 54,000 cases reported to the United Kingdom Public Health Laboratory Service during 2001 (1). It is also a major problem in developing countries, particularly among young children (7). In contrast to many other food-borne bacterial pathogens, the majority of cases of campylobacteriosis in humans are considered to be sporadic, with few cases of disease traceable to point sources. It is thought that the consumption of inadequately cooked contaminated meat, particularly that from poultry, is a major source of human infection, but this has not been established unequivocally (8). Investigations into the epidemiology of human infection by this bacterium have been complicated by a number of factors, including its high genetic and antigenic diversity (2) and its wide, perhaps ubiquitous, distribution. In addition, serological typing methods have lacked reproducibility among laboratories and discriminatory power, with a large number of isolates reported as untypeable (12, 28, 41).

*C. jejuni* is an apparently harmless commensal of the gastrointestinal tract of many domestic and wild animals, especially birds (5). It is also readily isolated from a range of environmental locations, including soil, surface water, and the sand of bathing beaches, probably as a consequence of contact with various contamination sources, including animal feces (3, 5, 20, 26, 30). Environmental reservoirs of *C. jejuni* can act as sources of infection for humans; for example, wild birds pecking milk

bottle tops have been shown to cause outbreaks of human campylobacteriosis (24). However, the contribution of such sources to the overall burden of human disease and to the colonization of farm animals has not been established. Whether only some of the *C. jejuni* present in environmental reservoirs are pathogenic to humans (20) and whether all animals and microenvironments harbor the same strains are important questions that remain incompletely addressed. Population genetic approaches that examine differentiation between populations provide a novel approach to resolving this problem.

Multilocus sequence typing (MLST) is a molecular isolate characterization technique that exploits recent advances in high-throughput molecular technology to generate accurate isolate characterization data efficiently and inexpensively. Data are highly reproducible among laboratories and can be shared electronically via the Internet (10). It has sufficient resolution to accommodate the high levels of diversity present in many bacterial pathogen populations but can also rationalize this diversity into groups of isolates with related genotypes. MLST has been successfully employed to characterize a variety of bacteria, including *C. jejuni* (11, 35), but to date studies of *C. jejuni* have focused on isolates obtained from human disease and retail food products, with relatively few environmental isolates having been investigated.

In the present work, MLST was used to characterize 112 isolates from a variety of farm and environmental sources with the aim of establishing the genetic diversity of *C. jejuni* in the farm setting and comparing this diversity with that found among collections of human disease isolates. There was small but significant differentiation between the two populations, although many of the genotypes found among isolates derived from human disease were present in the environmental sam-

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TABLE 1. Allelic diversity among 112 *C. jejuni* isolates from farm animal and environmental sources

Locus	Fragment size (bp)	No. of alleles	No. of variable sites <sup>b</sup>	% Variable sites <sup>b</sup>	$d_N/d_S$ ratio <sup>b</sup>	Pairwise $F_{ST}$ <sup>a</sup>	<i>P</i> values <sup>c</sup>
<i>asp</i>	477	5	13	2.7	0.045	0.044	0.005
<i>gln</i>	477	8	17	3.6	0.093	0.028	0.000
<i>glt</i>	402	12	16	4	0.048	0.094	0.000
<i>gly</i>	507	11	28	5.5	0.045	0.053	0.000
<i>pgm</i>	498	11	34	6.8	0.026	0.081	0.000
<i>tkt</i>	459	9	26	5.7	0.008	0.051	0.000
<i>unc</i>	489	6	71 (4)	14.5 (0.8)	0.143 (0.000)	0.015	0.135
All loci	3,309	30	207 (140)	6.3 (4.2)	0.030 (0.037)	0.005	0.000

<sup>a</sup> Comparison with the 91 human disease isolates from stool cultures in the United Kingdom during 1999.

<sup>b</sup> Figures in parentheses exclude *uncA* allele 17, which may have come from a different *Campylobacter* species (11).

<sup>c</sup>  $F_{ST}$  *P* values calculated at a 0.05 significance level.

ples. The results indicated the potential for combining MLST data with population genetic analyses in elucidating the relative importance of the various possible sources of human infection by *C. jejuni*.

#### MATERIALS AND METHODS

**Bacterial isolates.** A total of 112 *C. jejuni* isolates obtained from poultry, cattle, sheep, and environmental sources from northwestern England during the 1990s were investigated. These comprised 16 isolates from the whole intestine of broiler birds from a poultry processing plant supplied by 35 farms located within a 150-mile radius (38); 12 isolates from fresh feces of turkey chicks kept on a dairy farm (39); 14 isolates from the small intestines of adult beef cattle at an abattoir receiving cattle and sheep from northwestern England, northern Wales, and southwestern Scotland; 9 isolates from fresh feces of newborn calves (32); 9 isolates from the small intestines of lambs at the same abattoir as the beef cattle (33); 14 isolates from intestinal contents of sheep at slaughter; 10 isolates from fresh feces of sheep grazing on salt marsh; 9 isolates from fresh feces of sheep grazing on upland fell (21); 10 isolates from slurry storage tanks (34); and 9 isolates from starling feces collected on the same dairy farm as the isolates from turkey chicks (31).

**Microbiological isolate characterization.** The isolates had been presumptively identified as thermophilic *Campylobacter* species on the basis of microscopy and catalase and oxidase reactions. Isolates from the sheep and poultry were biotyped by using the modified scheme of Bolton et al. (4). Some isolates from sheep grazing on salt marsh and fell pastures were biotyped by using the MAST ID Camp Identification System (Mast Diagnostics, Bootle, United Kingdom). Where necessary, identification of some of the isolates to species level was confirmed by nucleotide sequencing of an 812-bp segment of 23S RNA, using the 43a and 69a primers designed by van Camp et al. (36).

**DNA preparation.** The isolates, which had been stored at  $-80^{\circ}\text{C}$  in a cryopreservative medium, were revived on Columbia agar (CM 0331; Oxoid Ltd., Basingstoke, United Kingdom) with 5% horse blood in a microaerophilic atmosphere produced by using gas-generating sachets (CN0025A; Oxoid Ltd.; and 96125; bioMerieux, Basingstoke, United Kingdom) at  $42^{\circ}\text{C}$  for 48 h. A suspension of approximately  $10^6$  cells per ml was prepared in sample buffer, and chromosomal DNA was extracted by using IsoQuick nucleic acid extraction kits (ISC Bioexpress, Kaysville, Utah) and the rapid DNA extraction protocol according to the manufacturer's instructions.

**MLST.** The previously published protocol for *C. jejuni* MLST was used (11). Briefly, fragments of seven housekeeping genes (aspartase A, *aspA*; glutamine synthetase, *glnA*; citrate synthase, *gltA*; serine hydroxymethyl transferase, *glyA*; phosphoglucomutase, *pgm*; transketolase, *tkt*; and ATP synthase subunit, *uncA*) were amplified by PCR, and the nucleotide sequence of the amplicons was determined with the published oligonucleotide primers and reaction conditions. Nucleotide sequence extension reaction products were separated and detected on an ABI Prism 3700 or an ABI Prism 377 automated DNA analyzer. Nucleotide sequences were determined at least once on each DNA strand and were assembled with the STADEN software package (29). Allele numbers and sequence types (ST) were assigned by using the *Campylobacter* MLST database (<http://campylobacter.mlst.net/>).

**Data from human disease isolates.** Data from 91 human disease isolates of *C. jejuni*, isolated from cases of gastroenteritis from stool cultures in northwestern

England during 1999, were obtained from the PubMLST isolate database (<http://campylobacter.mlst.net>) by using the "Search database—advanced queries" feature with the following criteria: id, <1316; country, United Kingdom; year, 1999; source, human stool. These data included sequence types for 40 previously published (10) and 51 unpublished isolates.

**Data analysis.** The STs were assigned to clonal complexes as described previously (10). Central genotypes were defined by using UPGMA cluster analysis and the BURST algorithm (13), both implemented with the computer program START (18), and split decomposition analysis was implemented with the program SPLITSTREE (17). Isolates were defined as belonging to a clonal complex if they shared at least four alleles with the central ST. The ratio of nonsynonymous to synonymous substitutions ( $d_N/d_S$ ), and numbers of fixed differences and shared polymorphisms were calculated by using the DNASP software package version 3.53 (25). The pairwise  $F_{ST}$  and test of significance calculations were performed by using the Arlequin software package, version 2.000 (27). For some of the analyses the gene sequences for each of the MLST loci were concatenated to give a single continuous nucleotide sequence of 3,309 bp for each isolate.

#### RESULTS

**Diversity of MLST allele sequences.** It was possible to determine the nucleotide sequences at each of the MLST loci from all of the farm and environmental isolates with previously published methods and reagents. The number of unique sequences at each locus varied from 5 for the *aspA* locus to 12 for the *gltA* locus, with the percentage of variable sites in this data set ranging from 2.7% for the *aspA* locus to 14.5% at the *uncA* locus. The apparent high diversity of the *uncA* locus was caused by a single allele, allele 17, which occurred 18 times in the data set. Exclusion of this allele gave diversity for this locus of 0.8%. The ratio of nonsynonymous to synonymous substitutions ( $d_N/d_S$ ) observed ranged from 0.000 to 0.143 (Table 1). All except two of the MLST allele sequences found in this isolate collection had been described previously.

**Diversity of sequence types.** The 112 isolates contained a total of 30 different STs (Table 2). The most common ST was ST-45, which was represented by 26 isolates, with several STs occurring only once in the data set (Table 2). The four most predominant STs (ST-45, ST-42, ST-61, and ST-262) represented approximately half of the isolates (61 out of 112 isolates, or 54%). Virtually all of the isolates (110 out of 112 isolates, or 98%) were assigned to 1 of 10 previously described clonal complexes, with the remaining two isolates unassigned as they did not share four alleles in common with any of the previously recognized central genotypes. The clonal complexes were represented by between 2 isolates (ST-52 complex) and 30 isolates (ST-45 complex). The number of STs within each

TABLE 2. Distribution of STs among 112 *C. jejuni* isolates from farm animals and their environment and their resolution into clonal complexes

Clonal complex	ST no.	No. of isolates <sup>a</sup>
21	21	1
	19	1
	53	7
	262	13
	376	1
	518	1
	519	1
	520	1
22	22	3
	512	1
42	42	10
	517	1
45	45	26
	100	1
	137	1
	514	1
	515	1
48	48	4
	38	3
	205	1
	216	1
52	52	2
61	61	12
	81	2
177	521	3
206	206	6
257	257	4
	513	1
Unassigned	688	1
	690	1

<sup>a</sup> The total numbers of isolates for each clonal complex are as follows: complex 21, 26; complex 22, 4; complex 42, 11; complex 45, 30; complex 48, 9; complex 52, 2; complex 61, 14; complex 177, 3; complex 206, 6; complex 257, 5; and the unassigned complex, 2.

clonal complex ranged from one (ST-52, ST-177, and ST-206 complexes) to eight (ST-21 complex). With the exception of the ST-21 complex, the previously assigned central genotype was the most predominant ST present in each of the clonal complexes identified in this sample. A total of 12 STs were novel to this study.

**Distribution of genotypes among isolation sources.** The most common clonal complexes among the farm isolates were ST-45 complex (30 isolates), ST-21 complex (26 isolates), ST-61 complex (14 isolates), and ST-42 complex (11 isolates) (Table 2). The remaining clonal complexes were represented by nine or fewer isolates, with the ST-52 complex, represented by two isolates, being the smallest. The number of clonal complexes identified in each isolation source varied from two for turkey chicks, broiler chicks, and slurry to six for adult beef cattle at slaughter and sheep at slaughter (Table 3). The ST-21 complex was the most widely distributed clonal complex, present in 8 of the 10 different isolation sources. The ST-52 and ST-177 complexes were the least widely distributed and were each identified in one isolation source. The ST-45 complex was predominant among turkey and broiler chick isolation sources and was absent from ovine isolation sources. Conversely, ST-42 and ST-61 complexes were predominant among ovine and bovine sources but were absent from avian sources. Two clonal complexes were identified in both turkey and broiler chicks, although only the ST-45 complex was common to both isolation sources in this study. Adult cattle and calves shared the ST-21, ST-45, and ST-48 complexes, but each had further complexes. Sheep at slaughter, sheep grazing on salt marsh, and sheep grazing on fell (hill) land shared three clonal complexes, ST-21 complex, ST-45 complex, and ST-61 complex. Adult sheep and lambs shared the ST-61 and ST-48 complexes, but only the ST-61 complex was found in all ovine groups. Isolates belonging to the ST-21 complex, ST-45 complex, and ST-257 complex were present in starlings, which were the only source from which the ST-177 complex was recovered. Both of the unassigned STs (ST-690 and ST-688) were from bovine isolation sources.

**Comparison of genotypes with human disease isolates.** The gene flow analysis (pairwise  $F_{ST}$ ) between the 112 isolates in this study and the 91 isolates from human disease gave values (0.015 to 0.094) that ranged from significantly different to zero

TABLE 3. Frequency and distribution of clonal complexes among farm and environmental sources of *C. jejuni*

Source (n)	Clonal complex													
	21	22	42	45	48	52	61	177	206	257	353	443	354	U <sup>a</sup>
Turkey chicks (12)	— <sup>b</sup>	—	—	11	—	—	—	—	—	1	—	—	—	—
Broiler chicks (16)	1	—	—	15	—	—	—	—	—	—	—	—	—	—
Calves (9)	3	—	—	2	2	—	—	—	—	1	—	—	—	1
Adult beef cattle at slaughter (14)	2	—	1	1	4	—	1	—	4	—	—	—	—	1
Slurry (10)	7	3	—	—	—	—	—	—	—	—	—	—	—	—
Lambs at slaughter (9)	—	1	—	—	2	—	6	—	—	—	—	—	—	—
Sheep at slaughter (14)	5	—	3	—	1	2	1	—	2	—	—	—	—	—
Sheep grazing in salt marsh (10)	5	—	3	—	—	—	2	—	—	—	—	—	—	—
Sheep grazing in fell (9)	1	—	4	—	—	—	4	—	—	—	—	—	—	—
Starlings (9)	2	—	—	1	—	—	—	3	—	3	—	—	—	—
Humans (91)	13	—	—	13	3	6	6	—	—	22	4	4	4	16

<sup>a</sup> U, unassigned isolates.

<sup>b</sup> —, zero.

for all of the alleles, except for *uncA* (Table 1) and for the concatenated sequences. A total of 183 polymorphisms were shared between the two data sets, and there were no fixed nucleotide differences between them.

## DISCUSSION

The contributions of the various possible sources of infection to the total disease burden of human campylobacteriosis have not been definitively established. In addition to the problems inherent in analyzing a sporadic zoonotic infection, this has been to a large extent due to the lack of a reliable and portable scheme for isolate characterization that enables comparisons among different studies. The *Campylobacter* MLST scheme was devised to address this problem and has been shown to be effective for the analysis of isolates from human disease and retail food (11). The analyses described here demonstrate that this scheme can be applied to isolates from the farm environment without modification, allowing direct and unambiguous comparison among *C. jejuni* isolates from a variety of sources (10, 11).

The nucleotide diversity present in the farm-derived isolates was similar to that described for human disease and retail food isolates (10) in terms of the number of alleles, nucleotide variability, and  $d_N/d_S$  ratio at each locus (Table 1). The largest degree of variability was seen at the *uncA* locus with the inclusion of allele 17, which may have originated in a different *Campylobacter* species, such as *Campylobacter coli* (11). All but two of the alleles, 16 of the 30 STs, and all of the clonal complexes identified in the farm and environment isolates had been previously described among the human disease and retail food isolates (10, 11), with the majority of alleles and STs identified in this study being commonly found among isolates on the *Campylobacter* PubMLST database.

The data were consistent with the idea that particular genotypes, indicated by clonal complex, are associated with given host sources, as suggested previously by using MLST, serotyping, and pulsed-field gel electrophoresis (10, 14, 28, 40). Isolates belonging to the ST-45 clonal complex were dominant among turkey and broiler chick samples and were absent from sheep and lamb samples. In contrast, ST-61 and ST-42 complexes were dominant among sheep isolates but were absent from poultry isolates: similar trends have been reported for retail food sources (10). The ST-21 complex appeared to have a wide distribution; this clonal complex probably corresponds to the large stable cluster of isolates capable of colonizing a wide range of hosts identified with other techniques (14, 28).

Several other observations concerning the distribution of clonal complexes among isolation source, while based on smaller numbers of isolates, warrant further investigation. Wild birds have been implicated in spreading infection in the farm environment on numerous occasions, although the extent of their contribution is as yet unknown (6, 9, 19, 20, 23, 37). The results from this investigation suggest that a range of *C. jejuni* genotypes may be prevalent in wild birds. Starlings were found to carry *C. jejuni* belonging to STs from complexes associated with poultry and environment sources as well as the ST-21 complex; however, none of the complexes potentially associated with cattle or sheep was present. This could be a reflection of host adaptation, behavioral patterns of the birds,

or small sample size. A larger number of clonal complexes were represented in animals at slaughter than in the other animal groups, perhaps reflecting different sampling sites or contamination within the slaughterhouse. Differences in clonal complex distribution between both adult cattle and calves and adult sheep and lambs could reflect the different conditions and farming practices in which the animal age groups are kept or could reflect host immunological maturity (20, 21, 32, 33).

Of the 30 STs, 8 accounted for 73% (82 out of 112) of the isolates, implying that these genotypes may be particularly stable. Possible explanations of such stability remain to be determined but could reflect stabilizing selection imposed by, for example, niche adaptation. Members of the ST-21 complex in particular might be well adapted for long-term survival given their apparently ubiquitous distribution. This idea was further supported by the observation that they were one of only two genotypes isolated from slurry to which a potentially large number of different genotypes were added on a regular basis (34). With the exception of ST-21 complex, the most common genotype in each of the major clonal complexes was the central ST, which is usually the most common ST among the clonal complexes in the *Campylobacter* PubMLST database. In contrast to isolate data available in the database, ST-262 was the most common ST-21 complex genotype in this study, found among slurry, sheep, and cattle isolates, while ST-53 isolates, the second most common type in the complex, were present among calves, sheep, chicks, and starlings. There were 12 (10% of the total number of isolates) STs unique to this investigation, which all differed from the central genotype of the clonal complex at one or two loci. These could be specific host-adapted STs but are more likely to represent recent variants from the persistent central genotypes.

A number of studies have indicated that isolates from human disease and farm animals are very similar (12, 14–16, 19). In the largest MLST study of human disease published to date, six clonal complexes accounted for more than 60% of human disease isolates, namely, the ST-21, ST-45, ST-206, ST-61, ST-48, and ST-257 complexes (10). The same clonal complexes were also predominant among retail meat isolates in the *Campylobacter* PubMLST database, although the frequency of each varies according to isolation source. These clonal complexes were all present among the farm and environmental isolates. Further, while the two isolate collections shared many polymorphisms, there were no fixed differences between them. These observations are consistent with the farm populations of *C. jejuni* being a source for food contamination and human infection. The slightly different clonal complex composition of the human disease and farm isolate collections was reflected by the nonzero values of pairwise  $F_{ST}$  values for most loci and for the concatenated sequences. This is consistent with other populations of *C. jejuni* contributing to human disease. The high degree of discrimination possible with such analyses presents the prospect of employing MLST data to improve estimates of the contribution of different *Campylobacter* populations to the human disease burden.

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