

Multilocus Sequence Typing Confirms Wild Birds as the Source of a *Campylobacter* Outbreak Associated with the Consumption of Raw Peas

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From August to September 2008, the Centers for Disease Control and Prevention (CDC) assisted the Alaska Division of Public Health with an outbreak investigation of campylobacteriosis occurring among the residents of Southcentral Alaska. During the investigation, pulsed-field gel electrophoresis (PFGE) of *Campylobacter jejuni* isolates from human, raw pea, and wild bird fecal samples confirmed the epidemiologic link between illness and the consumption of raw peas contaminated by sandhill cranes for 15 of 43 epidemiologically linked human isolates. However, an association between the remaining epidemiologically linked human infections and the pea and wild bird isolates was not established. To better understand the molecular epidemiology of the outbreak, *C. jejuni* isolates ($n = 130$; 59 from humans, 40 from peas, and 31 from wild birds) were further characterized by multilocus sequence typing (MLST). Here we present the molecular evidence to demonstrate the association of many more human *C. jejuni* infections associated with the outbreak with raw peas and wild bird feces. Among all sequence types (STs) identified, 26 of 39 (67%) were novel and exclusive to the outbreak. Five clusters of overlapping STs ($n = 32$ isolates; 17 from humans, 2 from peas, and 13 from wild birds) were identified. In particular, cluster E ($n = 7$ isolates; ST-5049) consisted of isolates from humans, peas, and wild birds. Novel STs clustered closely with isolates typically associated with wild birds and the environment but distinct from lineages commonly seen in human infections. Novel STs and alleles recovered from human outbreak isolates allowed additional infections caused by these rare genotypes to be attributed to the contaminated raw peas.

Campylobacter jejuni is the most commonly implicated pathogen associated with bacterial gastroenteritis worldwide (1). With an estimated 1.3 million cases per year, *Campylobacter* infection is one of the most common causes of diarrheal illness and hospitalizations in the United States (2).

Although the majority of *Campylobacter* infections are sporadic, outbreaks do occur. Between 1997 and 2008, there were 262 *Campylobacter* outbreaks, with a total of 9,136 cases, 159 hospitalizations, and 3 deaths in the United States (3). The great majority of outbreaks are food borne, and dairy products caused the largest number (50%) of illnesses in these outbreaks, followed by produce (10%) and poultry (4%).

Infection with *C. jejuni* is zoonotic and most commonly associated with food-producing animals (4), including poultry (5), cattle, and sheep (6). *C. jejuni* is also widely disseminated in the natural environment, which is often contaminated by feces from wildlife, including wild birds and mammals (7–9).

Although fresh produce is less often identified as a source of outbreaks, campylobacters have been isolated from various types of fresh produce, including radishes, cabbage, spinach, bean sprouts, lettuce, parsley, and potatoes (10–13), from samples collected from farms (10), local outdoor markets (10, 13) and supermarkets (11). *C. jejuni* survives on fresh produce for up to 3 days after contamination at different temperatures, which is long enough for produce to be distributed and eaten (14). In a retrospective case-control study in Wales, the consumption of salad vegetables was concluded to be second only to consumption of chicken as a risk factor for *Campylobacter* infection (15).

In August 2008, the CDC assisted the Alaska Division of Public Health with an investigation of a *Campylobacter* outbreak that occurred among the residents of Southcentral Alaska; an epidemiological link was identified between illness and the consumption of raw peas, harvested from a field frequented by wild sandhill cranes (*Grus canadensis*) (16). Pulsed-field gel electrophoresis (PFGE) testing of *C. jejuni* isolates from human, pea, and wild bird samples subsequently confirmed the link. There was substantial diversity in PFGE types: 25 different *C. jejuni* PFGE types were found among the 55 human isolates tested. Four clusters were identified that encompassed 15 human isolates, 2 pea isolates, and 3 fecal isolates from sandhill cranes. It was unclear, however, whether and how the remaining epidemiologically linked infections that were not matched with pea and wild bird samples were associated with the outbreak.

We exploited a rare opportunity to explore the underlying dynamics of disease transmission in a produce-associated *Campylobacter* outbreak linked to wild birds by characterizing *C. jejuni* isolates from human, pea, and wild bird samples by using multi-

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locus sequence typing (MLST) (16), with the aim to confirm the source of the outbreak and attribute the remaining epidemiologically linked infections that were not indistinguishable by PFGE from pea and wild bird samples from the outbreak. Studies of well-characterized outbreaks can provide important insights into understanding the routes and vehicles of disease transmission (17, 18).

MATERIALS AND METHODS

***C. jejuni* isolates.** A total of 59 isolates from humans were included. Forty-three outbreak-associated isolates from stool specimens from epidemiologically linked cases were available from the initial epidemiologic investigation (16). A total of 16 sporadic *C. jejuni* isolates (including 6 isolates from the initial study) from epidemiologically unrelated illnesses during the same period and in the same area were collected. From the initial study, 40 pea and 31 wild bird *C. jejuni* isolates derived from 16 environmental samples were also included for analysis (16).

MLST. *C. jejuni* isolates were characterized by MLST; the internal sequences of the seven housekeeping loci were amplified and sequenced by use of the primers and reaction conditions described in a previously published scheme (19). BioNumerics v6.1 (Applied Maths) was used for sequence editing and assembly. The *Campylobacter* MLST database was used for sequence type (ST) and clonal complex assignment (<http://pubmlst.org/campylobacter>) (20).

Penner serotyping and antimicrobial susceptibility testing. Penner serotypes were determined by standard methods for heat-stable antigens (21), using a panel of 25 antisera which represent common serotypes in the United States (22). Antimicrobial susceptibility testing was performed at the CDC by using the National Antimicrobial Resistance Monitoring System (NARMS) standard panel of nine antimicrobials (azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin, and tetracycline) and broth microdilution (Sensititre; Trek Diagnostics), which was performed according to the manufacturer's instructions and interpreted using CLSI criteria when available.

Data analysis. The genetic relatedness among unique STs from 130 *C. jejuni* isolates in the study was visualized, along with strains (887 STs; $n = 7,044$ isolates) from humans, chickens, wild birds, and environmental waters from the *Campylobacter* PubMLST database (20) as a comparison. A neighbor-joining tree was constructed from a matrix of pairwise differences calculated between the 3,309-bp concatenated nucleotide sequences from the seven MLST housekeeping genes. A consensus tree was derived using MEGA software v5.1 (23), with 1,000 bootstrap replicates. The degrees of linkage disequilibrium within the data set and subsets were measured by calculating the standardized index of association (I_A^S) (24). If $I_A^S = 0$, then alleles at multiple loci are in absolute linkage equilibrium, indicating a freely recombining or panmictic population structure, while if $I_A^S \neq 0$, it suggests the presence of linkage disequilibrium, indicating that recombination has been rare enough or absent to allow the emergence of a clonal population structure. I_A^S was computed using the START2 package (25). Population differentiation based on estimates of gene flow between *C. jejuni* subpopulations within the data set was carried out using 3,309-bp concatenated nucleotide sequences from the seven MLST loci, using DnaSP software v5.10.01 (26). Gene flow is measured by a fixation index (F_{ST}) value that falls between 0 and 1, where a value of 0 indicates that two populations are genetically indistinguishable and a value of 1 represents completely differentiated populations.

RESULTS

Novel genetic diversity. Among 130 *C. jejuni* isolates characterized by MLST, 39 STs and 11 clonal complexes were identified, comprising 26 novel ($n = 101$; 78% of all isolates) and 13 known ($n = 29$; 22% of all isolates) STs. Only one of the novel STs (ST-5068) belonged to one of the 11 clonal complexes identified (Table 1). Among the novel STs, 24 of 26 (92%) were composed of newly

identified alleles, often observed at multiple MLST loci. ST-5054 was composed entirely of novel alleles at all seven loci. Forty-six novel alleles (31%) were identified among a total of 145 alleles in the data set.

Outbreak molecular epidemiology. Most of the human outbreak-associated isolates (31 of 43 [72%]) and pea and wild bird isolates (67 of 71 [94%]) were represented by novel STs, while most of the comparable sporadic human isolates (13 of 16 [81%]) were represented by known STs. From a total of 39 STs identified in our data set, 20 STs were seen only among human isolates, and 14 STs were seen only among pea and wild bird isolates (Table 1). However, five clusters with five different STs were identified that contained both human outbreak-associated isolates and pea and wild bird isolates (clusters A to E) (Table 1). The clustering of isolates showed strong agreement between MLST, PFGE, and Penner serotyping results. Clusters A to D corresponded to the four combined SmaI-KpnI PFGE patterns identified in human isolates that were indistinguishable from pea and wild bird isolates in the initial investigation (16). Cluster E isolates contained isolates from all three sample types, including two human outbreak-associated isolates, three isolates from peas, and two isolates from wild bird feces. The two human outbreak-associated isolates from cluster E were unrestricted by PFGE using SmaI but were grouped by an indistinguishable KpnI-restriction PFGE pattern in the initial investigation (16).

ST-5062 did not belong to a cluster but was the most common subtype found among human outbreak-associated isolates from nine patients. The SmaI-KpnI PFGE restriction patterns and Penner serotype (HS:41) were also indistinguishable among eight of these nine patients. One of these eight patients developed Guillain-Barré syndrome (GBS). The isolate from the ninth patient had a different SmaI-KpnI PFGE restriction pattern and Penner serotype (HS:8).

Apart from one human isolate from cluster D, found to be resistant to ciprofloxacin and nalidixic acid, all isolates within the five clusters were pan-susceptible to the nine antimicrobials tested.

Population structure. To provide additional molecular evidence for the link between wild birds and human illness during the outbreak, we explored the underlying *C. jejuni* population structure between the human outbreak-associated isolates and pea and wild bird isolates that did not cluster during the outbreak.

The presence of novel alleles was a distinct feature among the five clusters of human outbreak-associated isolates and pea and wild bird isolates identified. However, novel alleles were also widely distributed among human outbreak-associated isolates and pea and wild bird isolates that did not cluster during the outbreak. In particular, four novel alleles (*glnA*-387, *glyA*-419, *tkt*-443, and *tkt*-449), from clusters A, B, C, and E, were also found among human outbreak-associated isolates and pea and wild bird isolates that did not cluster (Table 1).

We visualized the *C. jejuni* population structure of our data set (all 39 STs) in conjunction with isolates ($n = 7,044$) from the PubMLST database (887 STs) that originated from humans (green), chickens (pink), wild birds (dark blue), and environmental waters (light blue) by using a neighbor-joining tree (Fig. 1A). A host association of *C. jejuni* clades was apparent, as distinct clades with predominantly "human and chicken isolates" and "wild bird and environmental water isolates" were evident. *C. jejuni* STs from our human outbreak-associated isolates and pea and wild bird isolates collected during the outbreak were genetically closely

TABLE 1 Genetic diversity and frequency distribution of 130 *C. jejuni* isolates from humans, peas, and wild birds from the outbreak^{esf}

Sequence type	Clonal complex ^a	MLST allelic profile ^b							PFGE profile ^c		Penner serotype ^d	No. of isolates				
		<i>aspA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkl</i>	<i>uncA</i>	SmaI	KpnI		Human		Wild bird	Pea	Total
												Outbreak	Sporadic			
Human, pea, and wild bird clusters																
ST-5061* (cluster A)	UA	10	387 ‡	61	423	39	449 ‡	1	DBRS 16.1283	DBRK02.0549	HS19	1			1	2
ST-5059* (cluster B)	UA	10	31	25	49	57	7	313	DBRS 16.0127	DBRK02.0546	HS8	5	1		1	7
ST-5048* (cluster C)	UA	10	383	106	419 ‡	57	443 ‡	1	DBRS 16.1256	DBRK02.0522	HS18	3			4	7
ST-137 (cluster D)	ST-45	4	7	10	4	42	7	1	DBRS 16.0749	DBRK02.0524	HS15	5			4	9
ST-5049* (cluster E)	UA	180	172	322	47	39	180	3	Unrestricted	DBRK02.0548	HS29, HS21	2		2	3	7
Pea and wild bird isolates																
ST-5047*	UA	180	389	95	47	541	442	3						5	4	9
ST-5057*	UA	1	389	325	422	541	180	6						4	5	9
ST-5050*	UA	178	7	95	6	354	88	1							6	6
ST-5056*	UA	178	385	95	129	545	180	48						6		6
ST-5052*	UA	1	31	323	420	394	89	6						4	1	5
ST-5055*	UA	10	280	277	49	394	443 ‡	6						5		5
ST-5053*	UA	1	2	95	62	472	400	147						1	3	4
ST-5054*	UA	285	384	324	421	542	445	312						3		3
ST-5073*	UA	178	8	327	49	544	28	6							3	3
ST-5058*	UA	1	2	34	90	543	443 ‡	6						1	1	2
ST-5051*	UA	10	385	106	129	39	444	3							1	1
ST-5060*	UA	10	387 ‡	95	423	39	7	1							1	1
ST-5072*	UA	290	389	5	418	39	441	6							1	1
ST-5074*	UA	10	386	61	419 ‡	39	451	1							1	1
Human outbreak-associated isolates																
ST-5062*†	UA	287	6	34	424	394	446	172			HS41	8				9
											HS8	1				
ST-468	UA	10	2	50	62	91	73	45				3				3
ST-5069*	UA	10	6	61	427	548	447	6				3				3
ST-5063*	UA	10	308	106	47	57	447	1				2				2
ST-5065*	UA	1	172	5	426	39	231	6				2				2
ST-5064*	UA	1	82	95	425	546	448	314				1				1
ST-5067*	UA	286	172	5	426	39	443 ‡	6				1				1
ST-5066*	UA	10	74	276	47	39	449 ‡	51				2				2
ST-45	ST-45	4	7	10	4	1	7	1				2				2
ST-42	ST-42	1	2	3	4	5	9	3				1				1
ST-48	ST-48	2	4	1	2	7	1	5				1				1
Human sporadic isolates																
ST-5066*	UA	10	74	276	47	39	449 ‡	51					1			1
ST-45	ST-45	4	7	10	4	1	7	1					3			3
ST-42	ST-42	1	2	3	4	5	9	3					1			1
ST-48	ST-48	2	4	1	2	7	1	5					1			1
ST-19	ST-21	2	1	5	3	2	1	5					1			1
ST-52	ST-52	9	25	2	10	22	3	6					1			1
ST-353	ST-353	7	17	5	2	10	3	6					1			1
ST-403	ST-403	10	27	16	19	10	5	7					1			1
ST-508	ST-508	1	6	60	24	12	28	1					1			1
ST-572	ST-206	62	4	5	2	2	1	5					1			1
ST-991	ST-692	37	52	57	26	107	29	23					1			1
ST-2875	UA	2	29	4	48	10	25	57					1			1
ST-5068*	ST-42	1	2	3	4	5	9	315					1			1
Total												43	16	31	40	130

^a UA, unassigned status for a given sequence type.

^b Newly identified alleles are shown in bold.

^c PFGE profiles for the restriction enzymes SmaI and KpnI for the five identified clusters (A to E).

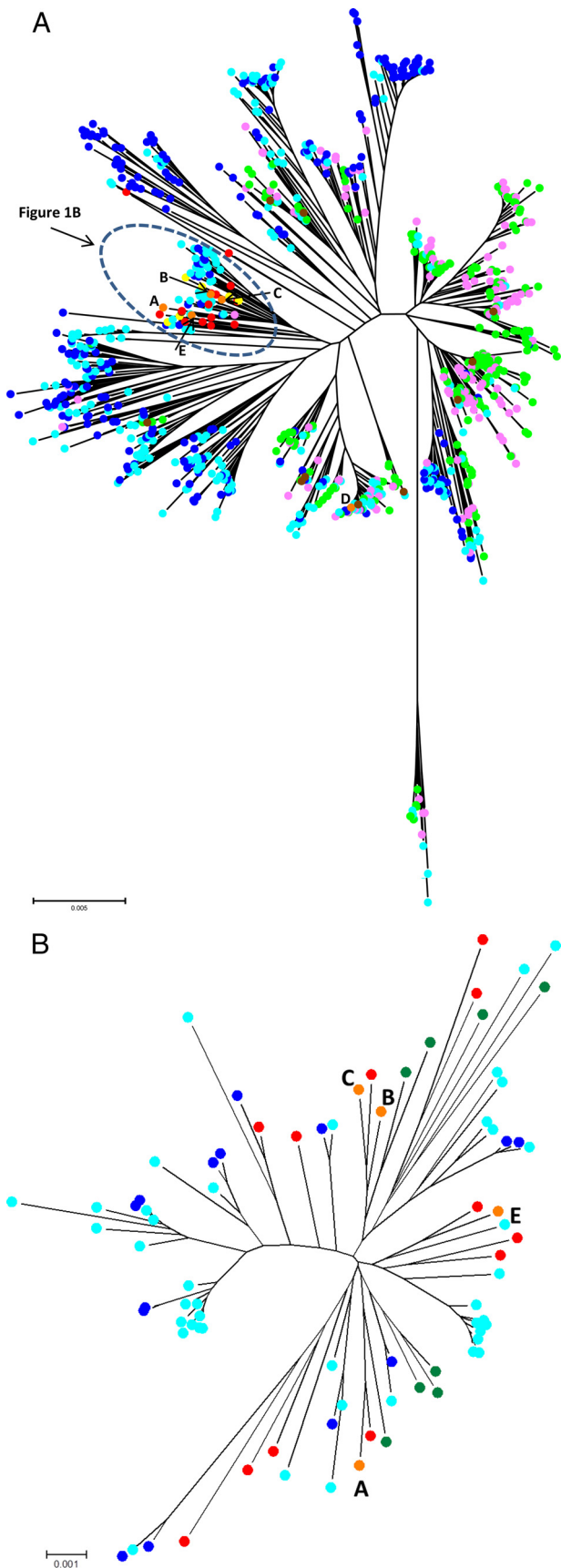
^d Penner serotyping was performed on representatives of human and environmental isolates from clusters A to E and on all ST-5062 isolates, including one from a GBS case.

^e *, novel STs; †, ST associated with one case of Guillain-Barré syndrome; ‡, overlapping novel alleles found in human and environmental isolates.

^f The numbers of alleles per locus (% novel) were 14 (28.5), 23 (26), 22 (22.7), 24 (41.6), 22 (31.8), 24 (41.6), and 16 (25) for the *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkl*, and *uncA* genes, respectively.

related (yellow, human outbreak-associated isolates; red, pea and wild bird isolates; orange, clusters A, B, C, and E) (Fig. 1B). Additionally, these isolates clustered within clades associated with the “wild bird and environmental water isolates” in the PubMLST

database. Cluster D isolates (ST-137, ST-45 complex; orange) were grouped among members of a genetically diverse complex frequently associated with avian sources, including chickens and wild birds (8, 27). In contrast, sporadic human *C. jejuni* isolates



from Alaska (brown) were well dispersed among clades associated with “human and chicken isolates.”

We also assessed the genetic similarity between the human outbreak-associated isolates and pea and wild bird isolates and their genetic divergence relative to the sporadic human isolates. The current outbreak data set was divided into subsets of novel STs (representing outbreak, pea, and wild bird isolates) and known STs (representing sporadic human isolates). Differences in the degree of clonality between these subsets were detected by calculating the standardized index of association (I_A^S) as a measure of linkage disequilibrium. The I_A^S values ($P < 0.001$) for the subsets using representative STs were 0.0526 for the subset of novel STs and 0.3057 for the subset of known STs. The difference in I_A^S values indicated that there were distinct *C. jejuni* subpopulations with differing degrees of clonality. The I_A^S value of 0.0526 for the novel ST subset denotes a subpopulation with a higher rate of recombination and a less clonal population structure than those of the known ST subset, which had a higher I_A^S value. The gene flow between the novel and known ST subsets appears to be limited, as the pairwise F_{ST} value of 0.192 indicates considerable population differentiation.

DISCUSSION

Given the association with a wild bird source and an unusual transmission vehicle, the 2008 Alaska *Campylobacter* raw pea outbreak offered a unique opportunity to extend our current understanding of the epidemiology of *C. jejuni* infections.

In this investigation, we sought to examine the association between the epidemiologically linked human *C. jejuni* infections and pea and wild bird samples that could not be linked using PFGE data from the initial study (16).

We explored genetic differences between *C. jejuni* isolates from pea and wild bird samples and those from human infections from the outbreak. Among the human isolates characterized in this study, most human outbreak-associated isolates belonged to novel STs and were therefore highly suggestive of unique infective sources. Similarly, all but one ST (ST-137) identified from pea and wild bird samples were novel. In contrast, the majority of sporadic human isolates belonged to known STs and clonal complexes that are globally widespread among human infections (<http://pubmlst.org/campylobacter/>). Despite the high genetic divergence normally shown among environmental *C. jejuni* isolates, we were still able to observe five clusters of overlapping STs among the genetically similar human outbreak-associated isolates and pea and wild bird isolates. In addition, the occurrence of ST-5049 in all three sample sources (cluster E) provides a definitive link that illustrates the transmission route of *C. jejuni* during the outbreak.

We attributed additional human cases from the outbreak that are likely to be linked to wild bird sources by exploring the under-

FIG 1 (A) The genetic relatedness and diversity of 39 unique STs from 130 *C. jejuni* isolates in the study were visualized, along with 887 STs ($n = 7,044$ isolates) from various sources, available in the PubMLST database, for comparison. Isolates from the Alaska outbreak included isolates from peas and wild birds (red), humans (yellow), and overlapping clusters A to E (orange). Isolates from the MLST database included isolate collections from humans (green), chickens (pink), environmental waters (light blue), and wild birds (dark blue). (B) Expanded view of the genetic relatedness of clusters A, B, C, and E with closely related isolates from the circled portion of panel A.

lying *C. jejuni* population structure. Based on the possession of identical novel alleles, we linked additional possible human outbreak-associated isolates without identical sequence type matches to those from the peas and wild birds. In our data set, approximately one-third of alleles and 67% of STs were novel and could not be grouped into clonal complexes. In particular, ST-5054, with three wild bird isolates, was composed entirely of novel alleles at seven MLST loci. This finding was similar to that of a study of New Zealand river waters, where 55% of *C. jejuni* isolates consisted of novel STs, and ST-2381 was composed of six new alleles (28). In several other studies, the occurrence of isolates with novel alleles and STs unassigned to clonal complexes has similarly emerged as a dominant characteristic of genetically divergent *C. jejuni* isolates found primarily among wildlife and environmental sources (28–30). By clearly demonstrating the dominance of novel *C. jejuni* strains from wild birds and the peas they contaminated (and fed on), our study lends further support to the hypothesis of the existence of environmental/wildlife-associated *C. jejuni* strains (30). A distinct distribution of genotypes between wildlife and farmed animals could therefore signify independent disease transmission routes to humans from these sources (29). However, the ecology of *C. jejuni* among environmental sources is complex, so the precise transmission pathways leading to human infection from these sources have remained difficult to determine. Nonetheless, the absence of the *C. jejuni* ST-21 complex among human outbreak-associated isolates and pea and wild bird isolates in the current study, a lineage which is thought to be ubiquitous and adapted to a wide range of sources (8), is consistent with this notion.

From the radial neighbor-joining tree constructed, we have shown that human outbreak-associated isolates that did not group in one of the five identified clusters (clusters A to E) with pea and wild bird isolates were genetically closely related and were located within clades associated with wild bird and environmental isolates in the PubMLST database. Similarly, four of the five clusters (clusters A, B, C, and E) identified in the study were also located within the same wild bird/environmental clade. The fact that cluster D was not placed in these clades is not an unusual observation. ST-137 isolates from this cluster belong to the ST-45 complex, which has frequently been associated with human infections and avian sources (8).

By assigning novel STs to represent wildlife and environmental isolates and known STs to represent common lineages seen in human infections, we observed genetically distinct *C. jejuni* subpopulations with different rates of recombination and limited genetic mixing. The difference between I_A^S values obtained for the subsets suggests a *C. jejuni* population structure that consists of a subpopulation of highly successful epidemic clones (human infection/food isolates) superimposed on the background of a genetically divergent subpopulation that has a higher rate of recombination and a less clonal structure (wildlife/environmental isolates). This “epidemic” population structure was first proposed for *Neisseria meningitidis* and may also apply to *C. jejuni* (31). Indeed, given the higher rate of recombination and weaker ancestral signal, it is not a surprising observation that rare and novel STs have continually been found among environmental *C. jejuni* isolates (28, 30, 32–34) that remain unassigned to clonal complexes. Based on the pairwise F_{ST} value calculated (0.192), we have also shown evidence for limited gene flow between the known and novel ST subsets. This can be compared to values of

0.026 (35) and 0.019 (36) between human and poultry isolates, 0.199 between cattle and wild birds, 0.009 between wild birds and farmland environment (29), and 0.932 between *C. jejuni* and *C. coli* (37).

From a public health perspective, we have shown that despite considerable genetic divergence from lineages commonly seen in human infections, *C. jejuni* from wild birds in this study had the capacity to cause gastroenteritis, GBS, and a large-scale outbreak under favorable circumstances. However, several possible explanations can be speculated for *C. jejuni* strains that did not overlap between human outbreak-associated isolates and those found in peas and wild birds. First, certain *C. jejuni* strains from this outbreak may have poor pathogenicity against humans, given the genetic divergence from common strains circulating in human infections. Second, immunity among humans who have had prolonged exposure to strains found in peas and wild birds may not lead to illness after the consumption of contaminated peas. Third, *C. jejuni* strains genetically identical to those found in humans were simply not captured against the background of a highly diverse genetic pool in the wild bird population, due to the sample size. Lastly, the survival of *C. jejuni* strains on peas may have been short-lived, limiting the infectious dose required to cause an infection.

It is worth noting that only one of eight patients who shared the same ST (ST-5062), SmaI-KpnI PFGE profile, and Penner serotype (HS:41) developed GBS. This observation indicates that the development of GBS after *Campylobacter* infection could vary depending on host factors, even when individuals are infected with seemingly genetically identical strains. Therefore, a correlation between MLST genotypes and the onset of GBS could be difficult to determine, though several studies have found significant associations (38, 39). Nonetheless, our result has added further support to the described association between infection with HS:41 and the development of GBS, even from a wild bird source. Furthermore, patients with specific HLA alleles have been shown to be more likely to be predisposed to GBS (40).

From the antimicrobial susceptibility testing performed, only one isolate belonging to a known ST (ST-137) was found to be resistant, showing resistance to fluoroquinolones. Among isolates with novel STs, none showed resistance to any of the nine antimicrobials. This suggests that *C. jejuni* in wild birds may not have been exposed to antimicrobials that are often used in animals reared for food production. Indeed, the incidence of resistance to antimicrobials among wild bird *C. jejuni* isolates in Sweden was previously shown to be low (41).

We explored the unique opportunity to investigate the rare occurrence of a *Campylobacter* outbreak linked to produce and wild birds. Although poultry has been identified as the major source of campylobacteriosis from source attribution studies, the source of a considerable proportion of illnesses remains unidentified (42, 43). Our study has provided new insights into the disease transmission dynamics from the environmental reservoir, highlighting the significance of *C. jejuni* from produce and/or environmental sources and its potential to cause human disease under favorable circumstances. This study was made possible through timely sample collection and close collaboration between epidemiologists, sanitarians, and microbiologists who worked on the investigation. Additional studies involving isolation and characterization of *C. jejuni* from the environment would be helpful

for building a more complete picture for source attribution efforts.

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