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Multilocus Sequence Typing of *Campylobacter jejuni* Isolates from Humans, Chickens, Raw Milk, and Environmental Water in Quebec, Canada▽

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Molecular strain typing is essential for deciphering the epidemiology of *Campylobacter jejuni* infections. We applied two different methods, multilocus sequence typing (MLST) and analysis of the *flaA* short variable repeat (SVR), to 289 isolates (163 human, 56 chicken, 34 raw milk, and 36 environmental water isolates) collected in the province of Québec, Canada, over 3 years; in addition, the analysis included the pulsed-field gel electrophoresis (PFGE) typing results for a subset of 131 isolates studied previously. MLST defined 96 sequence types (STs) and 20 clonal complexes (CCs), including 49 STs (73 isolates, 25%) and 39 alleles not previously documented in an international database. The frequency of new STs was significantly higher among water isolates than among isolates from other sources (18/36 [50%] and 55/253 [22%], respectively; $P < 0.001$). Nine of the 10 most prevalent CCs included isolates from humans and at least one other source; five CCs comprised exclusively or mostly human and chicken isolates. However, water and milk were the predominant nonhuman sources among the remaining CCs, suggesting that sporadic *C. jejuni* infections in humans may frequently arise from sources other than chickens. All three typing systems were discriminatory (discriminatory index > 0.9). Among 131 isolates analyzed by PFGE, each of the 20 types represented by two or more isolates corresponded to a single CC. In contrast, among the 14 most prevalent types detected by analysis of the *flaA* SVR (5 to 27 isolates each), 8 (57%) included isolates that represented multiple different CCs. The basis for these discordant results was uncertain. Antimicrobial resistance was randomly distributed among the CCs and appeared to be more closely related to the source of an isolate than its genotype. Although MLST is labor-intensive and expensive, it remains the single best method for the genotyping of *C. jejuni* isolates and deciphering the epidemiologic relationships among isolates.

Campylobacter jejuni is the leading reported cause of bacterial gastroenteritis in developed countries (2). It is also the leading notifiable cause of enteric food- and waterborne diseases in Canada, with 11,543 cases reported in 2002 (http://dsol-smed.phac-aspc.gc.ca). In the province of Quebec, nearly 3,000 cases of diarrheal illness are annually attributed to *Campylobacter* enteritis, more than the combined total number of cases caused by *Salmonella* and *Shigella* species, *Escherichia coli* O157:H7, and *Yersinia enterocolitica* (6).

The organisms colonize a range of hosts, including domestic animals and wild birds, and fecal shedding readily contaminates groundwater (2). While outbreaks are well documented, most clinical cases are isolated, sporadic infections for which the source is rarely apparent. The consumption of contaminated food, especially poultry, has been considered the most prevalent cause (1); however, recent studies have implicated environmental water and unpasteurized milk as being potentially important (28).

Given the limitations of conventional clinical epidemiological approaches (22, 28, 30), recent investigations and surveillance studies have emphasized molecular strain-typing methods. Pulsed-field gel electrophoresis (PFGE), a highly discriminatory technique that has been effectively applied to other enteric pathogens (42), can successfully confirm *C. jejuni* outbreaks suspected by epidemiological surveillance (15) but has proven only modestly successful in linking small clusters and sporadic cases to particular sources (17, 29).

Multilocus sequence typing (MLST) has emerged as the state-of-the-art method for the resolution of bacterial population genetics (9, 24). An MLST system for *C. jejuni* has recently been developed (10) and has been used to indicate that the species is genetically diverse and has a weakly clonal population structure, marked by frequent intra- and interspecies horizontal genetic exchange (10, 11, 25). Some MLST-defined lineages of *C. jejuni* have been linked to restricted geographical areas (12) or to particular ecological niches, such as bathing beaches (10); wild birds (5); and chickens, pigs, bovines, or sheep (25). Although the results of MLST are highly reproducible, portable, and easy to interpret, MLST is complex and expensive to perform.

A more practical, but still robust, alternative to MLST for strain typing is needed. To date, flagellin is the only virulence gene of *C. jejuni* that has proven to be sufficiently diverse to be informative. A simple and effective typing system was developed on the basis of primers directed at highly conserved regions of the *fla* locus, which provided a PCR product that could be analyzed for restriction fragment length polymorphisms (11, 34). However, previous studies revealed that the

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flagellin genes can undergo recombination, which limits the stability of the locus for strain typing (16).

flaA, the gene encoding the primary structural flagellin protein of *C. jejuni*, has a short variable repeat (SVR) region of 150 bp (27). Analysis of the SVR sequences provided reproducible and discriminatory strain-typing results comparable to those provided by conventional *fla* typing but required appreciably less effort.

We applied both MLST and *flaA* SVR typing to a collection of 289 *C. jejuni* isolates cultured from samples from humans, chickens, raw milk, and environmental water collected in Québec over a 3-year period; this report describes the congruence among the genotypic relationships defined by these two DNA-based strain-typing methods and considers their relative utility for epidemiologic and surveillance studies of *C. jejuni*. In addition, the analysis includes the PFGE typing results for a subset of 131 isolates studied previously (29, 30), as well as the profiles of resistance to ciprofloxacin, erythromycin, and tetracycline.

MATERIALS AND METHODS

Isolates. Only *C. jejuni* isolates were included in this study. Isolates were obtained as described previously (21) from human stools ($n = 163$), fresh whole retail chicken ($n = 56$), raw milk ($n = 34$), and environmental water ($n = 36$). The human isolates originated from fecal samples from patients with diarrhea submitted to the Eastern Townships hospital microbiology laboratories between 1998 and 2003 (8 samples in 1998–1999, 153 in 2000–2001, 32 in 2002, and 69 in 2003). For subjects with recurrent infections, only the first *C. jejuni* isolate was included in the study. Human isolates were considered to be acquired internationally if the subject had traveled abroad during the entire 10-day period before the onset of symptoms. Isolates for which this information was not available were excluded from the study. Overall, 139 isolates were acquired locally and 24 were acquired internationally, including 9 from Europe (France, $n = 5$; Spain, $n = 2$; and United Kingdom and Eastern Europe, $n = 1$ each), 10 from Central and South America (Peru, $n = 3$; Haiti, $n = 2$; and Argentina, Bolivia, Dominican Republic, Mexico, and Mexico or Argentina, $n = 1$ each), plus 1 isolate each from Chad, Tunisia, and Indonesia. For two isolates, the country of origin was uncertain.

The water isolates were obtained from river and tributary water samples collected in the Eastern Townships between 13 May and 12 August 2003 (23). In brief, each sample represented about 500 ml of water collected in a sterile Nalgene bottle that was transported on ice, held at 4°C, and tested within 24 h. The water was filtered through a sterile 0.45-μm-pore-size membrane filter; some samples required prefiltration with a 1.5-μm-pore-size membrane. The filters were transferred into a Whirl-Pak bag containing 100 ml of Park-Sanders enrichment broth with 0.5 ml of supplement A (0.2% vancomycin and 0.2% trimethoprim lactate) and 5 ml of supplement B (0.064% sodium cefoperazone in brucella broth) and incubated for 4 h at 37°C in a microaerobic atmosphere and then at 42°C for an additional 44 h. One milliliter of the suspension was transferred into a second Park-Sanders broth (10 ml) and incubated at 42°C for 24 h in a microaerobic atmosphere. Then, 100 μl of the final enrichment broth was plated on Karmali agar and incubated in a microaerobic atmosphere at 42°C for 48 h. Plates that were negative for *Campylobacter* at that time were reincubated for an additional 24 h. Isolates were identified to the species level by routine phenotyping methods (32) and by two PCR methods (7, 14). Isolates which had a hippurate-negative phenotype but in which a hippurate gene was detected by PCR were identified as *C. jejuni* (38).

The chicken isolates were cultured from fresh whole retail chickens purchased in grocery stores in the Eastern Townships (37 isolates in 2000–2001 and 19 in 2003) (28, 29). The raw milk isolates were cultured in Québec Province in 2000–2001 and were provided by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec.

Culture of isolates and preparation of genomic DNA. All *C. jejuni* isolates were grown on 5% (vol/vol) defibrinated sheep blood tryptic soy agar (Oxoid Inc., Nepean, Ontario, Canada) in a microaerobic atmosphere at 42°C for 24 to 48 h. Genomic DNA was extracted by transferring a single colony into 25 μl of 0.5 N NaOH. After 5 min, 25 μl of 1 M Tris (pH 8.0) and 450 μl of sterile distilled water were added. The DNA extracts were stored at −20°C.

MLST. MLST was carried out as previously described by Dingle et al. (10) with modified amplification conditions (denaturation at 94°C for 30 s; annealing at 50°C [*uncA*], 55°C [*aspA*, *gltA*, *glyA*, *pgm*, and *tkt*], or 60°C [*glnA*] for 30 s; and extension at 72°C for 1 min). For some isolates, the primers of the extended MLST system were used (31). The PCR products were visualized by 1% (wt/vol) agarose gel electrophoresis. Sequencing reactions were done in collaboration with Genome Quebec (http://www.genomequebec.mcgill.ca). The sequences were compared and analyzed with the BioNumerics (version 3.5) program (Applied Maths). Allele numbers, sequence types (STs), and clonal complexes (CCs) were assigned by submitting the DNA sequence to the *C. jejuni* MLST database website (http://pubmlst.org/campylobacter).

***flaA* SVR sequencing.** A fragment of 641 bp of the *flaA* gene containing an SVR was amplified by using the consensus forward primer for *flaA* (5′-ATG GGA TTT CGT ATT AAC AC) (43) and primer FLA625 RU (5′-CAA GTC CTG TTC CAA CTG AAG) (27). These primers were also used for sequencing. A fragment of 348 bp (corresponding to nucleotides 266 to 613 of the *flaA* gene of *C. jejuni* ATCC 33560) was used as a reference for comparison of the different alleles. Allele numbers were determined from the existing *flaA* SVR database (http://hercules.medawar.ox.ac.uk/flaA/).

PFGE fingerprint analysis. The PFGE typing results for KpnI digests of a subset of 95 human and 36 chicken isolates were determined in previous studies (29, 30).

Antimicrobial susceptibility testing. Agar dilution antimicrobial susceptibility tests were performed with ciprofloxacin, erythromycin, and tetracycline by agar dilution according to the guidelines of the Clinical and Laboratory Standards Institute (formerly the NCCLS) (33), as described previously (21). Antimicrobial susceptibility results were not available for two locally acquired and six internationally acquired human isolates because the stored isolates were not viable.

Phylogenetic analyses. Linkage analysis was performed by calculating the index of association (I_A) by using the algorithm of Smith et al. (40), which is included in the START2 package, version 0.5.13 (19). If there is a complete linkage equilibrium, i.e., a random association between alleles of different loci, which indicates a freely recombining population, then I_A is equal to 0. If there is linkage disequilibrium, which indicates a clonal population structure in which recombination has been rare or absent, then I_A is significantly different from 0 (40). The START2 package program was used to calculate the ratio of nonsynonymous to synonymous substitutions (d_N/d_S) and to develop a tree by the unweighted pair group method with arithmetic means (UPGMA). We determined the founding genotype for each clonal complex using the eBURST program (13) by querying the *C. jejuni* MLST database website and cross-checking with the tree developed by UPGMA.

Statistical analysis. Proportions were compared with the program Statistix for Windows (version 7.1; Analytical Software, Tallahassee, FL) by using the chi-square and Fisher's exact two-tailed tests and a significance level of 5%. The discrimination index (DI) was determined by the method of Hunter and Gaston (18).

RESULTS

Population structure. A total of 96 STs were identified among the 289 isolates analyzed (Table 1). Seventy-nine STs, representing 264 (91.3%) isolates, were assigned to 20 previously described CCs. The remaining 25 isolates were distributed among 17 STs which could not be assigned to any of the known lineages; these included 14 (39%) of the water isolates. The 10 most prevalent CCs (representing all CCs with 5 or more isolates) comprised 239 (83%) of all isolates. Within each CC, a single ST accounted for 30% to 100% of the isolates representing that CC; collectively, these dominant ("modal") STs accounted for 141 (49%) isolates (Table 1). The distribution of isolates in the tree developed by UPGMA was consistent with the isolate distribution resolved by eBURST analysis (data not shown).

Allelic diversity. A total of 194 alleles were identified across all seven loci, ranging from 24 alleles at *aspA* to 38 at *pgm* (Table 2). Overall, 39 (20%) of the alleles were previously unreported (last database query, December 2007); the frequency of new alleles at a locus ranged from 12% (*aspA* and

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TABLE 1. Distribution of 289 *C. jejuni* isolates among CCs and STs

CC	No. of isolates	No. of STs	Modal ST[a]	No. (%) isolates of CC in modal ST
21	76	13	21	44 (58)
42	17	3	42	11 (65)
45	53	9	45	35 (66)
48	5	4	48	2 (40)
49	7	4	467	3 (43)
61	15	3	61	13 (87)
353	32	17	353	10 (31)
508	10	1	132	10 (100)
607	13	4	1212	6 (46)
1275	11	4	637	7 (64)
Miscellaneous	25	17	NA[b]	NA
Unclassified	25	17	NA	NA
Total	289	96	10 (10)[c]	141 (49)

[a] Modal ST, ST within the CC represented by the largest number of isolates.
[b] NA, not available.
[c] The value in parentheses is in percent.

glnA) to 28% (*tkt* and *uncA*). The proportion of variable sites per allele ranged from 6.5% for *glnA* to 18.6% for *uncA*. The I_A for the complete data set was 3.519; when only one example of each ST was included, the I_A was 1.6779. Both values are significantly different from 0 and consistent with a predominantly clonal population with a moderate degree of horizontal recombination (40). The d_N/d_S ratios varied across the seven loci, ranging from 0.0125 for *uncA* to 0.0812 for *glyA*.

Previously unreported STs. Overall, 49 (51%) STs representing 73 (25%) isolates were previously unreported (Table 3; these have been submitted to the international database). Twenty-eight (57%) of the new STs resulted from new allele sequences, and the remainder resulted from new combinations of previously described alleles (Table 3). Of note, ST-1227, represented by one water isolate, had new allele sequences detected in all seven genes. Most (38/49; 78%) of the new STs were represented by a single isolate. Among the four sources studied, water had the highest proportion of isolates representing new STs (18/36; 50%), whereas 22% (36/163) of the isolates from humans, 26% (15/56) of the isolates from chickens, and 12% (4/34) of the isolates from raw milk represented new STs ($P < 0.001$, chi-square test). Thirty-five (71%) of the new STs could be assigned to known CCs (Table 2).

Association between CCs and source of isolation. Isolates from all four sources were represented only in the two largest CCs (CCs 21 and 45) (Fig. 1). Nine of the 10 most prevalent CCs included isolates from humans and at least one other source. Five CCs exclusively comprised mostly human and chicken isolates; among the remaining CCs, water and milk were the predominant nonhuman sources. CC 1275 comprised exclusively water isolates and accounted for 31% (11/36) of all isolates from that source. Among the 24 isolates acquired outside of Canada, 21 were distributed among seven CCs; the remaining isolates were not associated with any existing CC. Eight international isolates were found in CC 353, which represented 25% of the isolates in this CC; an additional six isolates were found in CC 21.

Distribution of antibiotic resistance by isolate source. The most common antimicrobial susceptibility pattern detected was susceptibility to all three antibiotics tested (Fig. 2). This was observed for 150 (53%) of the 281 isolates evaluated but was significantly less prevalent among human and chicken isolates (43% and 39%, respectively) than raw milk and water isolates (88% and 89%, respectively) ($P < 0.0001$, chi-square test). Tetracycline was the antibiotic to which resistance was most commonly observed (41%); only 21 (7.5%) isolates were resistant to ciprofloxacin, and only 16 (5.7%) were resistant to erythromycin. Erythromycin resistance was the most prevalent among isolates from chickens (9/56; 16%), whereas ciprofloxacin resistance was the most prevalent among those from humans (19/155, 12%). Of note, resistance to ciprofloxacin was significantly more frequent among the human isolates acquired abroad than among those acquired locally (9/18 and 10/127, respectively; $P < 0.0001$, Fisher's exact test). Tetracycline resistance was also observed in 44% of the human isolates acquired abroad. There was no apparent association between antibiotic resistance and the CC or ST (data not shown).

Association between *flaA* SVR type and CC. A total of 91 *flaA* SVR types were identified among 289 isolates. There were 47 (52%) *flaA* SVR types that corresponded to unique isolates. The 14 (15%) most prevalent *flaA* SVR types included at least 5 isolates each, for a total of 156 (54%) isolates (Table 4). These included six *flaA* SVR types in which each *flaA* SVR type was associated with only a single CC. Within each of the remaining eight most prevalent *flaA* SVR types, there was a single most common (modal) CC which represented all but one to three isolates (Table 4). Similarly, 23 of the 30 *flaA* types represented by two to four isolates each were associated with a single CC. Thus, among all 242 isolates with *flaA* types represented by more than a single isolate, the *flaA* SVR type was predictive of the CC for 215 (89%). There was no apparent association between the *flaA* SVR type and either antibiotic resistance or source (data not shown).

TABLE 2. Allelic diversity among 289 *C. jejuni* isolates

Locus	Fragment size (bp)	No. of alleles	No. (%) of new alleles[a]	No. of variable sites[b]	% of variable sites[b]	d_N/d_S[b]
aspA	477	24	3 (12)	72	15.1	0.0431
glnA	477	26	3 (12)	31	6.5	0.0370
gltA	402	26	6 (23)	36	9.0	0.0555
glyA	507	23	5 (22)	58	11.4	0.0812
tkt	459	32	9 (28)	56	12.2	0.0597
pgm	498	38	6 (16)	73	14.7	0.0449
uncA	489	25	7 (28)	91 (41)	18.6 (8.4)	0.0125 (0.0176)

[a] Number of new alleles identified in this study and submitted to the international MLST database.
[b] The data in parentheses are the percentages determined by exclusion of the data for *uncA* allele 17, which may have come from a different *Campylobacter* species.

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TABLE 3. New STs observed in this study

CC	New ST[a]	No. of isolates	Source[b]	Allele[c]						
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
21	1208	1	H	2	1	1	3	2	1	**103**
21	1209	2	H	2	1	**137**	3	2	1	5
21	1214	1	C	2	1	1	53	2	1	5
21	1459	1	H	2	1	1	2	2	1	5
21	1461	1	H	2	7	12	3	2	1	5
22	1686	1	H	118	3	6	4	3	3	17
45	1215	4	C (2), H (2)	4	7	10	4	121	7	1
45	1217	1	C	4	7	**139**	4	1	7	1
45	1222	4	RM	4	7	**140**	4	1	7	1
45	1228	1	W	4	7	10	4	1	7	**102**
45	1685	1	H	4	7	10	4	121	7	17
48	1460	1	H	6	4	5	2	11	1	5
49	1220	1	C	3	1	5	2	11	11	6
52	1235	1	H	9	17	2	10	**227**	3	6
52	2393	1	H	**181**	17	2	10	11	3	6
61	1244	1	H	1	1	2	2	**225**	3	17
61	1684	1	H	2	4	2	2	6	**181**	5
179	1207	2	H	12	6	**137**	**176**	40	32	3
353	1210	3	H	7	17	5	2	**224**	3	6
353	1211	1	H	7	17	5	2	10	3	3
353	1218	1	C	7	17	12	2	11	3	6
353	1232	1	H	7	17	5	10	11	3	6
353	1233	1	H	7	17	5	10	10	**177**	6
353	1462	1	H	7	4	5	2	11	3	**106**
353	1480	2	H	7	17	5	10	2	**177**	6
353	2394	1	H	**182**	17	5	2	10	3	6
353	2395	1	H	7	4	5	2	11	3	6
443	2396	1	H	24	2	2	15	10	3	12
460	1213	1	C	7	30	2	2	89	59	6
607	1212	6	C (4), H (2)	8	2	5	53	11	3	**105**
607	1216	1	C	9	2	5	53	11	3	1
692	991	1	W	37	52	57	26	107	29	23
1275	1223	2	W	27	33	22	49	43	9	31
1275	1225	1	W	27	33	22	49	43	7	31
1275	1231	1	W	27	2	22	104	43	86	31
UA	1206	1	H	2	59	**136**	105	126	25	23
UA	1219	3	C (2), H (1)	7	17	5	10	11	61	1
UA	1221	1	C	2	84	5	10	119	**178**	26
UA	1224	2	W	18	22	22	97	115	**175**	47
UA	1226	5	W	1	6	61	**176**	40	**180**	1
UA	1227	1	W	**122**	**163**	**135**	**175**	**219**	**176**	**101**
UA	1229	1	W	97	121	97	125	174	152	**100**
UA	1230	1	W	1	6	61	4	40	**180**	1
UA	1234	1	H	28	34	27	33	**223**	**182**	33
UA	1236	1	W	94	117	97	125	174	147	**100**
UA	1237	1	W	18	21	22	104	134	101	60
UA	1456	1	H	2	59	4	**187**	131	24	57
UA	1457	1	H	2	**165**	73	**147**	**220**	**190**	**104**
UA	1458	1	H	63	**164**	**141**	**188**	27	**179**	18

[a] All isolates were from Canada, except as follows: STs 1233, 1480, and 2396 were from Peru; ST 2395 was from Bolivia; ST 2394 was from Bolivia or Argentina (indeterminate); STs 1234 and 1458 were from Haiti; and ST 2393 was from Indonesia.

[b] UA, unassigned; C, chicken; H, human; RM, raw milk; W, water. Numbers in parentheses are the number of isolates from each source when more than one source was represented.

[c] New alleles identified in this study are in boldface.

Associations between PFGE types and CCs. PFGE analysis of KpnI restriction digests was performed for 131 isolates. A total of 87 PFGE types were identified; 67 (77%) of these were represented by a single isolate. The remaining 20 types comprised two to six isolates each; each of these PFGE types was associated with a single CC (Table 5). Thus, among the 64 isolates with PFGE types represented by more than a single isolate, the PFGE profile was 100% predictive of the CC.

Relationships among PFGE type, *flaA* SVR type, and ST. The relationships among the three typing systems are depicted graphically in Fig. 3. For three CCs, all PFGE types represented by two or more isolates are shown, as well as the ST and *flaA* types associated with all of those isolates. There were no simple linear relationships among the typing systems. Individual STs could be associated with multiple PFGE types (e.g., ST-21 and ST-262 in CC 21); conversely, a single PFGE type could

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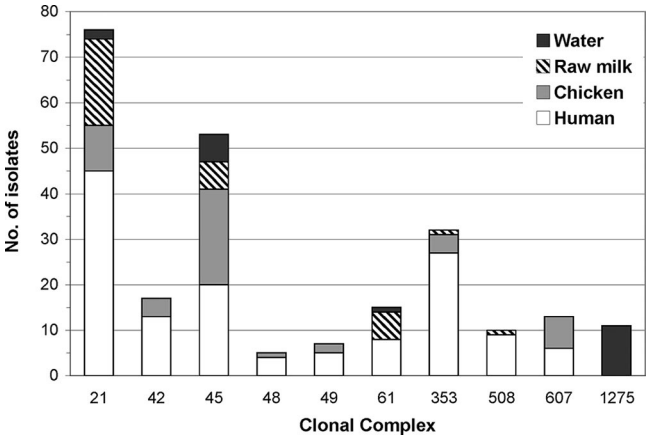

FIG. 1. Distribution of sources for *C. jejuni* isolates representing the 10 most prevalent CCs detected.

comprise isolates with different STs (e.g., PFGE types 1, 2, and 19 in CC 45). Similarly complex relationships were observed for the *flaA* SVR types. The discriminatory power of the typing methods was similar across the 289 isolates studied; the DIs were 0.97 for typing of the *flaA* SVR, 0.95 for MLST (ST), and 0.88 for MLST (CC). Among the 131 isolates for which PFGE typing results were available, the DIs were 0.99 for PFGE, 0.96 for typing of the *flaA* SVR, 0.95 for MLST (ST), and 0.85 for MLST (CC).

DISCUSSION

Each of the molecular methods considered here—MLST, typing of the *flaA* SVR, and PFGE—has previously been used to type *C. jejuni* isolates either in outbreak investigation settings (3, 37), from strain collections from various sources and geographical areas (36), or in human population-based studies (11, 12). The distinctive features of this study were the application of all three molecular techniques to a collection of isolates from diverse sources, including humans, whole retail chickens, raw milk, and environmental water, from the same

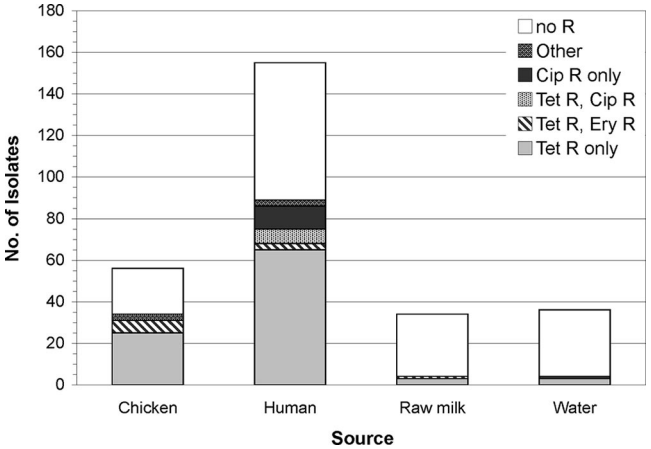

FIG. 2. Distribution of antibiotic susceptibility patterns for *C. jejuni* isolates by source. R, resistant; Tet, tetracycline; Cip, ciprofloxacin; Ery, erythromycin.

TABLE 4. Relationship between *flaA* SVR types and CCs

flaA SVR type	No. of isolates	Modal CC	No. (%) of isolates in modal CC	Nonconforming CC(s) (no. of isolates)
36	27	21	25 (93)	48 (1), 607 (1)
49	15	21	13 (87)	42 (2)
361	5	21	4 (80)	48 (1)
792	5	21	5 (100)	
359	10	42	10 (100)	
21	18	45	16 (89)	443 (2)
2	16	45	15 (94)	1275 (1)
8	11	45	11 (100)	
42	14	61	13 (93)	45 (1)
34	9	353	6 (67)	48 (1), 460 (2)
45	6	353	6 (100)	
14	5	353	3 (60)	607 (2)
172	10	508	10 (100)	
621	5	1275	5 (100)	
Miscellaneous[a]	86	NA[b]	73 (85)	NA
Total	242[c]		215 (89)	

[a] *flaA* types represented by 4, 3, or 2 isolates (8, 10, and 12 types, respectively). For *flaA* types with two isolates of different CCs, both isolates were considered nonconforming.
[b] NA, not available.
[c] There were 47 isolates with unique *flaA* types.

geographical region and testing of antimicrobial resistance as a clinically important phenotype.

The Eastern Townships comprise 102 municipalities (80% rural) in an area of about 10,000 km^2 with approximately 300,000 inhabitants. All of the chicken and water isolates analyzed in this study originated from the Eastern Townships, as did 83% of the human isolates; the raw milk isolates were cultured from samples collected elsewhere in Québec Province. Among the 289 isolates, MLST analysis at seven loci identified 194 alleles, which in turn defined 96 STs; almost 10% of the isolates could not be assigned to known lineages. Thus, even though the great majority of our isolates originated from a relatively small area, the genetic heterogeneity was comparable to that observed in the *Campylobacter* MLST database. These findings suggest that the high degree of diversity among the genotypes of the *Campylobacter* population observed on an international level is also reflected among the genotypes from a relatively small, regional microcosm.

Consistent with this, we observed 39 (20%) new alleles and 49 (51%) new STs. This genotypic diversity was distributed among isolates from all four sources examined. Isolates from humans were found in all but one of the CCs represented by more than one isolate, suggesting that potentially pathogenic strains are not restricted to specific lineages. Five of the most prevalent CCs (CCs 42, 48, 49, 353, and 607), which represented about 25% of all isolates, comprised exclusively or predominantly human and chicken isolates. However, water and raw milk were the predominant nonhuman sources of the remaining CCs, suggesting that sporadic *C. jejuni* infections in humans may frequently arise from sources other than chickens. Population-based studies, such as the study of Sopwith et al. (41), are needed to better define the role of the additional sources and the influences of seasonal and regional factors (28). We have recently initiated a 3-year study across the Eastern Townships to correlate the genotypes of the *Campylobacter*

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TABLE 5. Relationship among PFGE types, CCs, and STs for 131 isolates of *C. jejuni*

PFGE type[a]	No. of isolates	CC	ST(s) represented[b]	*flaA* type(s) represented[b]
3	2	21	806	41
5	6	21	50 (5), 1461	36 (5), 265 (1)
14	6	21	21 (5), 262	49 (5), 37 (1)
15	2	21	21	49
16	2	21	262	54
17	3	21	21	361
18	4	21	21	36
Unique	13	21	21 (5), 50 (4), others (4)	36 (5), 49 (3), 265 (2), others (3)
9	2	42	459	359
10	5	42	42	359 (3), 607 (2)
68	2	42	604	359
Unique	4	42	42 (3), 604	49, 359, 440, 607
1	5	45	45 (2), 1215 (3)	2 (4), 778 (1)
2	2	45	45, 1215	2
19	3	45	45	21
20	2	45	45	21
Unique	14	45	45 (10), 583, 766, 1217, 1685	2 (3), 8 (3), 21 (3), 22 (2), 42, 5, 70
13	3	61	61	42
Unique	1	61	1684	122
11	2	179	1207	779
8	2	257	929	16 (1), 873 (1)
Unique	1	257	929	16
6	2	353	353 (1), 405 (1)	780
Unique	13	353	353 (3), others (10)	45 (3), 780, others (9)
7	6	508	132	172
Unique	2	508	132	172
4	3	607	924 (2), 1216 (1)	790
Unique	2	607	607, 1212	756, 781
Other	17	NA[c]	NA	NA

[a] PFGE types are shown for all 22 types represented by two or more isolates. "Unique" indicates PFGE types represented by single isolates within the CC. "Other" indicates PFGE types represented by single isolates within the remaining CCs.

[b] The STs and *flaA* types represented within the PFGE type; numbers in parentheses indicate the number of isolates. Numbers are omitted when either a single ST or *flaA* type was observed among isolates of a given PFGE type or when the ST or *flaA* type was represented by only a single isolate. "Others" indicate STs or *flaA* types within the CC associated only with unique PFGE types.

[c] NA, not available.

isolates from clinical and other sources with geographic and temporal factors.

Excluding the human isolates, some CCs appeared to be preferentially associated with particular ecologic niches, although others represented all the sources examined. These observations support the hypothesis that some genotypes are associated with specific hosts, as suggested by the information in the international *C. jejuni* MLST database as well as that from other studies (5, 9, 10, 25, 26).

In this context, the water isolates raised especially interesting questions. Most of the unassigned STs were composed exclusively of water isolates, which also accounted for a disproportionate number of new alleles. We speculate that this reflects the exceptional diversity of environmental isolates; an alternative explanation is the relatively limited number of Canadian environmental isolates previously available in the international MLST database. CC 1275 in our series was exclusively composed of water isolates and in the database includes isolates from wild birds (61%), as well as environmental water (13%) and sand from bathing beaches (10%). Similarly, CCs 177 and 179 include isolates from both the environment (sand) and wild birds (5, 10, 25). Beaches typically have high concentrations of wild birds, particularly seagulls, as did the areas where we obtained the environmental water isolates. These observations raise two hypotheses. First, some environmental isolates might have preferentially adapted to colonize the intestines of wild birds and, given the opportunity, are capable of causing human disease. We are continuing to collect seagull and environmental water isolates from the same Eastern Townships area to explore this possibility. A second hypothesis is that some lineages represented among the environmental isolates may be largely nonpathogenic; this might be further investigated by virulence factor studies.

Several molecular strain-typing systems have been described for *C. jejuni*; however, there have been relatively few comparative studies, and the typing method that would be most effective at answering particular questions remains unresolved. In this study of a large collection of *C. jejuni* isolates from diverse sources, MLST and typing of the *flaA* SVR demonstrated comparable discriminatory powers. PFGE profiles were available for only ~45% of the isolates. Within this subset of isolates, the DIs calculated for *flaA* SVR typing and MLST were similar to the DI for the total study collection, while the DI for PFGE was higher. However, each of these molecular typing systems has particular strengths and limitations, as well as significant

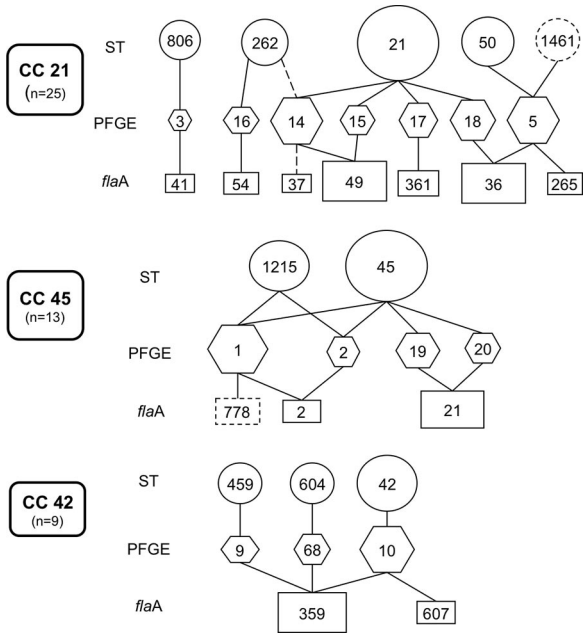

FIG. 3. Relationships among genotypes of *C. jejuni* as defined by ST (circles), *flaA* SVR typing (rectangles), and PFGE (hexagons). For each of the three CCs indicated, all PFGE types represented by two or more isolates within the CC, as well as the STs and *flaA* SVR types associated with those isolates, are shown. Dashed borders indicate that the enclosed types were represented by only a single isolate among the entire series of 289 isolates. The sizes of the symbols are roughly proportional to the number of isolates represented; see Table 5 for the precise numbers.

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operational differences. The method of choice may vary depending on the specific question at hand.

MLST is now the established standard for the analysis of population genetics. MLST provides objective, sequence-based data that are portable and readily analyzed, and MLST has an established, unified nomenclature that greatly facilitates integration of the findings from different studies (12). However, MLST, which examines seven loci, requires appreciable effort, time, and expense. Furthermore, in this series, the two most prevalent types (ST-21 and ST-45) represented over 25% of the isolates, which may significantly limit the ability to resolve specific epidemiologic relationships. For this reason, it would be interesting to generate other CCs with the subgroup founding genotypes identified by eBURST analysis. When we analyzed by eBURST analysis all the isolates included in CC 21 to date, we identified five subgroup founding genotypes (ST-19, ST-50, ST-53, ST-104, and ST-262) which met the criteria for the formation of new CCs. First, each CC had an abundant central genotype; and second, each central genotype had many single-, double-, and triple-locus variants (9). Creation of these new CCs would probably help provide an understanding of the specific epidemiologic relationships among the isolates in the CCs with larger numbers of isolates.

Although the discriminatory power of typing of the *flaA* SVR, which is also based on nucleotide sequencing, was comparable to that of MLST, the isolates were more evenly distributed among the different types by typing of the *flaA* SVR. Such diversity is potentially more useful for distinguishing among epidemiologically unrelated isolates (3, 9). However, among the *flaA* SVR types represented by two or more isolates, 10% of the isolates were discordant with respect to the CC, suggesting that the *flaA* SVR type does not consistently correlate with the chromosomal genotype. Other studies have also suggested that *flaA* SVR typing alone is poorly suited for investigation of the molecular epidemiology of *C. jejuni* isolates (8, 9, 11, 12, 35).

PFGE remains the most practical method for focused clinical molecular epidemiology studies, including the identification and tracking of outbreak strains. The variation among the PFGE profiles reflects not only nucleotide sequence changes affecting the target restriction sites but also chromosomal insertions, deletions, and rearrangements. Consequently, PFGE typically has a higher "clock speed" (i.e., is able to detect chromosomal changes that occur more rapidly) and greater discriminatory power (4). On the other hand, the high degree of diversity of the PFGE types within each CC may make the method less effective in long-term longitudinal studies of the epidemiology of *Campylobacter* (36). The major limitations of PFGE are the subjectivity of the visual interpretations of the patterns, the frequent need to confirm computer-detected matches, and the difficulties of sharing typing results among different laboratories.

Linkage analysis of these isolates indicated a moderately clonal structure, consistent with the findings of prior studies of *C. jejuni* (40). However, the relationships among the genotypes defined by the three typing systems were complex and nonlinear (Fig. 3). At this time, we cannot resolve whether the rapid divergence of sequences or relatively frequent horizontal recombination events, previously noted among *C. jejuni* isolates, contributes to the different typing results. Also, as discussed before (39), we cannot detect departure from clonality in our isolate population using the I_A value.

Antimicrobial resistance was randomly distributed among the CCs and appeared to be more closely related to the source of the isolate than to its genotype. Resistance to erythromycin and ciprofloxacin were more frequent among isolates from chickens and from patients with recent international travel, respectively. These results suggest that the emergence of resistance among *C. jejuni* isolates reflects antimicrobial selection pressure among isolates from different lineages rather than the diffusion of a unique clone. In their studies of *C. jejuni* chicken isolates collected in Senegal, Kinana et al. similarly observed that quinolone resistance was variable within STs (20). Of interest, they also noted the highest rate of quinolone resistance among isolates in CC 353. Our findings also have important implications on the use of antimicrobials in food animals; because campylobacters may be transferred from animals to humans via food or water, the emergence of multidrug resistance (resistance to fluoroquinolones and macrolides) in campylobacter strains from the food chain raises concerns that the treatment of human infections will be compromised (21).

The strongest observation from these studies is the rich genetic diversity of *C. jejuni*. This suggests that the sources for sporadic clinical infections are also likely to be diverse and that robust molecular stain typing tools will be required to decipher these relationships. *flaA* SVR typing, which involves the sequencing of only a single locus, would facilitate large-scale studies. However, the discordant results observed by us and others, although they were infrequent, remain unexplained and potentially problematic. At this time, MLST appears to be the single most effective tool for the molecular typing of *C. jejuni* strains and is uniquely suitable for use in extended, collaborative investigations.

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