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Comparative Genotyping of *Campylobacter jejuni* by Amplified Fragment Length Polymorphism, Multilocus Sequence Typing, and Short Repeat Sequencing: Strain Diversity, Host Range, and Recombination

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Three molecular typing methods were used to study the relationships among 184 *Campylobacter* strains isolated from humans, cattle, and chickens. All strains were genotyped by amplified fragment length polymorphism (AFLP) analysis, multilocus sequence typing (MLST), and sequence analysis of a genomic region with short tandem repeats designated clustered regularly interspaced short palindromic repeats (CRISPRs). MLST and AFLP analysis yielded more than 100 different profiles and patterns, respectively. These multiple-locus typing methods resulted in similar genetic clustering, indicating that both are useful in disclosing genetic relationships between *Campylobacter jejuni* isolates. Group separation analysis of the AFLP analysis and MLST data revealed an unexpected association between cattle and human strains, suggesting a common source of infection. Analysis of the polymorphic CRISPR region carrying short repeats allowed about two-thirds of the typeable strains to be distinguished, similar to AFLP analysis and MLST. The three methods proved to be equally powerful in identifying strains from outbreaks of human campylobacteriosis. Analysis of the MLST data showed that intra- and interspecies recombination occurs frequently and that the role of recombination in sequence variation is 50 times greater than that of mutation. Examination of strains cultured from cecum swabs revealed that individual chickens harbored multiple *Campylobacter* strain types and that some genotypes were found in more than one chicken. We conclude that typing of *Campylobacter* strains is useful for identification of outbreaks but is probably not useful for source tracing and global epidemiology because of carriage of strains of multiple types and an extremely high diversity of strains in animals.

Campylobacter jejuni is the most frequently isolated bacterial pathogen in cases of human gastroenteritis in developed countries. In The Netherlands *C. jejuni* was isolated from 2% of the cases of gastroenteritis in 1999, yielding an incidence of 6.8 cases per 1,000 person-years. This amounts to about 100,000 cases of campylobacteriosis annually in The Netherlands population of nearly 16 million (5). In comparison, the Central Public Health Laboratory Service reported 54,169 cases in 2001 in England and Wales (2). However, a recent community-based survey showed that the true incidence may be up to 500,000 cases per year (8.3 cases per 1,000 person-years), which is similar to the incidence seen in The Netherlands (34). Campylobacteriosis is also a frequently occurring infection in the United States, with an estimated incidence of 2.5 million cases each year (9.4 cases per 1,000 person-years) (26). These numbers exceed the incidence of *Salmonella* infection seen in these countries by a factor of 2 or 3.

Typically, *C. jejuni* infection in humans is associated with sudden onset of fever, abdominal cramps, and bloody diarrhea (21). Although the disease is self-limiting, occasional more severe sequelae and prolonged disease may result from infection. Complications may involve reactive arthritis (16), Guillain-Barré syndrome, and Miller-Fisher autoimmune syndrome (28).

Campylobacter spp. are widespread in the environment and constitute part of the natural intestinal flora of many mammalian species and birds. This includes not only domestic farm animals such as cattle, sheep, and pigs but also pet animals like cats and dogs. However, contaminated poultry meat probably constitutes the most important source for *C. jejuni* infection in humans. The handling or consumption of raw or undercooked meat products has been implicated in outbreaks among humans. Yet, the majority of *C. jejuni* infections are sporadic, with the source of infection remaining unidentified in most cases (1).

Although *C. jejuni* infections in humans are highly prevalent, knowledge of the pathogenicity of *C. jejuni* strains is still limited. It is unclear whether certain types of *C. jejuni* strains are specific for particular hosts or whether they are associated with specific disease manifestations in humans. Furthermore, the sources and routes of transmission remain unclear in most cases of campylobacteriosis. To be able to answer questions concerning source tracing and global epidemiology, it is important to use robust and well-differentiating typing methods.

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TABLE 1. *Campylobacter* strains used in the comparative molecular analyses

Host	Origin	No. of isolates			No. of strains by geographic origin	
		Total	*C. jejuni*	*C. coli*	The Netherlands	Other countries
Human	Dutch gastroenteritis patients	55	53	2	54	1
	Guillain-Barré syndrome patients	18	18		17	1
	CAMPYNET collection	9	9		1	8
	Type strain NCTC 11186	1	1			1
Poultry	Dutch farms	53	53		53	
	CAMPYNET collection	6	5	1	1	5
Cattle	Dutch farms	25	25		25	
	CAMPYNET collection	6	6		2	4
Other	Various animals	11	10	1	9	2
Total		184	180	4	162	16

Many research groups have used typing methods to characterize *Campylobacter* strains. A search of the *Campylobacter* literature published between 1995 and 2001 by use of the keyword "typing" returned more than 100 titles of papers on this subject. The typing methods range from phenotypic methods like serotyping to genotyping by pulsed-field gel electrophoresis and many others. In particular, the phenotypic typing methods pose many problems associated with a lack of typeability, high costs, the need to use labor-intensive procedures, and poor reproducibilities. The use of molecular genotyping methods may solve some of these difficulties. The various methods used to genotype *C. jejuni* have been reviewed in detail (37). In the study presented here we used amplified fragment length polymorphism (AFLP) analysis (3, 8, 14, 22, 23, 36), multilocus sequence typing (MLST) (6, 12, 32), and sequence analysis of the clustered regularly interspaced short palindromic repeats (CRISPRs) (18, 20) to genotype a collection of *C. jejuni* strains. The purpose of the study was to compare the three methods for use in epidemiology, to search for associations between genotypes and hosts, and to assess the role of recombination in *C. jejuni* strain variation.

MATERIALS AND METHODS

Bacterial strains. The collection of epidemiologically unrelated *Campylobacter* strains used in this study consisted of 83 strains isolated from humans, 59 strains isolated from poultry, 31 strains isolated from cattle, and 11 strains isolated from various other hosts. The 83 human strains were from patients with gastroenteritis enrolled in a case-control study among general practitioners in The Netherlands (4), from Dutch patients with Guillain-Barré or Miller-Fisher syndrome (7, 10). and from the CAMPYNET collection (http://www.svs.dk/campynet/). In addition, type strain NCTC 11168 was included because the genome of this strain has been sequenced (30). The poultry and cattle strains were isolated from geographically dispersed farms in The Netherlands. Furthermore, a small number of poultry and cattle isolates from the CAMPYNET collection were added to these groups. The last group of strains, designated "other," comprised *Campylobacter* strains from more exotic sources in this case: cats ($n = 2$), dogs ($n = 4$), wild birds ($n = 2$), a lynx ($n = 1$), a pig ($n = 1$), and a sheep ($n = 1$). Virtually all strains were *C. jejuni*; however, two strains isolated from humans, one poultry strain, and one strain from a pig were identified as *Campylobacter coli*. All strains are listed in Table 1.

Isolation of chromosomal DNA. Genomic *Campylobacter* DNA was isolated from bacteria grown on blood agar plates and purified as described before (8).

AFLP typing. Strains were typed by the AFLP method for *Campylobacter* genotyping by a protocol adapted from the AFLP microbial fingerprinting method of Applied Biosystems (Foster City, Calif.) (8). Briefly, AFLP fragments were created with *Hin*dIII- and *Hha*I-restricted genomic *Campylobacter* DNA by selective PCR with two PCR primers each with a single A extension. The final products were separated on a sequencing gel with an ABI 373A automated DNA sequencer (Applied Biosystems). After data collection with Genescan software (Applied Biosystems), the gels were normalized with an internal fluorescently labeled (6-carboxy-'*x*'-rhodamine) size standard included in each sample. Densitometric curves were processed with GelCompar (version 4.1) software and imported into Bionumerics software (version 2.5; Applied Maths, Kortrijk, Belgium). The levels of genetic similarity between AFLP patterns were calculated with the Pearson product-moment correlation coefficient. For cluster analysis of AFLP banding patterns, the unweighted pair group method with average linkages was used.

MLST. MLST was performed as described by Dingle et al. (6) by using sequences obtained from seven housekeeping genes. For a number of samples, either the PCR did not yield a product or the PCR product could not be sequenced with the primers described by Dingle et al. (6) and alternative primers were used. The oligonucleotide primers used to amplify and sequence the genes are shown in Table 2. The amplification reactions were performed in a 25-μl volume comprising approximately 10 ng of *Campylobacter* chromosomal DNA; 0.4 μM each primer; and the HotStar master mixture (Qiagen GmbH, Hilden, Germany), which includes deoxynucleoside triphosphates, buffer, and polymerase. The reaction conditions were one cycle at 95°C for 15 min to denature the DNA and activate the HotStar *Taq* DNA polymerase, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final elongation step at 72°C for 7 min. PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems).

MLST allele and ST assignment. MLST alleles and sequence types (STs) were assigned to the isolates by using the *Campylobacter* PubMLST database at Oxford University that is accessible on the Internet at http://phoenix.ceid.ox.ac.uk/campylobacter/. Similarity between STs was calculated by using the categorical numerical similarity coefficient and the complete linkage clustering of Bionumerics software (version 2.5). In addition, the program BURST (E. J. Feil and M.S. Chan, http://www.mlst.net/new/data_analysis/index.htm) was used to verify the results of the analysis with Bionumerics software. The members of a lineage were defined as groups of two or more independent isolates with an ST that shared identical alleles at five or more loci. The ST with the largest number of strains within a lineage was defined as the founder of that group (13). Each lineage was named after the ST identified as the putative founder of the group, followed by the word "complex" (e.g., ST-122 complex). Bionumerics software (version 3.0) was used for group separation (19). Group separation was performed by the Jacknife method with the maximum similarity setting and equal distribution over the groups when identical values were found for different groups.

Analysis of CRISPR region. The CRISPR regions were amplified with the primer pair CAMPDRF (AGCTGCCCTTATGGTGGTG) and CAMPDRR (AAGCGGTTTTAGGGGATTGT), which targeted the region flanking the CRISPR. The amplification reactions were performed in a 25-μl volume comprising approximately 10 ng of *Campylobacter* chromosomal DNA, 0.4 μM each primer, and the HotStar PCR master mixture. The reaction mixture was heated at 95°C for 15 min to activate the *Taq* DNA polymerase. After activation the

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TABLE 2. Oligonucleotide primers for *Campylobacter* MLST[a]

Gene	Forward primer			Reverse primer			Amplicon size (bp)
	Name	Sequence	Coordinates	Name	Sequence	Coordinates	
aspA	asp-A1F	AAAGCTGCAGCTATGGC	160–176	asp-A2R	TTCCAGTGTTCATTGCCTC	1279–1297	1,137
	asp-A3F	ATGAGGTTTATTATGGAGTGC	50–70	asp-A2R	TTCCAGTGTTCATTGCCTC	1279–1297	
	asp-A9F*	AGTACTAATGATGCTTATCC	418–437	asp-A10R*	ATTTCATCAATTTGTTCTTTGC	1337–1358	
	asp-S3F*	CCAACTGCAAGATGCTGTACC	561–581	asp-S6R*	TCAATTTGCGGTAATACCATC	1165–1184	
glnA	gln-A1F*	TAGGAACTTGGCATCATATTACC	92–114	gln-A2R*	TTGGACGAGCTTCTACTGGC	1377–1396	1,304
	gln-S1F	GCTCAATTCATGGATGGC	173–190	gln-S4R	GCATACCATTGCCATTATCTCCG	801–823	
	gln-S2hFc	GAAGAAACATTTCAAACTGG	136–155	gln-S3hFc	CTGGCATTCCATTTGATGG	152–170	
gltA	glt-A1F*	GGCCTTGACTTCTACAGCTACTTG	138–161	glt-A2R*	CCAAATAAAGTTGTCTTGGACGG	1227–1249	1,111
	glt-S1F*	GTGGCTATCCTATAGAGTGGC	212–232	glt-S6R	CCAAAGCGCACCAATACCTG	767–786	
	glt-S2hFc	TTAATGCACCGTGGCTATCC	202–221				
glyA	gly-A1F*	GAGTTAGAGCGTCAATGTGAAGG	49–71	gly-A2R*	AAACCTCTGGCAGTAAGGGC	1081–1100	1,051
	gly-A2hFc	GGTCTTGAAATGATAGCGAG	70–89	gly-A2hRc	GGAGTTCCAAGTCTTAATCC	1060–1079	
	gly-S3F*	AGTTAATACAGGTGTTTATGCGG	297–307	gly-S4R*	AGGTGATTATCCGTTCCATCGC	911–932	
pgm	pgm-A1F	TTGGAACTGATGGAGTTCG	11–29	pgm-A2R	AAGAGCTTAATATCTCTGGCTTCTAG	1258–1283	1,272
	pgm-A3F	TCAGGGCTTACTTCTATAGG	181–200	pgm-A4R	AACTTAATATCTCTGGCTTC	1261–1280	
	pgm-A7F*	TACTAATAATATCTTAGTAGG	114–134	pgm-A8R*	CACAACATTTTTCATTTCTTTTTC	1285–1308	
	pgm-S3F	GCTTATAAGGTAGCACCTACTG	559–580	pgm-S2R*	TCCAGAATAGCGAAATAAGG	1214–1233	
tkt	tkt-A5F	TTTAAGTGCTGATATGGTGC	45–64	tkt-A4R	CATAGCGTGTTCTCTGATACC	1162–1182	1,851
	tkt-A1F	TACAAGAACAGGCAAATACTCTAAG	17–41	tkt-A2R	GAAACGCTAAATCCAAAGCG	1849–1868	
	tkt-S1F	TGCACCTTTGGGCTTAGC	90–107	tkt-S4R*	ACTTCTTCACCCAAAGGTGCG	771–791	
uncA	unc-A4R	AAAGCTGATGAGATCAGTTC	10–29	unc-A3F	CTCATTAATGGCTTTAGCTAG	1465–1485	1,475
	unc-A7F*	ATGGACTTAAGAATATTATGGC	125–146	unc-A8R	ATAAATTCCATCTTCAAATTCC	1362–1383	
	unc-S3F	AAAGTACAGTGGCACAAGTGG	605–625	unc-S4R*	TGCCTCATCTAAATCACTAGC	1195–1215	
	unc-S3aFd	AAAGCACAGTGGCTCAAGTTG	605–625	unc-S3cFe	GCACAGTGGCTCAAGTTGT	608–626	

[a] An A in the primer name indicates that it was used for amplification, an S in the primer name indicates that it was used for sequencing. An asterisk denotes that this primer was described before in the paper of Dingle et al. (6).

TABLE 3. Host specificity of *Campylobacter* genotypes measured by group separation

Host	% Closest relative by AFLP analysis			% Closest relative by MLST		
	Cattle	Human	Poultry	Cattle	Human	Poultry
Cattle	29	58	13	41	51	8
Human	5	75	20	30	50	20
Poultry	0	39	61	15	38	47

following touchdown PCR protocol was applied: denaturation at 95°C for 30 s; primer annealing at 69°C for 30 s; extension at 72°C for 1 min, with lowering of the primer annealing temperature 2°C every 2 cycles until 59°C was reached; and another 30 cycles with a primer annealing temperature of 59°C, followed by a final elongation step at 72°C for 7 min. PCR amplifications were performed in a GeneAmp PCR system. Nucleotide sequencing of both strands was performed with the PCR primers. Each spacer sequence was given a number resulting in a type code for the various CRISPR regions similar to the ones used for MLST typing; e.g., CRISPR type (CT) 45 (CT-45) contains spacers 165-116-031-032. CRISPR regions that were found in three or more strains and that shared one or more of the spacer sequences were designated CRISPR groups, e.g., the CT 1 group.

DNA sequencing. PCR products were checked for integrity on ethidium bromide agarose gels and purified with the Qiaquick PCR purification kit (Qiagen GmbH). For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology with the protocol of the manufacturer (Applied Biosystems) was used. Unincorporated dye terminators were removed with the Multiscreen assay system (Millipore, Molsheim, France), according to the protocol of the manufacturer, and the reaction products were separated and detected with an ABI Prism 3700 automatic DNA sequencer (Applied Biosystems). Sequence assembly and editing were performed with the Seqman module of the DNAstar package (DNAstar Inc., Madison, Wis.), and subsequently, the edited sequences were imported into Bionumerics software (version 2.5) for further analysis.

RESULTS

AFLP patterns of *C. jejuni* strains from different hosts. AFLP analysis yielded highly polymorphic and diverse patterns consisting of approximately 50 bands. At a cutoff of 90%, clustering yielded 116 different AFLP types among the 184 strains. Strains with AFLP patterns with similarities above 90% are genetically highly related and usually represent epidemiologically related isolates (7, 8). The largest cluster of AFLP patterns with similarities of 90% and above consisted of 14 strains; 7 of these were isolated from humans, 4 were isolated from poultry, 2 were isolated from cattle, and 1 was isolated from a cat. In addition, there were six small clusters that each contained three to four strains and that comprised only strains isolated from humans and one cluster of three poultry strains. In a further effort to determine whether the AFLP patterns could be separated into host-specific groups, a statistical method called group separation was applied (19). Three groups were defined on the basis of the origins of the strains: poultry, human, and cattle strains. After the clustering, each pattern within the three groups was compared by calculating the maximum similarities with the patterns of the members of each group. This resulted in a percentage of cases in which a pattern was most related to a pattern of a strain belonging to one of the groups. The analysis showed that both the poultry and the human isolates were most related to other members of the same host group (Table 3). The patterns of about 75% of the human strains were found to be most closely related to the patterns of the other human strains, and the patterns of 20% of the human strains were more similar to the patterns of the strains isolated from poultry. The patterns of only 5% of the human strains were more similar to the patterns of the strains from cattle. Similarly, 61% of the AFLP patterns of the chicken strains were most closely related to those of other chicken strains, and 39% of the AFLP patterns of the chicken strains were most closely related to the patterns of the human strains. The patterns of the cattle strains were less host specific, as the AFLP patterns of only 29% of the cattle strains were found to be most similar to those of other strains from the cattle group; and the AFLP patterns of more than half (58%) of the cattle strains were most closely related to those of human strains, and the AFLP patterns of 13% of the cattle strains were most closely related to those of poultry strains. Remarkably, none of the patterns of the poultry strains were found among the patterns of the cattle strains.

Distribution of MLST alleles among different hosts. The allele sequences for seven MLST loci were determined, and the frequencies at which these alleles were present were stratified by the host from which the strains were isolated (Fig. 1). Most alleles were present at similar frequencies among the three host groups. Exceptions were *aspA* allele 1, which was rare in chicken strains (2%) but which was predominant in cattle strains (23%) and human strains (18%). Such pronounced differences were not found for alleles of the *glnA* locus. In poultry strains *gltA* allele 5 was present in 41% of the poultry isolates, whereas this allele was found in only 18 and 7% of the human and cattle strains, respectively. Also, marked differences in the distribution of the *pgm* locus were detected. Among the cattle strains, 23% carried *pgm* allele 6, and 6% of the *Campylobacter* strains isolated from humans also contained the same allele. However, the latter allele was completely absent from the poultry strains tested. When the data from the *Campylobacter* PubMLST database (http://phoenix.ceid.ox.ac.uk) were compared to those of this study, a similar, although not identical, distribution of alleles was found.

MLST STs, ST complexes, and host specificities of STs. Among the 184 strains tested, 117 different STs were found. Strains from cattle and humans had similar moderate degrees of ST diversity: 20 STs among 31 strains (65% diversity) and 53 STs among 83 strains (64% diversity), respectively (Table 4). In contrast, 54 different STs were found among the 59 strains isolated from poultry, resulting in 92% diversity. Of the 117 STs, 101 (86%) were found in only a single type of host, yet 11 of the 20 (55%) different STs found among cattle strains were also found among human strains, whereas only 6 of the 54 (11%) different STs found among chicken strains were identical to those found among strains from humans.

When the criteria similar to those suggested by Feil et al. (13) were used, 11 different clonal complexes were identified. If complexes were defined as described by Dingle at al. (6), in which STs with four identical loci are considered a complex, virtually the same distribution was found; however, two complexes (ST-48 and ST-122) merged under the conditions of Dingle et al. (6). Due to the high degree of ST diversity, only 58% (34 of 59) of the poultry strains could be assigned to 1 of the 11 ST complexes. In contrast, 81% (25 of 31) of the cattle strains and 78% (65 of 83) of the human strains belonged to one of the ST complexes. Half of the strains grouped in the four ST complexes ST-21, ST-45, ST-46, and ST-122 (Table 4).

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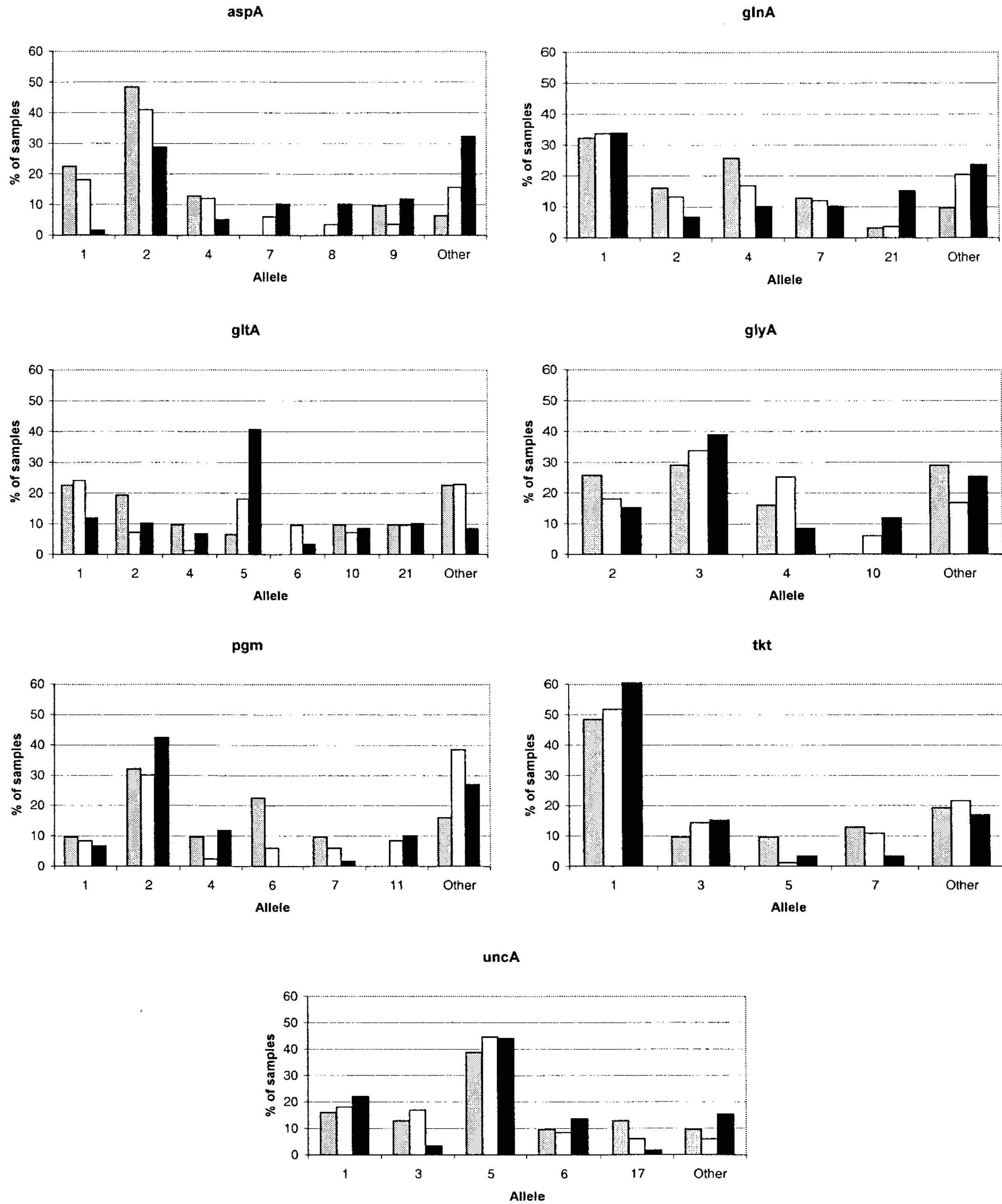

FIG. 1. Frequencies of alleles of the seven genes used for MLST. The frequency is expressed as the percentage of samples that carry a particular allele. Only those alleles that were found at frequencies of 10% or more among the samples are depicted; alleles that were present at lower frequencies are displayed as "other" alleles. Gray bars, alleles found among strains isolated from cattle; white bars, alleles found among strains isolated from humans; black bars, alleles found among strains isolated from poultry.

The most abundant ST, ST-53, was assigned to the ST-21 complex as defined by Dingle et al. (6). In total, 22 STs belonged to this ST-21 lineage, comprising 51 strains (28%). Using a different set of strains, Dingle at al. (6) also identified ST-21 as the most predominant ST complex (29%). The strains of the ST-21 complex appeared to be evenly distributed among the different hosts. Although the number of strains was small, the results suggest that there may be some host-specific ST

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TABLE 4. Distribution of MLST STs and ST lineages among the four host groups

Host	No. of strains	No. of STs	No. of STs not shared with other hosts	No. of STs not present in humans	No. of STs shared by cattle and humans	No. of STs shared by poultry and humans	No. of strains of:										
							ST-21 complex	ST-45 complex	ST-46 complex	ST-122 complex	ST-61 complex	ST 42 complex	ST 48 complex	ST 257 complex	ST 22 complex	ST 44 complex	ST 52 complex
Cattle	31	20	9	9			9	4	1		4	2	2	3			
Human	83	53	38		11	6	27	9	2	5	4	5	4	1	5	2	1
Poultry	59	54	47	48			13	3	7	4			1	3		2	1
Other	11	11	7	8			2	1		1						1	1

complexes. Examples are the ST-61 complex, which was not found among the strains isolated from poultry; the ST-122 complex, which was not found among the strains isolated from cattle; and the ST-22 complex, which was found exclusively among the strains isolated from humans.

Group separation analysis showed that 41% of the cattle strains had MSLT profiles that were most similar to the profiles of other cattle strains (Table 3). Yet, 51% of the cattle strains were more related to human strains, and only 8% of the cattle strains were found to have their closest relatives in the group of poultry isolates. Half of the human strains were most related to other human strains, and of the remaining half, 30% of the human strains were more related to cattle strains and 20% were more related to poultry strains. The MLST profiles of the poultry isolates were most related to each other (47%) and to the human isolates (38%), but only 15% of all poultry strains had profiles that were most related to those of cattle strains. We also used the *Campylobacter* PubMLST database (http://phoenix.ceid.ox.ac.uk) profiles of all strains for which the source was indicated as cattle, human stools, and chickens for group separation analysis. In the Oxford University (PubMLST) data set, 52% of the cattle strains were most related to human strains and 17% were most related to poultry strains. Furthermore, only 6% of the poultry strains had MLST profiles that found their most similar counterpart among the cattle strains.

Typing of *Campylobacter* strains using interspaced short repeat sequences. The sequenced genome of strain NCTC 11186 has been shown to carry repetitive tandem DNA sequences which were designated CRISPRs (18). This region, located at positions 1,455,126 to 1,455,424 of the *C. jejuni* genome (30), comprises five 34-bp direct repeats interspersed with four 31-bp unique spacer sequences (Fig. 2). Analysis of this repeat region was included to determine its value for genotyping of *Campylobacter* strains. Of the 184 strains tested, 19 (10%) did not yield a PCR product, indicating either that the strain did not contain the CRISPR region or that polymorphism in the flanking region prevented proper annealing of the primers. Sequencing of the PCR products revealed that 28 (15%) of the strains contained a CRISPR locus carrying a single repeat unit and thus no spacer sequence (Table 5). The remaining strains, including two of the four *C. coli* strains, carried CRISPR regions in which the number of direct repeats varied between two and eight, with an average number of five repeats. Although the numbers of CRISPR regions in the *Campylobacter* strains were relatively small, they were highly polymorphic in spacer composition. For comparison, the number of spacers in the CRISPR region of *Mycobacterium tuberculosis* varied from 6 to 47, yet the number of different spacers in a set of more than 1,000 strains tested was limited to approximately 50 (35). In contrast, 170 different spacer sequences were detected among the 137 *Campylobacter* strains that carried one or more spacers. Due to the high degree of polymorphism, most of the CTs (78 of 90) were found to be unique to a particular host group. Similarly, 111 of the 170 spacer sequences were found in strains originating from only one of the host groups. Therefore, it was impossible to find an association between the various host groups and the CTs or spacers. The strains could be further differentiated by adding the CT to the MLST allelic profile as an extra locus. This resulted in an expansion of the

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┌ 1,455,126                    AGTGGAGTAATTAGCCCTAGTGGAGTTTCAATCCACTAGGGTTTA
TTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAA TATGGCAGTTTTTAAAAGAGCTTGGCGGTTG
TTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAA TTTCCAAAGTTTCATTAGTTGAATTTAACTG
TTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAA CTACAAGAATGAGGATGATGATATTTTACAG
TTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAA ATGAGTGTGCTAAAAAAAATGGACTTAAATG
TTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAA AGATATTTACCAGATAATGAAAATTTCGGGG
                                   └ 1,455,424
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FIG. 2. DNA sequence of the short tandem repeat region found in *C. jejuni* strain NCTC 11168. The boldface characters indicate the repeat sequence in the CRISPR, the characters in the regular typeface denote the regions flanking the CRISPR, and the characters in italics indicate the spacer sequences. The numbers denote the coordinates of the start and end positions of the repeat region in the genome of NCTC 11168 (30).

number of different MLST STs from 117 to 158. As an example, the 12 strains of ST-53 could be further differentiated into 10 different STs when the CT was added as the eighth locus of the MLST profile.

Congruence between typing methods. Comparison of the clustering results of the various typing methods showed that categorical clustering of MLST profiles and clustering of AFLP patterns with the Pearson product-moment correlation coefficient resulted in 61% congruence. The congruence increased to 86% when MLST clustering was performed based on the DNA sequences of the alleles rather than on the allelic profiles. For these comparisons the strains containing highly divergent MLST sequences obtained by interspecies transfer were omitted. Due to the nature and high degree of diversity of the CTs, the congruence between clustering based on MLST or AFLP analysis and clustering based on CRISPR sequencing could not be calculated. However, there was a clear association between the composition of the CRISPR locus and the ST complex or AFLP group, as the members of several CT groups were found predominantly in certain ST complexes and AFLP clusters (Table 6). Furthermore, all members of the ST-48 complex had a CRISPR with a single direct repeat, and six of the seven members of the ST-42 complex did not yield a PCR product in the CRISPR PCR.

Typing of *Campylobacter* outbreak strains. Among the 184 strains used in this study, 3 strains represented three different outbreaks of *C. jejuni* infections in humans (11, 15, 27). Three additional strains were available from each outbreak, and these samples were also typed by AFLP analysis, MLST, and CRISPR sequencing. As shown in Table 7, the strains belonging to the same outbreak had identical patterns by all three typing methods, yet the strain from each outbreak had its own characteristic AFLP type, MLST type, and CT.

Multiple *Campylobacter* strains in one chicken. Cecum samples taken from three laying hens from one farm were used to determine whether there was heterogeneity among the *Campylobacter* strains found in one animal. The cecum samples were streaked onto agar plates, and four colonies from each sample were used for species identification and typing by AFLP analysis, MLST, and CRISPR sequencing. One of colonies did not yield a proper AFLP pattern and was not used for further analysis. Analysis of the remaining colonies revealed that two of the three chickens carried both *C. jejuni* and *C. coli*. Furthermore, each of the three or four colonies taken from a single chicken were of a different genotype. However, there were three instances in which *C. jejuni* strains with identical typing patterns were found in two different chickens (Table 8).

Intraspecies recombination in *Campylobacter*. To determine whether intraspecies recombination occurs in *C. jejuni*, two computer programs were used to calculate the likelihood of recombination based on MLST data. Application of the homoplasy test (24) to the *C. jejuni* MLST data set resulted in values ranging between 0.16 for *pgm* and 0.31 for *tkt*, indicating frequent intraspecies recombination. The other statistical method used to measure the extent of linkage equilibrium due to recombination was determination of the index of association (I_A) (25). When the MLST data from this study were analyzed, an I_A of 2.21 was found for the complete set, an I_A of 1.45 was found when only the unique STs were used, and an I_A of 1.92 was found when only the founder STs of the clonal complexes were used. These values would indicate significant linkage disequilibrium ($P < 0.001$), which is normally found in clonal populations.

As the results of the homoplasy test and I_A were contradictory, we used an additional approach to determine whether recombination occurs in *C. jejuni*. The members of the ST-21 complex were compared at the sequence level, and for some of these strains, the number of nucleotide changes relative to the sequence of the founder ST are listed in Table 9. ST-376 differs from the founder type ST-21 at only a single base in the *glnA* gene and could represent a descendant of ST-21 with a single mutation. However, the likelihood that the 22-base differences in the *pgm* gene of ST-185 have been introduced by simple mutation of the parental ST-21 *pgm* gene leaving the other six MLST genes unaffected is virtually nil and is most likely the result of recombination. Many other examples support this conclusion. The second finding supporting the conclusion that recombination occurs comes from the observation that certain changes in genes are not linked to a particular founder type. For example, if the single nucleotide change in the *glnA* gene of ST-376 were introduced by mutation of the ST-21 *glnA* sequence, one would expect to find this allele predominantly in allelic profiles related to ST-21. However, *glnA* allele 2 was also found in 13 other allelic profiles that were totally unrelated to ST-21 (Table 9). This indicates that this allele is not a direct descendant of the *glnA* allele 1 of ST-21 but has been introduced in other profiles by intergenic recombination.

The approach described by Feil et al. (13) was used estimate the ratio of mutation to recombination. By this approach it was estimated that it is eight times more likely that an allele is changed by recombination than by mutation. Furthermore, the likelihood that an individual nucleotide of the housekeeping genes studied changes by recombination was calculated to be about 47 times higher than the chance that an individual nu-

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TABLE 5. Characteristics of CRISPRs found in *Campylobacter* strains

Host	No. of strains	No. of strains with amplifiable CRISPRs	No. of strains without a spacer	No. of different CRISPR types	No. of CRISPR types shared with human strains	No. of CRISPR types found in cattle, human, and poultry strains	No. of unique CRISPR types	No. of different spacers	No. of spacers shared with human strains	No. of spacers found in cattle, human, and poultry strains	No. of host-unique spacers
Cattle	31	30	5	21	2		14	52	12		16
Human	83	71	15	44		5	30	100		21	40
Poultry	59	55	5	35	3		23	92	22		50
Other	11	9	3	6			5	23			5
All	184	165	28	90	5	5	72	170			111

cleotide of the housekeeping genes studied changes by a point mutation. In addition, by using the algorithm proposed by Feil et al. (13), the recombinational replacement size was estimated to be about 3.3 kb. However, due to the small number of entries that could be used for later calculation, its validity is somewhat questionable.

Interspecies recombination in *Campylobacter*. The average sequence divergence among the different alleles of the loci used in this study varied from 1.3% for *gltA* (5 nucleotides) to 3.6% for *glyA* (18 nucleotides). However, 14 isolates in the set used in this study carried alleles with extreme sequence divergence ranging between 11.2 and 16.2%, corresponding to 45 and 82 nucleotide changes, respectively (Table 10). Two of the isolates from the CAMPYNET collection, CNET064 and CNET066, have been identified as *C. coli*. Nine *C. jejuni* strains had the same *uncA* allele as these two *C. coli* strains, and two *C. jejuni* strains (strains 185KU and NIV108980251) had an *aspA* allele that was identical to that of CNET066. In addition, in two other strains (strains C2441 and NIV108980251), one of the alleles also displayed extreme sequence divergence. These findings indicate that horizontal transfer from *C. coli* to *C. jejuni* has taken place. In contrast, in strain NIV108980171, which originally was identified as *C. jejuni*, six of the seven loci displayed extreme sequence divergence. It seems likely that this strain has been misidentified as *C. jejuni* and instead represents a *C. coli* strain that has received a *pgm* gene or a gene fragment from *C. jejuni* by interspecies horizontal transfer.

DISCUSSION

In this study we have used three molecular typing methods to characterize a set of 180 *C. jejuni* and 4 *C. coli* strains that originated from humans, cattle, and poultry. By AFLP analysis, MLST, or CRISPR sequencing, about two-thirds of the isolates yielded different genotypes due to extensive heterogeneity of the *Campylobacter* genome. This level of discrimination is comparable to that found in other studies on strain differentiation by AFLP analysis and MSLT. In our study, cluster analysis yielded a similar grouping of strains by either AFLP analysis or MLST. This congruence indicates that these methods, both of which are based on characterization of multiple loci in the genome, are equally suited for typing of *Campylobacter*. The AFLP method is cheaper, faster, and easier to perform than MLST; but interlaboratory comparison by AFLP analysis will be difficult because complex banding patterns are PCR based and therefore prone to variation. MLST is more expensive, but it does result in solid DNA sequence data that are not subject to experimental variation. Therefore, the latter method is particularly suited for the creation of large comparative databases by use of sequences generated by several research groups. Although the discriminative power of CRIPR sequencing was found to be comparable to that of AFLP typing and MLST, about 26% of the *Campylobacter* strains were nontypeable by CRISPR sequencing due to the presence of only a single repeat sequence or the lack of an amplifiable CRISPR locus. Therefore, CRISPR sequencing is not the method of choice for *Campylobacter* strain typing. However, CRISPR sequencing may be useful for subtyping of strains with common AFLP or MLST profiles.

By genogrouping of *Campylobacter* strains by AFLP analysis

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TABLE 6. Relationship between CRISPR type and MLST and AFLP types

CT group	No. of strains	No. of strains of the following ST complex:								No. of strains of the following AFLP type:														
		21	44	45	46	61	52	122	Not in complex	1	4	6	8	9	10	11	12	14	15	17	21	23	25	Other
5	19	17			1				1					5	1				12					1
7	14	8				5	1											6	2		5			1
8	11	1			9				1				9							1				1
1	8	1						6	1											5				3
2	7	1						5	1		4								1					2
10	7			6					1												1	2	3	1
9	6	6												1	4				1					
6	5	4							1						1		3							1
3	4								4			2				2								
4	4								4	3														1
11	4		3						1							4								

or MLST, we were unable to disclose a characteristic association between genogroups and host, consistent with previous attempts by others (6–8). However, a remarkable association emerged when we applied a statistical method called group separation. By this method, it was shown that the AFLP patterns of strains isolated from humans and poultry were most related to the patterns of other human and poultry strains. For virtually none of the strains was the nearest neighbor found among the cattle strains. Remarkably, more than half of the patterns for cattle strains were most related to those for human strains and only one-third of the patterns for cattle strains most closely resembled the patterns for other cattle strains. Similar but somewhat less pronounced results were obtained by using the MLST data. The apparent association between cattle and human strains might suggest that the guts of cattle and humans display similar types of selection for colonization, resulting in infection with strains of similar genotypes. Alternatively, humans may more frequently become infected with *C. jejuni* strains from cattle than strains from poultry. The latter hypothesis does not seem very likely, particularly because the strong relationship found by group separation was not a bidirectional one. The most likely explanation for this observation may be that cattle and humans are infected from a common source but that humans acquire infections from more diverse sources than cattle. The distribution of some of the MLST alleles further supports the idea that the cattle strains are more closely associated with human strains than with poultry strains. *aspA* allele 2 was found only once among the poultry strains, yet it was present in 22 and 18% of the cattle and human strains, respectively. A similar result was found for *pgm* allele 6, which was present in the cattle and human strains but absent from the poultry strains. As discussed later, chickens seem to be infected with multiple *Campylobacter* strains, and therefore, the *C. jejuni* population in poultry is likely to be much more diverse than the *Campylobacter* population that causes disease in humans. Perhaps the latter bacteria constitute a nonpredominant flora in poultry and therefore are underestimated by traditional bacteriological procedures, in which usually only a single or a few colonies are chosen for strain typing.

To determine the extent to which recombination occurs in *Campylobacter* strains, two methods, determination of I_A and the homoplasy test, were used to analyze the MLST data set. For the complete data set I_A was 2.21, which is comparable to the value of 2.01 found by Dingle et al. (6). When only one representative of each lineage was used, I_A dropped to 1.45, which is consistent with the linkage disequilibrium found in clonal populations. However, Dingle et al. (6) found that their data set yielded an I_A of 0.56, which would indicate a weakly clonal population. Suerbaum et al. (32) found an I_A of 0.256, probably as the result of analysis of the whole data set, and also concluded that this was indicative of a limited amount of recombination. Suerbaum et al. (32) also used the homoplasy test and found values ranging from 0.36 to 0.48, which are comparable to the values that we found. The I_A results of this and the other two MLST studies were in disagreement. Furthermore, the I_A and homoplasy test yielded conflicting results

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TABLE 7. Typing of strains belonging to three different outbreaks of infection with *C. jejuni*

Strain	ST	AFLP type	CRISPR spacer order	Country	Outbreak no.	Reference
CNET001	ST-53	2	3-4	Denmark	1	11
CNET002	ST-53	2	3-4	Denmark	1	11
CNET003	ST-53	2	3-4	Denmark	1	11
CNET004	ST-53	2	3-4	Denmark	1	11
CNET005	ST-45	3	19-20-21-22	Scotland	2	15
CNET006	ST-45	3	19-20-21-22	Scotland	2	15
CNET007	ST-45	3	19-20-21-22	Scotland	2	15
CNET008	ST-45	3	19-20-21-22	Scotland	2	15
CNET009	ST-21	1	23-24-25-26-27-2-4	Northern Ireland	3	28
CNET010	ST-21	1	23-24-25-26-27-2-4	Northern Ireland	3	28
CNET011	ST-21	1	23-24-25-26-27-2-4	Northern Ireland	3	28
CNET012	ST-21	1	23-24-25-26-27-2-4	Northern Ireland	3	28

TABLE 8. Typing results for *Campylobacter* colonies from cecal swab samples of three different laying hens from a Dutch hatchery

Chicken no.	Colony no.	Species	MLST allelic profile							AFLP type	CRISPR spacer order
			aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
1	1	*C. jejuni*	2	4	2	4	19	3	6	3	181-156-182-3-2-37
1	2	*C. jejuni*	24	21	2	2	89	59	6	5	Single direct repeat
1	3	*C. coli*	ND[a]	ND	30	ND	ND	ND	ND	1	No PCR product
1	4	*C. jejuni*	2	21	5	3	2	1	5	6	178-156-28-29-30
2	1	*C. jejuni*	24	21	2	2	89	59	6	5	Single direct repeat
2	2	*C. jejuni*	3	17	5	2	10	3	6	4	180-67-68-69
2	3	*C. jejuni*	2	4	2	4	19	3	6	3	181-156-182-3-2-37
3	1	*C. coli*	ND	ND	57	ND	ND	ND	ND	2	191-18
3	2	*C. jejuni*	2	21	5	3	2	1	5	6	178-156-28-29-30
3	3	*C. jejuni*	8	1	17	3	23	1	1	7	192-193-194-11-12-13-14-15
3	4	*C. jejuni*	24	21	2	2	89	59	6	5	Single direct repeat

[a] ND, the sequence was not determined because no PCR product was formed.

in our study. For this reason we compared the sequence data for members of the ST complexes with the sequences of their founder types, and this unambiguously showed that intra- and interspecies recombination in *Campylobacter* is a frequently occurring event, creating a panmictic population of strains.

Campylobacteriosis is a major problem in developed countries, with the incidence of disease in the population being as high as 1% per year. Disclosure of the sources of human infections is crucial to develop control strategies for campylobacteriosis. Many studies have shown that campylobacteriosis is primarily a food-borne disease and that handling and consumption of contaminated poultry meat are the major sources of human infection. In The Netherlands up to 60% of the broiler poultry flocks processed in slaughterhouses are infected with *Campylobacter* species (9). Cattle and pigs also show high infection rates (over 50%). However, at the retail level less than 1% of the beef and pork contain viable *Campylobacter* strains, whereas 36% of the poultry meat is contaminated with cultivable *Campylobacter* strains, indicating that the processing of the meat is important for the survival of the pathogen. In this study we conclusively showed that strains isolated from patients in an outbreak of campylobacteriosis are identical by any of the three methods used. However, the original animal source of infection was unknown for isolates from the three outbreaks investigated. The disclosure of the sources of infection may be extremely difficult because of the enormous reservoir of numerous extremely polymorphic *Campylobacter* strains in animals such as poultry and cattle, as shown in this and previous studies (6–8).

Another factor complicating the tracing of sources of human infection is the observation in this study of the carriage of strains of multiple types among individual chickens. Although mixed infections have been demonstrated by analysis of fecal samples from flocks (17, 33), we are not aware of reports of such mixed infections in individual animals. In this study we analyzed only a few colonies from individual chickens, and the majority of the isolates showed different molecular types. Therefore, it seems likely that a single chicken may harbor a multiplicity of at least four different strains, as disclosed during this study. Assuming that the predominant types in animals are

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TABLE 9. Examples of allelic profiles and their corresponding number of nucleotide changes providing evidence for intraspecies recombination in the housekeeping genes of *C. jejuni*

ST	*aspA*		*glnA*		*gltA*		*glyA*		*pgm*		*tkt*		*uncA*	
	Allele no.	NC[a]	Allele no.	NC	Allele no.	NC	Allele no.	NC	Allele no.	NC	Allele no.	NC	Allele no.	NC
Members of the ST-21 complex														
ST-021	2		1		1		3		2		1		5	
ST-376	2		2	1	1		3		2		1		5	
ST-053	2		1		21	2	3		2		1		5	
ST-185	2		1		1		3		1	22	1		5	
ST-333	2		1		21	2	2	17	2		1		5	
ST-104	2		1		1		3		7	20	1		5	
ST-348	33	55	1		21	2	3		2		1		5	
ST-343	4	8	1		5	1	3		2		1		5	
STs with *glnA* allele 2														
ST-316	9	3	2	1	4	5	62	19	4	6	1		6	1
ST-042	1	5	2	1	3	5	4	15	5	21	9	10	3	2
ST-321	*1*	5	2	1	12	1	4	15	5	21	1		3	2
ST-380	7	1	2	1	6	2	10	1	78	20	37	14	1	2
ST-304	6	3	2	1	42	4	4	15	68	21	58	13	34	3
ST-331	24	4	2	1	5	1	72	16	75	1	5	15	39	2
ST-054	17	8	2	1	9	7	5	17	24	18	2	15	4	3

[a] NC, number of nucleotides changes compared to the sequences of the houskeeping genes of NCTC 11168.

TABLE 10. Allelic profiles and sequence divergence of the housekeeping genes of *Campylobacter* strains with highly divergent sequences

Strain	ST[a]	CRISPR	Host	Country	Yr	% sequence divergence from NCTC 11168							Allele no.						
						aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
185KU	ST-348	31-32	Poultry	The Netherlands	1998	**11.5**[b]	0.0	0.2	0.0	1.2	0.0	0.0	**33**	1	21	3	2	1	5
CNET066	PT-820	No	Pig	The Netherlands	1998	**11.5**	**11.5**	**11.2**	**16.0**	**15.5**	**14.4**	**14.1**	**33**	**38**	**30**	**82**	**99**	**43**	**17**
CNET064	PT-821	No	Poultry	Denmark	?[c]	**11.5**	**11.3**	**11.2**	**16.2**	**15.1**	**14.8**	**14.1**	**33**	**39**	**30**	**79**	**98**	**77**	**17**
NIV108980171	ST-437	No	Human	The Netherlands	1998	**11.5**	**11.3**	**11.2**	**16.2**	3.2	**14.4**	**14.1**	**33**	**39**	**30**	**79**	39	**43**	**17**
CNET033	ST-60	31-32	Human	United Kingdom	?	1.0	2.1	0.7	3.5	0.6	0.2	**14.1**	1	4	2	16	6	3	**17**
302_KO_1252	ST-61	31-32	Cattle	The Netherlands	1998	1.0	2.1	0.7	3.4	0.6	0.2	**14.1**	1	4	2	2	6	3	**17**
315_KO_1297	ST-61	31-32	Cattle	The Netherlands	1998	1.0	2.1	0.7	3.4	0.6	0.2	**14.1**	1	4	2	2	6	3	**17**
CNET073	ST-61	31-32	Cattle	The Netherlands	1992	1.0	2.1	0.7	3.4	0.6	0.2	**14.1**	1	4	2	2	6	3	**17**
GB19	ST-61	32	Human	The Netherlands	1998	1.0	2.1	0.7	3.4	0.6	0.2	**14.1**	1	4	2	2	6	3	**17**
GB16	ST-61	0	Human	Belgium	1997	1.0	2.1	0.7	3.4	0.6	0.2	**14.1**	1	4	2	2	6	3	**17**
NIV108970197	ST-432	31–32	Human	The Netherlands	1997	1.0	2.1	0.7	3.4	0.6	0.0	**14.1**	1	4	2	2	6	1	**17**
325_KO_1343	ST-432	31	Cattle	The Netherlands	1998	1.0	2.1	0.7	3.4	0.6	0.0	**14.1**	1	4	2	2	6	1	**17**
C2441	PT-815	91-92-37	Poultry	The Netherlands	1992	**11.1**	0.2	1.0	3.7	0.0	3.3	0.2	**49**	2	4	62	4	5	6
NIV108980251	ST-382	183	Human	The Netherlands	1998	0.0	2.1	0.2	3.0	3.4	**14.6**	0.0	2	4	1	4	19	**63**	5

[a] The PT numbers represent provisional sequence types.
[b] Boldface indicates highly divergent sequences and alleles.
[c] ?, isolation date unknown.

not necessarily the ones most infectious for humans, traditional bacteriological isolation methods may be inadequate to disclose source animals. Mixed infections may further complicate source tracing, particularly if cross-contamination of meat occurs during processing in the slaughterhouse (29).

A recent study showed that the molecular types of sequential isolates from an episode of human infection are generally identical (31), suggesting that only a single strain of the putative mixture of strains in contaminated meat is able to cause infection in humans. The enormous variations in types and the carriage of multiple types in animals may even contribute to the apparent sporadic nature of most *Campylobacter* infections.

For a better understanding of the epidemiology of campylobacteriosis, more quantitative data on the carriage of multiple types of strains among animals is needed. Furthermore, although we now know that recombination is the major driving force for strain variation, the speed with which this variation occurs in nature is unclear. Therefore, future studies should focus on determination of the pace of molecular variation in the natural habitat of *C. jejuni.*

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REFERENCES

1. **Blaser, M. J.** 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. J. Infect. Dis. **176**(Suppl. 2)**:**S103–S105.
2. **Central Public Health Laboratory Service.** 2001. Common gastrointestinal infections, England and Wales. Commun. Dis. Rep. Wkly. **11:**7.
3. **Desai, M., J. M. J. Logan, J. A. Frost, and J. Stanley.** 2001. Genome sequence-based fluorescent amplified fragment length polymorphism of *Campylobacter jejuni*, its relationship to serotyping, and its implications for epidemiological analysis. J. Clin. Microbiol. **39:**3823–3829.
4. **De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. Van Leeuwen, A. I. M. Bartelds, and Y. Van Duynhoven.** 2001. Gastroenteritis in sentinel general practices, The Netherlands. Emerg. Infect. Dis. **7:**82–91.
5. **De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, W. J. B. Wannet, J. Vinje, F. Van Leusden, A. I. M. Bartelds, and Y. Van Duynhoven.** 2001. Sensor, a population-based cohort study on gastroenteritis in The Netherlands: incidence and etiology. Am. J. Epidemiol. **154:**666–674.
6. **Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14–23.
7. **Duim, B., C. W. Ang, A. Van Belkum, A. Rigter, N. W. J. Van Leeuwen, H. P. Endtz, and J. A. Wagenaar.** 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barré or Miller-Fisher syndrome. Appl. Environ. Microbiol. **66:**3917–3923.
8. **Duim, B., T. M. Wassenaar, A. Rigter, and J. Wagenaar.** 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. **65:**2369–2375.
9. **Dutch Inspectorate for Health Protection and Public Health.** 2001. Zoonoses and zoonotic agents in humans, food, animals and feed in The Netherlands. Dutch Inspectorate for Health Protection and Public Health, The Hague, The Netherlands.
10. **Endtz, H. P., C. W. Ang, N. Van Den Braak, B. Duim, A. Rigter, L. J. Price, D. L. Woodward, F. G. Rodgers, W. M. Johnson, J. A. Wagenaar, B. C. Jacobs, H. A. Verbrugh, and A. Van Belkum.** 2000. Molecular characterization of *Campylobacter jejuni* from patients with Guillain-Barré and Miller-Fisher syndromes. J. Clin. Microbiol. **38:**2297–2301.
11. **Engberg, J., P. Gerner-Smidt, F. Scheutz, E. Moller Nielsen, S. L. On, and K. Molbak.** 1998. Water-borne *Campylobacter jejuni* infection in a Danish town: a 6-week continuous source outbreak. Clin. Microbiol. Infect. **4:**648–656.
12. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology United Kingdom **144**(Pt 11)**:**3049–3060.
13. **Feil, E. J., J. M. Smith, M. C. Enright, and B. G. Spratt.** 2000. Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. Genetics **154:**1439–1450.
14. **Hanninen, M. L., P. Perko Makela, H. Rautelin, B. Duim, and J. A. Wagenaar.** 2001. Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electrophoresis genotypes studied by amplified fragment length polymorphism analysis, ribotyping, and serotyping. Appl. Environ. Microbiol. **67:**1581–1586.
15. **Harrington, C. S., F. M. Thomson Carter, and P. E. Carter.** 1999. Molecular epidemiological investigation of an outbreak of *Campylobacter jejuni* identifies a dominant clonal line within Scottish serotype HS55 populations. Epidemiol. Infect. **122:**367–375.
16. **Hughes, R. A., and A. C. Keat.** 1994. Reiter's syndrome and reactive arthritis: a current view. Semin. Arthritis Rheum. **24:**190–210.
17. **Jacobs-Reitsma, W. F., A. W. Van de Giessen, N. M. Bolder, and R. W. A. W. Mulder.** 1995. Epidemiology of *Campylobacter* spp. at two Dutch broiler farms. Epidemiol. Infect. **114:**413–421.
18. **Jansen, R., J. D. A. Van Embden, W. Gaastra, and L. M. Schouls.** 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. **43:**1565–1576.
19. **Jobson, J. D.** 1992. Applied multivariate data analysis, vol. I and II. Springer-Verlag, New York, N.Y.
20. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *Myco-*

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bacterium tuberculosis for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
21. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology United Kingdom **143**(Pt 1)**:**5–21.
22. **Kokotovic, B., and S. L. W. On.** 1999. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. FEMS Microbiol. Lett. **173:**77–84.
23. **Lindstedt, B. A., E. Heir, T. Vardund, K. K. Melby, and G. Kapperud.** 2000. Comparative fingerprinting analysis of *Campylobacter jejuni* subsp. *jejuni* strains by amplified-fragment length polymorphism genotyping. J. Clin. Microbiol. **38:**3379–3387.
24. **Maynard-Smith, J., and N. H. Smith.** 1998. Detecting recombination from gene trees. Mol. Biol. Evol. **15:**590–599.
25. **Maynard-Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90:**4384–4388.
26. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
27. **Moore, J. E., T. Stanley, R. Smithson, H. O'Malley, and P. G. Murphy.** 2000. Outbreak of *Campylobacter* food-poisoning in Northern Ireland. Clin. Microbiol. Infect. **6:**399–400.
28. **Nachamkin, I., B. M. Allos, and T. Ho.** 1998. *Campylobacter* species and Guillain-Barré syndrome. Clin. Microbiol. Rev. **11:**555–567.
29. **Newell, D. G., J. E. Shreeve, M. Toszeghy, G. Domingue, S. Bull, T. Humphrey, and G. Mead.** 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. Appl. Environ. Microbiol. **67:**2636–2640.
30. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. M. Van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665–668.
31. **Steinbrueckner, B., F. Ruberg, and M. Kist.** 2001. Bacterial genetic fingerprint: a reliable factor in the study of the epidemiology of human *Campylobacter* enteritis? J. Clin. Microbiol. **39:**4155–4159.
32. **Suerbaum, S., M. Lohrengel, A. Sonnevend, F. Ruberg, and M. Kist.** 2001. Allelic diversity and recombination in *Campylobacter jejuni*. J. Bacteriol. **183:**2553–2559.
33. **Thomas, L. M., K. A. Long, R. T. Good, M. Panaccio, and P. R. Widders.** 1997. Genotypic diversity among *Campylobacter jejuni* isolates in a commercial broiler flock. Appl. Environ. Microbiol. **63:**1874–1877.
34. **Tompkins, D. S., M. J. Hudson, H. R. Smith, R. P. Eglin, J. G. Wheeler, M. M. Brett, R. J. Owen, J. S. Brazier, P. Cumberland, V. King, and P. E. Cook.** 1999. A study of infectious intestinal disease in England: microbiological findings in cases and controls. Commun. Dis. Public Health **2:**108–113.
35. **Van Embden, J. D. A., T. Van Gorkom, K. Kremer, R. Jansen, B. A. M. Van Der Zeijst, and L. M. Schouls.** 2000. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. J. Bacteriol. **182:**2393–2401.
36. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, et al.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23:**4407–4414.
37. **Wassenaar, T. M., and D. G. Newell.** 2000. Genotyping of *Campylobacter* spp. Appl. Environ. Microbiol. **66:**1–9.

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