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Genomic Diversity of *Campylobacter coli* and *Campylobacter jejuni* Isolates Recovered from Free-Range Broiler Farms and Comparison with Isolates of Various Origins†

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In many industrialized countries, the incidence of campylobacteriosis exceeds that of salmonellosis. *Campylobacter* bacteria are transmitted to humans mainly in food, especially poultry meat products. Total prevention of *Campylobacter* colonization in broiler flocks is the best way to reduce (or eliminate) the contamination of poultry products. The aim of this study was to establish the sources and routes of contamination of broilers at the farm level. Molecular typing methods (DNA macrorestriction pulsed-field gel electrophoresis and analysis of gene polymorphism by PCR-restriction fragment length polymorphism) were used to characterize isolates collected from seven broiler farms. The relative genomic diversity of *Campylobacter coli* and *Campylobacter jejuni* was determined. Analysis of the similarity among 116 defined genotypes was used to determine clusters within the two species. Furthermore, evidence of recombination suggested that there were genomic rearrangements within the *Campylobacter* populations. Recovery of related clusters from different broiler farms showed that some *Campylobacter* strains might be specifically adapted to poultry. Analysis of the *Campylobacter* cluster distribution on three broiler farms showed that soil in the area around the poultry house was a potential source of *Campylobacter* contamination. The broilers were infected by *Campylobacter* spp. between days 15 and 36 during rearing, and the type of contamination changed during the rearing period. A study of the effect of sanitary barriers showed that the chickens stayed *Campylobacter* spp. free until they had access to the open area. They were then rapidly colonized by the *Campylobacter* strains isolated from the soil.

Thermophilic *Campylobacter* species (particularly *Campylobacter jejuni* and *Campylobacter coli*) have been recognized as major causes of acute diarrheal disease in humans (43) for the last 20 years. The incidence of *Campylobacter* infection is higher than that of salmonellosis in many western countries (49). *Campylobacter* rarely causes death or spectacular outbreaks of food poisoning (31), so these organisms do not trigger the same degree of concern as *Escherichia coli* O157:H7 or *Salmonella*. Nevertheless, *C. jejuni* is one of the most common causes of bacterial enteritis in humans (13, 26) and may lead to serious complications, such as Guillain Barré syndrome (51) or mucosa-associated lymphoid tissue (23). A recent retrospective Danish study indicated that the risk of death was significantly increased after infection with *Campylobacter*, especially in patients older than 55 years (20). The main source of *Campylobacter* infections highlighted in epidemiological studies is consumption of contaminated food, particularly raw or insufficiently cooked poultry products (11, 15, 18, 34).

The following factors contribute to the high correlation between poultry products and human infection: (i) chicken guts, particularly ceca, can be colonized at very high levels (about 10^9 organisms per g of cecal contents [5]) without symptoms; (ii) usually the entire flock is colonized once an infection becomes established in a poultry house (39), and thus, most flocks are contaminated on the day of slaughter (9, 22); and (iii) the *Campylobacter* spp. on the carcasses originate mainly from the guts of live birds, as shown by various studies in abattoirs (33, 44). Cross-contamination between different batches of broilers at the slaughterhouse is almost impossible to prevent due to the current slaughtering processes (38) and the high level of contamination of broilers. Thus, by reducing *Campylobacter* infection in broiler flocks, it should be possible to limit human campylobacteriosis (22). Total prevention of *Campylobacter* colonization of broilers at the farm level is the best way to prevent contamination of poultry products.

Despite various epidemiological studies, the origin of *Campylobacter* and its route of colonization in poultry are still incompletely understood. Some authors (2, 4, 22) reported that a poultry flock could be infected by one or a limited number of *Campylobacter* strains characterized by one or two typing methods. The prevalence of such strains could be explained by their greater ability to survive in a hostile environment and their better adaptation to colonization of the poultry gut. In these studies, the strains were defined by characterization of the *Campylobacter* isolates by biotyping and serotyping, which are not particularly discriminating.

Molecular methods, such as DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) or PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of different loci, are highly discriminatory (36). Several studies have demonstrated the ability of PFGE to discriminate subtypes

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within serotypes and to type strains that are untypeable with antisera (14, 41). In 1991, Yan et al. (50) demonstrated that DNA macrorestriction with the SmaI enzyme could be used to distinguish the two major species of *Campylobacter* and to define genotypes within these species. The discriminatory power of PFGE typing can be increased if two enzymes, e.g., SmaI and KpnI (14) or SacII (17), are used in combination. Recently, the recommendation (32) that a second enzyme should be used to determine relatedness between isolates has been emphasized (24).

In this study the role of biosecurity measures against *Campylobacter* was evaluated in epidemiological investigations of free-range broiler flocks reared for a minimum of 81 days. The poultry house density was lower than the density in conventional broiler flocks (11 chickens per m^2), and, in addition, access to a free-range area (1 ha) was provided after 6 weeks. The facility had to be vacant for 3 weeks for reasons of sanitation. The purpose of this study was to establish the routes of *Campylobacter* infection of chickens in this kind of rearing system by determining the genomic diversity of thermophilic *Campylobacter* strains on seven free-range poultry farms using macrorestriction combined with PFGE and analysis of the restriction fragment length polymorphism (RFLP) of different genomic regions amplified by PCR. The biodiversity of *Campylobacter* strains isolated from free-range poultry production systems was compared with the biodiversity of strains having various origins.

MATERIALS AND METHODS

Strains. The origins and dates of isolation of the *C. coli* and *C. jejuni* strains obtained from a laboratory collection are shown in Table 1.

Farms. This study was conducted from 1996 to 1999 and involved seven French broiler farms, designated farms A to G, belonging to different poultry companies. Only one of the separate broiler houses (houses 1 to 3) on each farm was chosen for the epidemiological study. The surface area of each poultry house was 400 m^2, with an open area of 1 ha. A maximum of 4,600 chickens were reared in each building. The all-in all-out system was used on all farms, which meant

TABLE 1. Characterization by PFGE and PCR-RFLP of the different loci of the strains with different origins belonging to the laboratory collection

Species	Strain	Animal	Year	Source	Geographic origin	PFGE profile		PCR-RFLP profile			Cluster-genotype[b]
						SmaI	KpnI	*hipO*	*flaA*	*pflA/gyrA* or rRNA gene[a]	
C. jejuni	CWH260199	Human	1999	Hospital	Western France	S78	K78	h25	fl10	m5	J35
	BOF	Human	1994	Hospital	Western France	ND[c]	K79	h6	fl6	m8	J36
	UA580	Human	1983	D. E. Taylor	Canada	S79	K80	h12	fl9	m12	J37
	ATCC 33560	Bovine	1991	Pasteur Institute	France	S80	ND	h26	fl22	m18	J38
	3J01	Poultry	1994	AFSSA[d]	Brittany, France	S81	K81	h6	fl9A	m15	J39
	3J4.5	Poultry	1994	AFSSA	Brittany, France	S82	K82	h19	fl17	m13	15-J40
	3J32.30	Poultry	1994	AFSSA	Brittany, France	S83	K83	h22	fl1	m6	J41
	A800	Poultry	1996	Aerial	Eastern France	S84	K84	h1	fl1	m1	1-J42
	A805	Poultry	1996	Aerial	Eastern France	S7	K5	h1	fl1	m1	1-J1
	A728	Poultry	1996	Aerial	Eastern France	S85	K85	h20	fl18	m3	5-J43
	A922	Poultry	1996	Aerial	Eastern France	S86	K86	h19	fl17	m3	15-J44
	A940	Poultry	1996	Aerial	Eastern France	S87	K87	h21	fl10	m3	J45
	A943	Poultry	1996	Aerial	Eastern France	S88	K88	h2	fl21	m17	J46
	A1020	Poultry	1996	Aerial	Eastern France	S89	K89	h24	fl20	m16	J47
	A1306	Poultry	1996	Aerial	Eastern France	S90	K90	h23	fl19	m4	J48
C. coli	CWH020399	Human	1999	Hospital	Western France	S62	K60		fl31	rc7	C35
	UA417	Human	1983	D. E. Taylor	Canada	S63	K61		fl23	rc4	C36
	A9821	Pork	1996	Aerial	Eastern France	S64	K62		fl30	rc9	14-C37
	A992	Pork	1996	Aerial	Eastern France	S64	K63		fl30	rc9	14-C38
	A1538	Pork	1996	Aerial	Eastern France	S65	K64		fl30	rc9	C39
	A1581	Pork	1996	Aerial	Eastern France	S66	K65		fl9A	rc2	C40
	A1642	Pork	1996	Aerial	Eastern France	S67	K66		fl9A	rc2	C41
	A1635	Pork	1996	Aerial	Eastern France	S68	K67		fl9A	rc2	C42
	A1649	Pork	1996	Aerial	Eastern France	S69	K68		fl10	rc2	15-C43
	A1578	Pork	1996	Aerial	Eastern France	S70	K69		fl10	rc2	15-C44
	A1575	Pork	1996	Aerial	Eastern France	S71	K70		fl32	rc2	15-C45
	A1552	Pork	1996	Aerial	Eastern France	S70	K71		fl33	rc2	15-C46
	ATCC 33559	Pork	1980	Pasteur Institute	France	S72	K72		fl9A	rc8	C47
	MJ4.3	Poultry	1994	AFSSA	Brittany, France	S73	K73		NC[e]	NC	C48
	A849	Poultry	1996	Aerial	Eastern France	S74	K74		fl25	rc2	C49
	A879	Poultry	1996	Aerial	Eastern France	S75	K75		fl4	rc2	16-C50
	A846	Poultry	1996	Aerial	Eastern France	S76	K76		fl4	rc2	16-C51
	A963	Poultry	1996	Aerial	Eastern France	S77	K77		Fl34	rc10	C52

[a] *pflA/gyrA* PCR-RFLP profile for *C. jejuni* strains and rRNA gene profile for *C. coli* strains.

[b] The number before the hyphen indicates the cluster to which the genotype belongs (e.g., in 15-J40 15 indicates cluster 15-J and in 14-C37 14 indicates cluster 14-C), and the number after the hyphen indicates the genotype. When there is no hyphen, the number indicates the genotype, which may belong to any cluster.

[c] ND, not digested. The isolate was refractory to digestion by the restriction enzyme.

[d] AFSSA, Agence Française de Sécurité Sanitaire des Aliments.

[e] NC, isolate was not characterized by the typing method used.

that the broiler houses were depopulated, left empty for at least 3 weeks, and then restocked simultaneously. The broiler houses were cleaned and disinfected within 3 days of depopulation. The total rearing period was 81 days. When the chickens were 6 weeks old, they had access to an open space during the day. Straw was used for litter, and the broilers were provided with chlorinated tap water and were fed with a minimum of 75% cereals.

Sampling. The buildings were investigated by sampling outside each house (near the entrance doors and soil in the closed pen) and inside either by taking soil samples or swabbing the walls and floors. When there were bovine feces on the ground of the open space or near the buildings, samples were taken. Animals were sampled by cloacal pressure (10 pools of 10 droppings). Feed and drinking water were also sampled. A set of samples was obtained almost every week on farms A, B, and C from just before arrival of the chicks until their departure for the slaughterhouse. Only three sets of samples were taken on the four other farms: on the day of arrival, on the day just before the chickens were freed (6 weeks), and on the day of their departure for the slaughterhouse.

Isolation of *Campylobacter* spp. Isolation of *Campylobacter* spp. was carried out on the day of sample collection.

Swabs were added to 150 ml of *Campylobacter* selective enrichment Preston broth. This medium consisted of NO_2 nutritive broth (Oxoid, Dardilly, France), 5% lysed horse blood (AES Laboratory, Combourg, France), and Preston antibiotic supplements (AES Laboratory). Ten grams of soil, litter, food, or feces was added to 90 ml of Preston broth. Five hundred milliliters of drinking water was filtered through a 0.2-μm Millipore filter. The filter was then transferred to 20 ml of Preston broth. All samples were plated onto two *Campylobacter* selective agar media: Virion medium made with Mueller-Hinton agar base (Merck, Coger, Paris, France) and Bacto agar (Difco, Fisher Scientific, Elancourt, France) with 5% (vol/vol) defibrinated horse blood (AES Laboratory) and antibiotic supplements (cefoperazone, rifampin, colistin, and amphotericin; Sigma Aldrich Chimie, La Verpillière, France) and Karmali medium (AES Laboratory) with selective supplement CV (AES Laboratory). The plates were incubated at 42°C for 72 h under a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2).

After the enrichment step, which consisted of incubation at 42°C for 24 h in a microaerobic atmosphere, the samples were streaked onto the Virion and Karmali selective agar media. The plates were incubated microaerobically at 42°C for 48 h. Three suspected colonies of *Campylobacter* spp. were isolated from each plate, placed onto blood agar (Mueller-Hinton agar base [Merck] supplemented with 5% defibrinated horse blood [AES Laboratory]), and incubated under similar conditions. Characteristic colonies were examined with a phase-contrast microscope for typical spiral-shaped cells and rapid motility. Approximately four isolates were collected from each positive sample, which resulted in collection of 2,880 isolates, which were frozen at −80°C in glycerol peptone broth before nearly one-half of them were genotyped. One or two isolates per sample were typed by molecular typing methods.

DNA preparation for PFGE and PCR-RFLP analysis. The bacterial lawn obtained from an overnight culture on blood agar was suspended by adding 2.5 ml of a Tris-NaCl solution (0.01 mol liter^{-1} Tris-HCl, 1 mol liter^{-1} NaCl, pH 7.6). Cells were harvested and washed twice with 2 ml of the Tris-NaCl solution.

For PCR, the pellet was resuspended in 200 μl of the same solution, and a rapid DNA extraction technique (Kit Fisher, Osi, France) was performed according to the manufacturer's instructions. The DNA was precipitated, pelleted, dried, and resuspended in 100 μl of TE buffer (0.01 mol liter^{-1} Tris-HCl, 0.001 mol liter^{-1} EDTA, pH 7.6) and then stored at 4°C. Dilutions were prepared from the resulting stock solutions and adjusted with TE buffer to an optical density at 260 nm of 0.1.

For PFGE, agarose plugs were prepared as described by Ragimbeau et al. (36). Each plug was then cut into four thin slices and stored in TE buffer at 4°C.

Restriction endonuclease digestion and PFGE conditions. One-quarter of a plug was used for restriction endonuclease digestion in each separate reaction using 40 U of either SmaI or KpnI (Boehringer) under the conditions recommended by the manufacturer in a 100-μl (final volume) mixture with incubation for 5 h at the appropriate temperature. PFGE was done using the CHEF-DRIII system (Bio-Rad Laboratories, United States). An agarose gel (1%) prepared in 0.5× TBE (45 mmol liter^{-1} Tris, 45 mmol liter^{-1} boric acid, mmol liter^{-1} EDTA) was subjected to electrophoresis for 23 h at 220 V and 14°C with ramped pulse times from 2 to 25 s for KpnI. Fragments generated by SmaI digestion were separated by electrophoresis for 24 h at 200 V and 14°C with ramped pulse times from 15 to 45 s for the first 22 h and from 2 to 8 s for the last 2 h.

***flaA* PCR-RFLP conditions.** PCR was performed using the RAA19 and pg 50 primers (1) and generated a 1,448-bp amplified product. The following reagents were used for this PCR (50-μl mixture): 1× PCR buffer II (Perkin-Elmer), 1.5 mmol liter^{-1} $MgCl_2$, 0.5 μmol liter^{-1} of each primer, 200 μmol liter^{-1} of deoxynucleoside triphosphates (Advantage ultrapure PCR deoxynucleoside mixure; Clontech, Ozyme, France), and 0.2 U liter^{-1} of AmpliTaq polymerase (Perkin-Elmer). The PCR was conducted with a Gene AMP 9600 system (Perkin-Elmer Instruments, Norwalk, CT) under the following conditions: 94°C for 1 min and then 30 cycles of 94°C for 15 s, 45°C for 30 s, and a 2-min ramp to 72°C for 30 s. The reaction was completed by a final extension of 10 min at 72°C.

Following PCR amplification, 5 μl of the reaction mixture was first checked for the presence of the amplicon on a 1% agarose gel (agarose standard; Eurobio). To study polymorphism of the *flaA* gene, 7.5 μl of PCR product was digested a 15-μl (total volume) mixture with 5 U of restriction enzyme DdeI (New England Biolabs, Ozyme, France). Digestion was performed with buffer 3 (New England Biolabs, Ozyme, France) at 37°C for 3 h according to the manufacturer's instructions.

***rib* rRNA gene PCR-RFLP conditions.** PCR was performed using the Rib5 (48) and Therm2 (12) primers, and this generated a 3,925-bp amplified product. The PCR (50-μl mixture) was carried out using an XL PCR kit (Perkin-Elmer) with 1× PCR buffer II, 1 mmol liter^{-1} magnesium acetate, 0.4 μmol liter^{-1} of each primer, 200 μmol liter^{-1} of deoxynucleoside triphosphates (Advantage ultrapure PCR deoxynucleoside mixture; Clontech, Ozyme, France), and 2 U liter^{-1} of rTth DNA polymerase XL (Perkin-Elmer). The PCR was conducted using the hot start technique and the following conditions: 95°C for 1 min and then 16 cycles of 94°C for 15 s, 52°C for 30 s, and a 4-min ramp to 68°C for 10 s, followed by 16 other cycles with a 5-min ramp to 68°C. The reaction was completed by a final extension of 10 min at 72°C.

Following PCR amplification, 5 μl of the reaction mixture was first checked for the presence of the amplicon on a 1% agarose gel (agarose standard; Eurobio). To study polymorphism of the *rib* rRNA gene, 7.5 μl of PCR product was digested in a 15-μl (total volume) mixture. For *C. jejuni*, the *rib* rRNA gene was digested with 8 U of AluI in buffer 1 (New England Biolabs, Ozyme, France) at 37°C for 3 h. For *C. coli*, the *rib* rRNA gene was digested with 10 U of HhaI and 5 U of BsiHKAI in the same tube using buffer 4 (New England Biolabs, Ozyme, France) for 2 h at 37°C and for 1 h at 65°C.

***hipO* PCR-RFLP conditions.** PCR was performed using the Hipu1 and Hipl3 primers (designed in our laboratory), and this generated various 2,800 to 5,000-bp amplified products. The PCR was carried out with the same reagents and conditions that were used for *rib* rRNA gene amplification; the cycling conditions were also the same except for the use of a ramp to 68°C of 4.5 min and 5.5 min.

Following PCR amplification, 5 μl of the reaction mixture was first checked for the presence of the amplicon on a 1% agarose gel (agarose standard; Eurobio). To study polymorphism of the *hip* gene, 8 μl of PCR product was digested in a 15-μl (total volume) mixture. A set of three enzymes (5 U of RsaI, 10 U of HhaI, and 6 U of MnlI) was used with buffer 2 at 37°C for 3 h.

***pflA*/*gyrA* PCR-RFLP conditions.** PCR was performed using the set of primers and the cycling conditions described by Ragimbeau et al. (36). Four enzymes were used (10 U of HhaI, 20 U of HindIII, 5 U of HinfI, and 5 U of DdeI) at the same time with the buffer, temperature, and time conditions described by Ragimbeau et al. (36).

Electrophoresis conditions. For all PCR-RFLP analyses, the digests were analyzed by submarine gel electrophoresis. A 2.5% agarose gel (agarose standard; Eurobio) was used with 1× TBE (89 mmol liter^{-1} Tris, 89 mmol liter^{-1} boric acid, 2 mmol liter^{-1} EDTA, pH 8.3). Electrophoresis was performed at 3 V cm^{-1} for 4 h.

Analysis of the patterns. The agarose gels were stained with ethidium bromide, and the images were captured using UV illumination with a video system (Gel DOC 1000 system; Bio-Rad). The electrophoretic patterns were compared by Molecular Analyst software fingerprinting (Bio-Rad). Similarities between the profiles, based on band positions, were derived by using the Dice correlation coefficient with a maximum position tolerance of 1%. Dendrograms were constructed to reflect the similarities between the strains in the matrix. Strains were clustered by the unweighted pair group method using the arithmetic mean (40).

The discriminatory power of the typing methods was calculated by using Simpson's index (D) (21), determined as follows:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} n_j(n_j - 1)$$

where N is the number of isolates tested that are not related, S is the number of different genotypes, and n_j is the number of isolates belonging to type j.

RESULTS

A total of 2,880 *Campylobacter* isolates were recovered during the epidemiological investigations of the seven poultry farms, and 1,225 isolates were identified; 42.6% of the isolates (522 isolates) belonged to *C. coli* (Table 2), and 57.4% (703 isolates) belonged to *C. jejuni* (Table 3).

PFGE analysis. Digestion of the genomic DNAs of the 1,225 *Campylobacter* isolates and the 33 collection strains using restriction enzyme SmaI, followed by PFGE analysis (SmaI-PFGE), yielded 90 profiles, which were designated S1 to S90 (Tables 1, 2, and 3; see Fig. SA1 in the supplemental material). Seven *C. jejuni* isolates recovered from poultry farm B and *C. jejuni* strain BOF (Tables 1 and 2) were refractory to SmaI. Each SmaI profile contained 4 to 14 bands that ranged in size from approximately 45 kbp to 545 kbp (see Fig. SA1 in the supplemental material). The genetic similarities between patterns were analyzed using the Dice coefficient and the unweighted pair group method using the arithmetic mean for cluster analysis, and a dendrogram was constructed (see Fig. SA1 in the supplemental material). Two major groups could be distinguished in this dendrogram; the first consisted of the 50 patterns of the *C. coli* isolates, and the second consisted of the 40 patterns corresponding to the *C. jejuni* isolates

TABLE 2. Characterization by PFGE and PCR-RFLP of different loci of the *C. coli* isolates collected on poultry farms

Farm	Year	Total no. of typed isolates	PFGE			PCR-RFLP			Cluster-genotype[a]
			SmaI	KpnI	No. of isolates	*flaA*	rRNA gene	No. of isolates	
A	1996	134	S1	K1	7	fl23	rc1	7	1-C1
			S2	K1	1	fl23	rc1	1	1-C1A
			S3	K1	1	fl23	rc2	1	1-C2
			S4	K2	1	fl24	rc2	1	2-C3
			S5	K3	119	fl25	rc2	56	3-C4
			S6	K4	5	fl25	rc2	1	3-C5
B	1997	224	S11	K10	124	fl23	rc2	33	C6
			S12	K11	3	fl25	rc2	3	4-C7
			S13	K12	1	fl25	rc2	1	4-C8
			S28	K18	1	fl25	rc2	1	5-C9
			S14	K13	7	fl25	rc2	4	5-C10
			S15	K14	57	fl25	rc2	36	5-C11
			S14	K18	2	fl25	rc2	2	5-C15
			S16	K15	18	fl23	rc3	14	6-C12
						fl24	rc3	2	6-C12A
			S17	K16	8	fl24	rc2	5	7-C13
			S18	K17	3	fl26	rc2	3	8-C14
C	1998	20	S29	K29	5	fl24	rc2	5	7-C16
			S31	K31	4	fl28	rc2	4	7-C18
			S30	K30	7	fl23	rc2	7	9-C17
			S32	K32	4	fl27	rc2	4	9-C19
D	1998	16	S38	K41	5	fl23	rc4	1	C20
			S39	K42	11	fl23	rc5	2	C21
E	1999	46	S40	ND[b]	1	fl29	rc3	1	C22
			S41	K43	17	fl25	rc2	5	2-C23
			S42	K44	20	fl23	rc3	5	10-C24
			S45	K47	4	fl23	rc3	2	10-C27
			S43	K45	2	fl28	rc2	2	11-C25
			S44	K46	1	fl28	rc2	1	11-C26
			S46	K48	1	fl23	rc6	1	C28
F	1999	37	S47	K49	11	fl28	rc2	3	12-C29
			S47	ND	1	fl28	rc2	1	12-C29A
			S51	ND	2	fl28	rc2	2	12-C33
			S49	ND	1	fl28	rc2	1	C31
			S50	K50	2	fl25	rc2	2	3-C32
			S18	K17	2	fl24	rc2	2	8-C14A
			S48	ND	14	fl23	rc2	6	13-C30
			S40	ND	4	fl23	rc2	4	13-C22A
G	1999	45	S40	ND	11	fl23	rc2	11	13-C22A
			S18	K17	9	fl24	rc2	9	8-C14A
			S52	ND	25	fl23	rc2	4	C34

[a] See Table 1, footnote *b*.
[b] ND, not digested. The isolate was refractory to digestion by the restriction enzyme.

TABLE 3. Characterization by PFGE and PCR-RFLP of different loci of the *C. jejuni* isolates collected on the poultry farms

Farm	Year	Total no. of typed isolates	PFGE			PCR-RFLP				Cluster-genotype[a]
			SmaI	KpnI	No. of isolates	*hipO*	*flaA*	*pflA/gyrA*	No. of isolates	
A	1996	67	S7	K5	17	h1	fl1	m1	14	1-J1
			S8	K6	13	h2	fl2	m2	11	J2
			S9	K7	31	h3	fl3	m3	20	J3
			S7	K8	4	h1	fl1	m1	2	1-J4
			S10	K9	2	h4	fl4	m4	2	J5
B	1997	358	S19	K19	174	h4	fl4	m4	50	2-J6
			S19	K20	26	h4	fl4	m4	3	2-J7
			ND[b]	K21	7	h6	fl6	m8	6	3-J8
						h6	fl4	m8	1	3-J8A
			S20	K22	101	h5	fl7	m5	48	4-J9
						h5	fl8	m5	14	4-J9A
						h5	fl6	m5	1	4-J9B
			S21	K23	1	h1	fl10	m7	1	5-J10
			S22	K24	12	h9	fl11	m3	3	J11
			S23	K25	21	h7	fl9	m9	16	6-J12
						h7	fl7	m9	1	6-J12A
			S24	K26	5	h1	fl1	m6	5	7-J13
			S24	K27	1	h1	fl1	m6	1	7-J14
			S25	K28	6	h8	fl1	m10	3	8-J15
						h8	fl9	m10	2	8-J15A
			S26	ND	3	h9	fl10	m11	2	9-J16
			S27	ND	1	h8	fl1	m10	1	9-J17
C	1998	225	S33	K33	1	h10	fl12	m13	1	J18
			S34	K34	154	h11	fl9	m10	73	10-J19
						h12	fl9	m12	4	10-J19A
			S35	K35	26	h12	fl9A	m12	9	11-J20
						h11	fl9	m10	5	11-J20A
						h13	fl7	m7	2	11-J20B
			S36	K36	23	h13	fl7	m7	20	12-J21
						h11	fl9	m10	1	12-J21A
			S37	K37	8	h14	fl9A	m6	8	13-J22
			S37	K38	3	h14	fl9A	m6	3	13-J23
			S37	K39	7	h14	fl9A	m6	5	13-J24
						h12	fl9A	m10	1	13-J24A
						h13	fl7	m7	1	13-J24B
			S37	K40	2	h14	fl9A	m6	2	13-J25
			S9	K7	1	h3	fl3	m3	1	J3
D	1998	19	S53	K51	6	h15	fl13	m4	1	J26
			S54	K52	5	h4	fl4	m1	2	J27
			S55	K53	1	h1	fl1	m1	1	1-J28
			S56	K54	4	h16	fl14	m14	2	J29
			S57	K55	1	h6	fl7	m13	1	14-J30
			S58	K56	2	h17	fl15	m3	2	J31
F	1999	11	S59	K57	8	h18	fl9A	m1	3	J32
			S60	K58	3	h6	fl16	m13	3	14-J33
G	1999		S61	K59	23	h1	fl10	m3	3	J34

[a] See Table 1, footnote *b*.
[b] ND, not digested. The isolate was refractory to digestion by the restriction enzyme.

(see Fig. SA1 in the supplemental material). Some patterns in these two main groups showed high levels of similarity (80 to 99%) (see Fig. SA1 in the supplemental material) and were closely related.

Macrorestriction by the KpnI enzyme (KpnI-PFGE) of the 1,258 *Campylobacter* isolates distinguished 90 different patterns, which were desiganted K1 to K90 (Tables 1, 2, and 3; see Fig. SA2 in the supplemental material). Four *C. jejuni* isolates recovered from poultry farm B and *C. jejuni* strain ATCC 33560, as well as 59 *C. coli* isolates (1 isolate from poultry farm E, 22 isolates from farm F, and 36 isolates from farm G) were refractory to KpnI (Tables 1, 2, and 3). The numbers of bands in these KpnI-PFGE patterns ranged from 8 to 17, and the sizes ranged from 40 kbp to 445 kbp. Analysis of the similarity of the different KpnI-PFGE patterns did not reveal any well-defined clusters related to the two species of *Campylobacter* (*C. coli* and *C. jejuni*) (see Fig. SA2 in the supplemental material). Nevertheless, some profiles of the two species showed high

degrees of genetic similarity (see Fig. SA2 in the supplemental material).

Combining the two restriction enzyme PFGE analyses permitted determination of 102 PFGE genotypes, 54 genotypes corresponding to *C. coli* (Tables 1 and 2) and 48 genotypes corresponding to *C. jejuni* (Tables 1 and 3). The similarity analysis showed that there were two main clusters, one cluster corresponding to the *C. coli* PFGE genotypes and one cluster corresponding to the *C. jejuni* PFGE genotypes (Fig. 1) (genotypes composed of only one macrorestriction were not included). Within these two clusters, some PFGE genotypes were close to each other (e.g., *C. coli* genotypes S1K1, S2K1, and S3K1 and *C. jejuni* genotypes S7K5 and S55K53 or S7K8 and S84K84 [Fig. 1]).

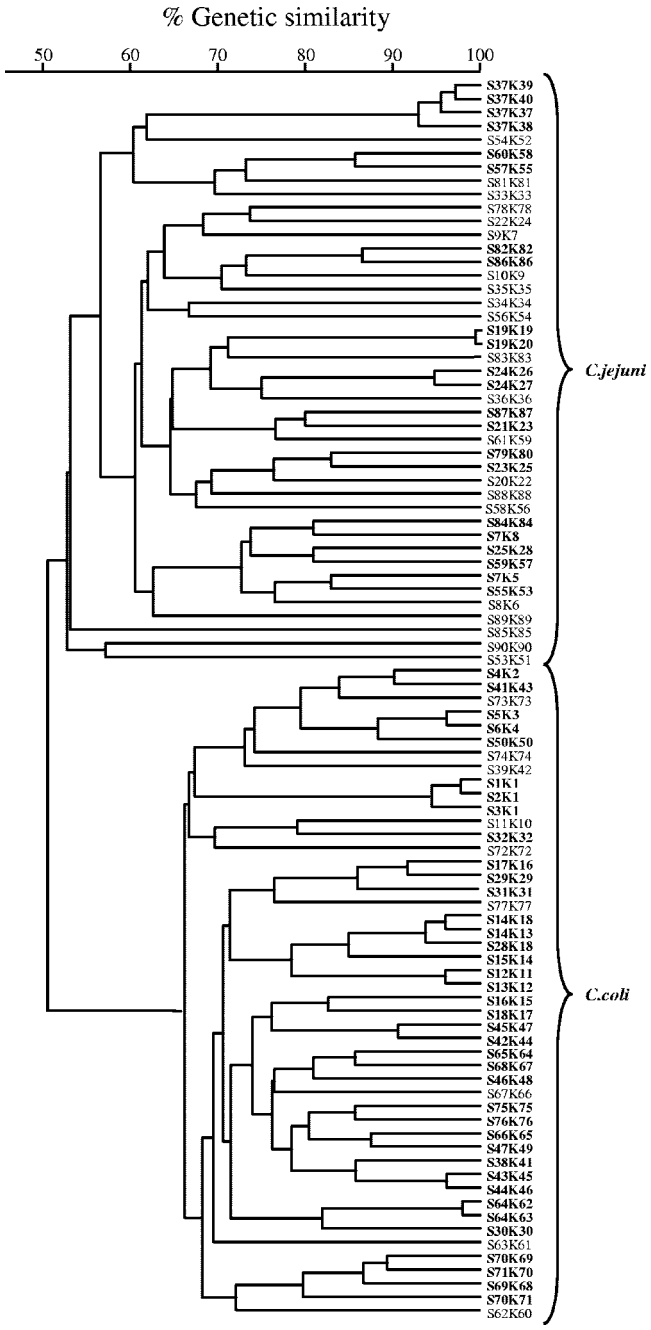

FIG. 1. Dendrogram showing the relatedness among the PFGE genotypes.

PCR-RFLP analysis. Of the 1,225 *Campylobacter* farm isolates, 242 *C. coli* and 360 *C. jejuni* isolates were chosen on the basis of PFGE genotype and sample origin.

The *C. coli* isolates and 17 collection strains were characterized by two PCR-RFLP typing methods, *flaA* and *rib* rRNA gene typing (Tables 1 and 2). Fifteen and ten different patterns were discriminated by *flaA* and *rib* rRNA gene typing, respectively (Tables 1 and 2; see Fig. SA3 in the supplemental material). By combining the results of the two PCR-RFLP typing methods, we distinguished 22 PCR-RFLP types for the 259 *C. coli* isolates.

For the 15 *flaA* types, two major profiles (fl23 and fl25) represented 78.8% of the *C. coli* isolates tested (Table 2). In addition, profiles fl24 and fl26 exhibited more than 85% similarity with profile fl25 (see Fig. SA3 in the supplemental material). Similarly, most (216) of the 259 *C. coli* isolates studied by *rib* rRNA gene typing had the same rc2 profile, and all the profiles were very similar, demonstrating the genetic homogeneity of this region in *C. coli*.

Three PCR-RFLP typing methods (*flaA*, *hipO*, and *gyrA*/*pflA* typing) were used to characterize the 360 *C. jejuni* isolates and the 15 collection strains (Tables 1 and 3). The *C. jejuni* isolates characterized by *flaA*, *hipO*, and *gyrA*/*pflA* typing gave 22, 26, and 18 profiles, respectively. The three typing methods did not classify the *C. jejuni* isolates in the same way (Tables 1 and 3), and 43 combined PCR-RFLP types were distinguished. Some identical PCR-RFLP combinations (h1fl1m1, h4fl4m4, and h6fl6m8 [Tables 1 and 3]) were found for isolates having different origins.

Some profiles obtained with the three PCR-RFLP methods differed by only a few DNA bands and showed high degrees of similarity (more than 80%). Thus, for the 22 *flaA* types, profiles fl9A-fl15-fl9, fl21-fl10, and fl7-fl8 were very similar (see Fig. SA3 in the supplemental material). Some *hipO* types were also closely related (h5-h9-h25, h1-h21-h22-h24, and h3-h7) (see Fig. SA3 in the supplemental material). The patterns obtained by *gyrA*/*pflA* typing also exhibited many similarities (m7-m4-m2, m13-m3, m6-m1-m16, and m17-m5-m12-m18) (see Fig. SA3 in the supplemental material).

Evidence of clusters obtained by analysis of all genotyping methods. A combination of all molecular typing methods led to definition of 116 genotypes for the two *Campylobacter* species, 57 genotypes corresponding to the *C. coli* isolates (genotypes C1 to C52) (Tables 1 and 2) and 59 genotypes corresponding to the *C. jejuni* isolates (genotypes J1 to J48) (Tables 1 and 3).

Some individual PFGE genotypes were divided slightly by the PCR-RFLP methods, and some similarities observed between similar PFGE genotypes were strengthened. This led to identification of clusters within the two species; 43 of the 57 *C. coli* genotypes could be grouped into 16 clusters (designated clusters 1-C to 16-C) (Tables 1 and 2), and 38 of the 59 *C. jejuni* genotypes could be grouped into 15 clusters (designated clusters 1-J to 15-J) (Tables 1 and 3).

Based on the identical PFGE genotypes and slight differences determined by PCR-RFLP analysis, eight clusters (clusters 3-J, 4-J, 6-J, 8-J, 10-J, 11-J, 12-J, and 13-J) (Table 3) were

then defined for the *C. jejuni* isolates, and three clusters (clusters 6-C, 8-C, and 13-C) (Table 2) were defined for the *C. coli* isolates.

On the other hand, some similarities revealed by PFGE were strengthened by PCR-RFLP typing, which also led to identification of clusters for the two *Campylobacter* species. Thus, 14 and 8 additional clusters were described for *C. coli* and *C. jejuni*, respectively (Tables 1, 2, and 3); e.g., within the *C. coli* isolates, genotypes C1, C1A, and C2 were considered members of the same cluster (cluster 1-C) (Table 2) as they produced similar SmaI profiles (S1, S2, and S3) (Fig. 1) and identical KpnI, *flaA*, and *rib* rRNA gene profiles. Similarly, within the *C. jejuni* isolates, those having genotypes J1, J4, J28, and J42 were placed in the same cluster (cluster 1-J) (Table 3). Although the isolates with genotypes J1 and J4 produced a significantly different KpnI profile, they produced the same SmaI, *hipO*-, *flaA*, and *pflA*/*gyrA* profiles. Isolates with the J28 and J42 genotypes also produced the same *hipO*, *flaA*, and *pflA*/*gyrA* profiles, and their PFGE genotypes (S55K53 and S84K84) were similar to PFGE genotypes J1 and J4 (S7K5 and S7K8, respectively) (Fig. 1 and Tables 1 and 3).

Some genotypes, such as C6 or J2 (Tables 2 and 3), were not included in any cluster.

Biodiversity of *Campylobacter*: comparison with the laboratory collection strains. Molecular characterization revealed 116 *Campylobacter* genotypes among the 1,225 isolates from the seven poultry farms and the 33 strains from the laboratory collection. As the frequencies of investigation were different for different farms (weekly for farms A, B, and C but only three times for farms D, E, F, and G), the numbers of isolates characterized from the farms differed considerably.

The two species of *Campylobacter* (*C. coli* and *C. jejuni*) were recovered at the same ratio throughout the study, and there was a slight preponderance of *C. jejuni* (57.4% versus 42.6%). However, large variations between different farms were apparent; e.g., 100% of the isolates from farm E belonged to *C. coli*, compared with only 8.2% of the isolates from farm C.

With the exception of farm E, all broiler flocks were contaminated by both *Campylobacter* species, and several genotypes within these species were described (Tables 2 and 3). On farm B, for example, 61.5% of the 582 isolates characterized belonged to *C. jejuni*, and 38.5% belonged to *C. coli*; 28 distinct genotypes were described, some of which could be further grouped into 13 clusters (Tables 2 and 3). In each species, some genotypes were predominant; 55.4% of the *C. coli* isolates collected on this farm were genotype C6 isolates (Table 2), and cluster 5-C accounted for 29.9% of the *C. coli* isolates studied (Table 2). The remaining *C. coli* isolates (14.7%) were grouped into six genotypes (genotypes C7 and C8 in cluster 4-C, genotypes C12 and C12A in cluster 6-C, and genotypes C13 and C14). On this farm, 55.9% and 28.2% of the *C. jejuni* isolates belonged to clusters 2-J and 4-J, respectively (Table 3). The remaining isolates (15.9%) grouped into five distinct clusters (clusters 3-J, 6-J, 7-J, 8-J, and 9-J) (Table 3) and two distinct genotypes (genotypes J10 and J11) (Table 3).

It was also apparent from the genotype distribution on the seven poultry farms that some isolates from different farms had identical or similar genotypes. A few similar or identical patterns were also observed in the collection strains. For *C. coli*, isolates belonging to cluster 8-C were collected in 1997 from farm B and in 1999 from farms F and G (Table 2). Isolates belonging to cluster 13-C were found on both farms F and G, and isolates belonging to cluster 3-C were collected from farm A during 1996 and from farm F in 1999 (Table 2). Similarly, some cluster 2-C isolates were collected from farm A in 1996 and from farm E in 1999, and some cluster 7-C isolates were found on farms B and C in 1997 and 1998, respectively (Table 2). Some strains in the laboratory collection were members of the same cluster. Thus, strains A9821 and A992 collected from pork during 1996 in eastern France belonged to cluster 14-C (Table 1). In the same way, strains A1649, A1578, A1575, and A1552, also collected from pork in eastern France in 1996, had similar profiles and seemed to belong to the same cluster (cluster 15-C) (Table 1). Strains A879 and A846 collected from poultry in eastern France in 1996 belonged to cluster 16-C (Table 1).

Within the *C. jejuni* isolates, genotype J3 was isolated from farm A in 1996 and from farm C in 1998 (Table 3). Genotype J1 was isolated from farm A and, during the same year, from a poultry farm located in eastern France (Tables 1 and 3). Moreover, this organism belonged to the same cluster as strains having genotypes J4, J28, and J42 that were collected from farms A and D in 1998 and from a poultry farm in eastern France in 1996, respectively. Some isolates belonging to cluster 14-J were collected from farms D and F in 1998 and 1999, respectively (Table 3). One isolate collected from farm B in 1997 belonged to cluster 5-J, the same cluster as strain A940 isolated from a poultry farm in eastern France in 1996 (Tables 1 and 3). Strains 3J4.5 and A922, which were isolated from geographically distant poultry farms (in Brittany and Alsace) and were studied in different years (1994 and 1996), belonged to the same cluster, cluster 15-J (Table 1).

Tracing *Campylobacter* spp. on the broiler farms. No *Campylobacter* strains were detected in the farm buildings on any of the seven poultry farms before arrival of the chickens (Table 4). The 1-day-old chicks and the transport cases were also free of *Campylobacter* spp. No *Campylobacter* was detected in any feed or drinking water samples. On farms A, B, and C, the chickens were contaminated by *Campylobacter* during the rearing period inside the farm buildings (Table 4). No *Campylobacter* was detected in the broiler droppings on farms D, E, F, and G before the chickens went outside into the open rearing space (Table 4). Nevertheless, all seven poultry flocks were contaminated by *Campylobacter* spp. before their departure for the slaughterhouse.

On farm A (Table 4), some *Campylobacter* strains (*C. coli* genotypes C1, C1A, and C2 and *C. jejuni* genotype J1) were detected in soil samples collected in the open rearing space on the first sampling day. Other strains of *C. coli* (genotype C3) and *C. jejuni* (genotype J2) were detected in soil samples and in bovine feces, respectively, in the second set of samples. *C. jejuni* genotype J5 was also detected in bovine feces during the epidemiological study of this farm. Broilers were contaminated between days 29 and 36 by *C. coli* genotype C4. This was the only genotype detected in the poultry droppings during the next two sampling times (Table 4). Nevertheless, just before their departure for the slaughterhouse, the chickens were found to carry several *Campylobacter* strains (*C. coli* genotype C4 and *C. jejuni* genotypes J1, J3, and J4). It should be noted that the birds were contaminated by the same *C. jejuni* strain (genotype J1) isolated from the soil on the first day of the

TABLE 4. Tracing of *Campylobacter* spp. on seven broiler farms

Farm	Chicken age (days)	Outside sampling	Bovine feces	Entrance hall	Inside environment	Chicken
A	1	1-C1, 1-C1A, 1-C2, **1-J1**[a]	–[b]	–	–	–
	8	C3	–	–	–	–
	15	–	–	–	–	–
	22	–	–	–	–	–
	29	–	J2	–	–	–
	36	1-J1, J2	J2	–	C4	3-C4
	43[c]	3-C4	J2	3-C4	C4, C5	3-C4
	50	3-C4, 3-C5	–	3-C4, **1-J1**	C4	3-C4
	78	3-C4, J3, 1-J4	J5	ND[d]	ND	3-C4, **1-J1,** J3, 1-J4
B	1	–	ND	–	–	
	8	–	ND	–	–	
	15	–	ND	–	–	
	22	–	ND	C6, 2-J6		C6, 2-J6, 3-J8, 4-J9
	29	5-C11, 2-J6, 4-J9	ND	C6, 2-J6, 4-J9	C6, 2-J6, 2-J7, 4-J9	C6, 5-C11, 2-J6, 2-J7, 4-J9
	36[c]	–	ND	–	–	–
	48	4-C7, 5-C10, 5-C11, 2-J6, 2-J7, 4-J9, J11, 6-J12, 7-J13	ND	5-C10, 4-J9, 6-J12, 7-J13, 7-J14	J6, J9	C6, 5-C11, 2-J6, 3-J8, 4-J9, 6-J12, 7-J13, 8-J15
	87	ND	ND	6-C12, 7-C13, 4-J9, 5-J10	ND	4-C7, 4-C8, 5-C10, 5-C11, 6-C12, 7-C13, 8-C14, 5-C15, 4-J9, J11, 6-J12, 7-J13, 8-J15, 9-J16, 9-J17
C	1	–	ND	–	–	–
	12	J18	ND	–	–	–
	27	10-J19, 11-J20, 12-J21	ND	10-J19	–	10-J19, 11-J20
	33	10-J19	ND	–	10-J19	10-J19, 11-J20, 12-J21
	40[c]	10-J19	ND	10-J19	–	10-J19, 12-J21, 13-J24
	48	10-J19, 12-J21	ND	–	–	10-J19, 11-J20, 12-J21, 13-J23, 13-J24, 13-J25
	68	10-J19	ND	–	–	10-J19, 12-J21, 13-J22, 13-J23, 13-J24, 7-C16, 9-C17
	83	ND	ND	ND	ND	10-J19, 11-J20, J3, 7-C16, 9-C17, 7-C18, 9-C19
D	1	C20	J26	–	–	–
	36[c]	–	ND	–	–	–
	86	ND	ND	–	–	C21, J27, 1-J28, J29, 14-J30, J31
E	1	C22, **2-C23, 10-C24,** 11-C25, 11-C26	ND	–	–	–
	41[c]	–	ND	ND	ND	–
	81	ND	ND	ND	ND	**2-C23, 10-C24,** 10-C27, C28
F	1	8-C14, 12-C29, **13-C22A, 13-C30,** C31	ND	–	–	–
	41[c]	–	ND	ND	ND	–
	81	ND	ND	ND	ND	**13-C22A, 13-C30,** 3-C32, J32, J33, 12-C29, 12-C29A, 12-C33
G	1	8-C14, **13-C22A**	ND	–	–	–
	41[c]	–	ND	ND	ND	–
	81	ND	ND	ND	ND	**13-C34,** J34

[a] Boldface type indicates genotypes and clusters that were isolated from the soil at the beginning of the rearing period and from animals before their departure for the slaughterhouse. See Table 1, footnote *b*.

[b] –, negative samples.

[c] After this date the chickens could go outside.

[d] ND, sampling not done.

rearing period and also detected in the entrance hall during sampling on day 50 of rearing.

No *Campylobacter* was detected in the environment outside the broiler building on farm B (Table 4) before the birds became contaminated. Some strains of *C. coli* (genotypes C6 and C11) and *C. jejuni* (genotypes J6, J7, J8, and J9) were isolated from birds on days 22 and 29. Before departure of the chickens for the slaughterhouse, eight distinct genotypes of *C. coli* and seven genotypes of *C. jejuni* were isolated from the 100 chickens sampled (10 samples of 10 individual droppings) (Table 4).

One *C. jejuni* strain on farm C (Table 4) was isolated from a soil sample in the second set of samples. This strain (genotype J18) was never recovered during the rearing period. Birds carried *C. jejuni* (genotypes J19 and J20) from day 27. During the same sampling, genotype J21 was also isolated from soil in front of the entrance door. This organism was isolated from the chicken droppings in the next set of samples (Table 4). *C. jejuni* genotype J19 was the genotype that was most frequently isolated from chicken droppings during most of the rearing period. On day 68, 2 *C. coli* isolates (genotypes C16 and C17) were detected among 18 isolates obtained from droppings; the 16 other isolates belonged to *C. jejuni* and were genotypes J19, J21, J22, J23, J24, and J25. However, just before their departure for the slaughterhouse, the chickens seemed to carry a majority of *C. coli*. Sixteen of the 20 isolates from droppings belonged to *C. coli* (genotypes C16, C17, C18, and C19).

On farm D (Table 4), *C. coli* genotype C20 and *C. jejuni* genotype J26 were isolated from soil and bovine feces, respectively, on the first day of the rearing period. At the end of this period, the chickens were contaminated by one *C. coli* strain (genotype C21) and five different *C. jejuni* strains (genotypes J27, J28, J29, J30, and J31).

On farm E (Table 4), several soil samples were *Campylobacter* positive on the first day of the rearing period. Twenty-three isolates were identified and characterized. They belonged to five *C. coli* genotypes, genotypes C22, C23, C24, C25, and C26. At the end of the rearing period, the chickens were contaminated by four distinct *C. coli* genotypes (genotypes C23, C24, C27, and C28). It is important to note that some isolates with identical genotypes (genotypes C23 and C24) were found in the soil at the beginning of the rearing period and in droppings before transport of the chickens to the slaughterhouse. Moreover, the genotype C27 isolates recovered from the droppings belonged to the same cluster as the genotype C24 isolates (cluster 10-C) (Table 2).

On farm F (Table 4), five distinct *C. coli* genotypes were isolated from soil samples on the first day of the rearing period (genotypes C14, C29, C22A, C30, and C31). Genotypes C22A and C30 belonged to the same cluster (cluster 13-C) (Table 2). Before their departure for the slaughterhouse, the chickens were contaminated by six *C. coli* genotypes (genotypes C22A, C30, C32, C29, C29A, and C33) and two *C. jejuni* genotypes (genotypes J32 and J33) (Table 4). As on farm E, the same or closely related genotypes (genotypes C29, C29A, C22A, and C30) were isolated in the soil on the first day and in the chicken droppings at the end of the rearing period.

On farm G (Table 4), two *C. coli* genotypes (genotypes C22A and C14) were isolated from the soil on the first day of sampling. The chickens were contaminated by *C. coli* genotype C34 isolates and *C. jejuni* genotype J34 isolates before their departure for the slaughterhouse (Table 4). It is important to note that the genotype C22A and C34 isolates belonged to the same cluster (cluster 13-C) (Table 2).

DISCUSSION

The best way to evaluate the genomic diversity of *C. coli* and *C. jejuni* is DNA sequencing. However, this technique is both time-consuming and expensive, so a number of subtyping methods have been developed to differentiate bacterial isolates beyond the species level. In this study, PFGE and PCR-RFLP methods were used to analyze the whole genome and specific sequences, respectively.

Thus, *Campylobacter* isolates were first characterized by PFGE using two restriction enzymes, SmaI and KpnI. Gibson et al. (14) showed that the divergence between some strains varied significantly according to the restriction endonuclease used and that matches between PFGE profiles obtained with at least two enzymes were required to prevent misinterpretation of strain affinities. In the same way, Lindmark et al. (24) showed that 19 isolates with identical SmaI profiles displayed 15 different profiles after digestion with KpnI. This clearly underlines the need to use a second enzyme when the relatedness between isolates is determined.

Characterization of selected *Campylobacter* isolates was completed by studying the polymorphism of three loci (*hipO*, *flaA*, and *gyrA*/*pflA*) for the *C. jejuni* isolates and two loci for *C. coli* (*flaA* and *rib* rRNA gene). Molecular characterization by PCR-RFLP typing is less discriminatory than macrorestriction using two restriction enzymes (102 PFGE genotypes versus 65 PCR-RFLP genotypes). Simpson's index of discrimination (21) for the different techniques was calculated using the poultry farm results (Table 5). Thus, KpnI-PFGE analysis gave better discrimination than the other techniques used in this study (SmaI-PFGE analysis, in particular) (27). Nevertheless, as previously described (50), SmaI-PFGE analysis was able to discriminate between *C. coli* and *C. jejuni*.

A total of 116 genotypes for the 1,258 *Campylobacter* isolates were defined by combined PFGE and PCR-RFLP typing. With minor changes in profiles, PCR-RFLP typing permitted further discrimination of the *Campylobacter* isolates characterized by PFGE typing. Thus, three PFGE genotypes for the *C. coli* isolates (S16K15, S18K17, and S40ND) displayed different PCR-RFLP genotypes. Eight PFGE genotypes for the *C. jejuni*

TABLE 5. Simpson's index of discrimination calculated using poultry farm data shown in Tables 2 and 3

Typing method	Enzyme or amplified gene	Simpson's index	
		Single method	Combined methods
Macrorestriction	SmaI	0.9237	0.9313
	KpnI	0.9317	
PCR-RFLP (*C. jejuni*)	*hipO*	0.8852	0.8943
	flaA	0.8468	
	gyrA/*pflA*	0.8741	
PCR-RFLP (*C. coli*)	*flaA*	0.7717	0.7972
	rRNA gene	0.2957	

isolates (NDK21, S20K22, S23K25, S25K28, S34K34, S35K35, S36K36, and S37K39) were slightly divided by PCR-RFLP typing. These differences could have been due to intra- and interstrain recombination in the different amplified sequences, as was shown previously for the flagellin gene (19). On the other hand, the PCR-RFLP typing methods strengthened some of the similarities observed between similar PFGE genotypes. Thus, by combining the different molecular typing methods some clusters could be defined within the two species; 43 of the 57 *C. coli* genotypes were grouped into 16 clusters (designated clusters 1-C to 16-C) (Tables 1 and 2), and 38 of the 59 *C. jejuni* genotypes were grouped into 15 clusters (clusters 1-J to 15-J) (Tables 1 and 3).

This study demonstrated the relative genetic diversity of *Campylobacter* isolates from poultry (84 genotypes for 1,225 isolates). In this study, six of the seven poultry flocks investigated were contaminated by both species of *Campylobacter*, while only *C. coli* was isolated from farm E. To our knowledge, no previous study has revealed multiple *Campylobacter* types in broiler flocks. In most previous studies, flocks were contaminated by only one species (generally *C. jejuni*), and when typing was used, the contamination was generally due to a single serotype or genotype of *Campylobacter* (24, 28, 30). Thus, during a 1-year epidemiological study of 287 poultry flocks, Berndston et al. (4) showed that 75 of 77 contaminated flocks were contaminated by *C. jejuni* and only 2 flocks were contaminated by *C. coli*. Only 11 distinct serotypes were detected for the *Campylobacter* isolates collected from these 77 poultry flocks. Moreover, most of the flocks were contaminated by a single *Campylobacter* serotype, and only four flocks carried more than one serotype. Perko-Mäkelä et al. (35) also found that chicken flocks were contaminated by a single *Campylobacter* species, generally *C. jejuni* (31 of 33 flocks). In another study, 24 flocks reared on the same farm were contaminated by a single species (*C. jejuni*) with the same serotype (serotype HS2) (3).

The greater diversity found in our study could have been due to the use of powerful discriminating techniques, to the characterization of a larger number of *Campylobacter* isolates, and to the kinds of rearing systems studied. Thus, on farm B, 11 *C. coli* genotypes and 17 *C. jejuni* genotypes were identified among the 582 poultry isolates tested. However, dominant genotypes were found for each species, and several genotypes showed relatedness; almost 85% of the isolates tested could be placed into two *C. coli* groups (genotype C6 and cluster 5-C) and two *C. jejuni* groups (clusters 2-J and 4-J). Similarly, on farm A, although five *C. jejuni* and six *C. coli* genotypes were found among the 201 isolates, 87.5% of these isolates could be grouped into two *C. jejuni* clusters (clusters 1-J and J3) and one *C. coli* cluster (cluster 3-C). On farm C, 62.8% of the isolates belonged to cluster 10-J. *C. coli* was much less frequent on this farm (8.2% of the isolates), and the isolates could be divided into two clusters (clusters 7-C and 9-C). Although fewer *Campylobacter* isolates were found on the four other farms, several genotypes were still apparent. Thus, on farm D, two distinct genotypes were found for the 16 *C. coli* isolates studied, and six genotypes were found for the 19 *C. jejuni* isolates. No relatedness was observed for these various isolates, but 73.3% of the isolates were members of two genotypes of *C. jejuni* (genotypes J26 and J27) and one genotype of *C. coli* (genotype C21). The genotypic heterogeneity observed on all seven poultry farms was probably due to the existence of different sources of *Campylobacter* in the environment.

The well-known genetic instability of *Campylobacter* species (46) could also explain our results, which included finding 16 clusters for the 57 *C. coli* genotypes and 15 clusters for the 59 *C. jejuni* genotypes. The *Campylobacter* species were naturally transformable and had the capacity to acquire exogenous DNA that could be integrated into the chromosome by illegitimate recombination (37, 45). Hanninen et al. (16) showed that *C. jejuni* could undergo genetic recombination (insertions, deletions, acquisition of foreign DNA, inversions, crossovers, etc.) during chicken intestine colonization. Similarly, Boer et al. (6) demonstrated that interstrain genetic exchange and intragenomic alterations occurred in vivo during *C. jejuni* infection, which might explain the genome plasticity observed for this pathogen. Our results corroborate these findings and also demonstrate the genetic instability of *Campylobacter* within a single poultry flock. Thus, on farm B, genotypes C9, C10, and C15 of *C. coli* probably originated from genotype C11. All four of these genotypes had identical *flaA* and *rib* rRNA gene profiles, whereas minor modifications were observed with the SmaI and KpnI profiles. Genotype C11, which was found more frequently throughout the rearing period, was considered the major genotype. Thus, genotypes C9, C10, and C15 could have been derived from genotype C11 by genomic rearrangements that could have occurred in vivo or in vitro. Indeed, they may have occurred in vitro during treatment of the samples. Recombination in genotypes J6 and J7 (cluster 2-J), collected during the same rearing period, could have occurred in vivo, because very small amounts of genotype J7 of *C. jejuni* were found at several times throughout the rearing period. This genetic instability can be explained by the fact that *Campylobacter* and other bacteria with small genomes (*Helicobacter*, for example) need to undergo genetic rearrangements in order to increase their potential and adapt to the environment (25, 29, 47).

Another important result of this study was that identical strains or strains belonging to the same cluster were observed on different farms and at different times. In certain cases, the farms belonged to the same poultry company and therefore received chickens from the same hatchery and food from the same factory. This was the case for strain C14, found on farms B, F, and G, for strain C22A found on farms F and G, and for strain J3 found on farms A and C. These results highlight the problems associated with a common source of chicks and a common source of feed. However, some strains that were identical or were members of a given cluster were also found on farms without any such connection (Table 4). These results suggest that some *Campylobacter* strains could be adapted to poultry; the relative genetic stability in space and time could have been the result of adaptation to environmental pressure (7, 25). Thus, Broman et al. (7) showed that some *C. jejuni* subtypes could be associated with different biotopes. Moreover, this hypothesis was supported by a multilocus sequence typing investigation, in which it was shown that certain clonal complexes could be found only among isolates from certain sources (e.g., sand samples) (10). However, in our study, the presence of a particular strain may have been entirely stochastic or may have been the result of pressures outside the present niche that influenced the migration.

Campylobacter spp. could be traced throughout the rearing period on all seven poultry farms in this study. On farms A, B, and C, on which no particular biosecurity measures were used by the farmers, chickens were contaminated by *Campylobacter* from the second week of rearing, as has been described in other studies (3, 22). The farmers on the four other farms (farms D, E, F, and G) had been informed of the need for biosecurity measures, such as systematic changing of boots before the rearing house is entered. The chickens on these farms were free from *Campylobacter* during the first 6 weeks of rearing inside the building. Nevertheless, all seven poultry flocks were contaminated by *Campylobacter* before their departure for the slaughterhouse.

The molecular typing methods used in this study revealed that the soil around the farm building was a source of *Campylobacter* contamination. On farm A, *C. jejuni* strain J1 was isolated from the soil on the first day, from the entrance hall on day 50, and from the poultry feces at the end of the rearing period. Similarly, the same strains were isolated on farms E, F, and G from the soil on the first day of sampling and from the birds before their departure for the slaughterhouse.

In this study, the soil was clearly identified as a potential source of *Campylobacter* contamination, but we also demonstrated that there were multiple sources of contamination as several *Campylobacter* strains were isolated from each poultry flock. This study also showed that the carriage of *Campylobacter* by the birds may change during the rearing period. Thus, broilers on farm C were primarily contaminated by *C. jejuni* strains at the beginning of contamination (day 27) until day 48. For the last two sets of samples (days 68 and 83), *C. coli* was isolated from the majority of the samples before departure of the chickens for the slaughterhouse. Several hypotheses could be advanced to explain these changes in chicken colonization by *Campylobacter*. First, the broilers were exposed to several sources of *Campylobacter* during the rearing period, and certain *Campylobacter* strains colonized the chickens. Then a physiological alteration (food change, additives, immune status, etc.) might have resulted in the establishment of another type of strains. Moreover, the method used to recover the *Campylobacter* isolates (direct plating, prior enrichment, choice of medium, etc.) may influence the subtypes of strains (30).

No *Campylobacter* was isolated from the soil in the second set of samples obtained before the end of the in-house period on farms D, E, F, and G, even when *Campylobacter* strains had been isolated from the soil in the first set of samples. One possible explanation for this is that the number of samples was not sufficient for the real soil contamination to be evaluated. This is unlikely, however, as some *Campylobacter* strains were isolated from the soil in the open area during the first sampling in the same number of samples on all four farms. The other explanation is that the *Campylobacter*-contaminated chickens from the previous batch were the source of soil contamination. On the first day of the rearing period, the open rearing area had been vacant for 3 weeks (the conventional sanitary procedure), and the *Campylobacter* strains excreted by chickens from the previous batch might still have been present. On the other hand, by the time of the second sampling, no chicken would have had access to the open rearing space for 9 weeks (3 weeks empty plus 6 weeks with the poultry in the house), by which time the *Campylobacter* strains previously present could have died and the number of soil samples taken would have been insufficient to detect the presence of the bacterium. Moreover, it is possible that, given the conditions of stress encountered in the soil, the *Campylobacter* strains might have transformed into viable but noncultivable forms and might have become cultivable after passage in the intestinal tract of chickens, as was observed in laboratory conditions by Cappellier (8). On these farms, the *Campylobacter* strains isolated from the soil and from the chickens at the end of the rearing period belonged to *C. coli*. It is known that survival is different from strain to strain (42) and that injuries can be repaired through intestinal passage. It would be interesting to compare the survival of the different strains and their capacities to recover the ability to grow.

This study is the first study to reveal relatively significant biodiversity within *Campylobacter* strains collected from broiler flocks. This biodiversity could have resulted from the fact that the flocks studied were free-range broilers exposed to multiple sources of contamination. The use of several methods to characterize the *Campylobacter* isolates might also explain this biodiversity. We also showed that a broiler flock colonized by one *Campylobacter* strain could be colonized by one or more other strains. In this study, we also observed that *Campylobacter* exhibited different related profiles that could be due to genomic rearrangements which could have occurred in vitro or in vivo throughout the rearing period. This work also demonstrated that the soil around the rearing facilities was a potential source of contamination and that biosecurity measures were useful for preventing or minimizing *Campylobacter* colonization of broilers.

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