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Evidence that Certain Clones of *Campylobacter jejuni* Persist during Successive Broiler Flock Rotations

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Through the national surveillance program for *Campylobacter* spp., nine broiler chicken farms that were infected with *Campylobacter jejuni* in at least five rotations in 1998 were identified. One additional farm, located at the island of Bornholm where divided slaughter is used extensively, was also selected. Twelve broiler houses located on 10 farms were included in the study. The *C. jejuni* isolates collected from the selected houses during the surveillance were typed using *fla* typing and macrorestriction profiling (MRP), and a subset of the isolates, representing each of the identified clones, was serotyped according to the Penner scheme. Pulsed-field gel electrophoresis typing using *Sma*I and *Kpn*I revealed that the majority of houses (11 of 12) carried identical isolates in two or more broiler flocks. Such persistent clones were found in 63% of all flocks (47 of 75). The majority of persistent clones (7 of 13) had *fla* type 1/1, but MRPs distinguished between isolates from different houses, and *fla* type 1/1 clones belonged to different serotypes. Seven houses carried persistent clones that covered an interval of at least four broiler flock rotations, or at least one half year. The dominant *fla* type (1/1) was represented by 44% of isolates, or by at least one isolate from 31 of 62 broiler flocks. This significantly exceeded the prevalence of *fla* type 1/1 *C. jejuni* isolates that we have estimated from other studies and suggests that isolates carrying this *fla* type are overrepresented in flocks with recurrent *Campylobacter* problems. The MRPs of clones belonging to *fla* type 1/1 serotype O:2 isolated from persistently infected flocks shared a high percentage of bands compared to the remaining isolates, indicating that some clones that have the ability to cause persistent infections in broiler farms are highly related to each other.

Campylobacter jejuni has become recognized as a major cause of human enteritis in many industrialized countries during the past 2 decades (4). In Denmark the incidence rate was 64 recorded cases per 100,000 inhabitants in 1998, and in 1999 it reached a peak, with 78 recorded cases per 100,000 inhabitants (1, 2). In developed countries the majority of human *Campylobacter* cases are sporadic and the consumption of undercooked poultry meat is considered an important source, whereas outbreaks are rare (4). In 1998 46% of Danish broiler flocks were infected with *C. jejuni* (26). Contamination control at the farm level would be one way of reducing consumer exposure; however, the epidemiology of *C. jejuni* in broiler flocks is still unclear.

It is well established that biosecurity measures such as improved hygiene barriers and staff education, together with pest and rodent control, reduce the frequency of infected flocks (3, 5, 26). However, full control of *C. jejuni* contamination has not been obtained yet. It has not been established whether common contamination reservoirs, for instance those that are attached to the broiler production chain (hatchery and abattoir, etc.), are of importance, although a British study (18) and a Danish study (26) point in that direction.

The diversity of *C. jejuni* is considerable, and investigations of contamination reservoirs therefore depend on the application of suitable typing tools. In recent years, various genotyping methods for *C. jejuni* have been described, with the most frequently used ones being PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *flaA* gene (*fla* typing) and macrorestriction profiling (MRP) using pulsed-field gel electrophoresis (PFGE) (25). *fla* typing has the advantage of being fast and cheap. PFGE-MRP, on the other hand, is more laborious but has the advantage of being significantly more discriminatory and better able to describe the significant genetic variation of *C. jejuni* (16, 21). Penner serotyping has less discriminatory power than the genotyping tools, but on the other hand, it is standardized and has been used successfully in several studies worldwide (12, 14, 17).

The present study focused on farms where the proportion of positive flocks was above average in 1998. The purpose was to evaluate the potential epidemiological importance of farm-associated clones that occur in successive rotations versus clones that are common between different farms. An additional aim was to characterize such clones, when identified, using serotyping, *fla* typing, and MRP.

MATERIALS AND METHODS

Selection of isolates and culture conditions. A total of 94 *Campylobacter* isolates were investigated. These isolates originated from a collection of fecal isolates sampled at the time of slaughter from healthy broiler chickens during 1998 as part of an ongoing surveillance program for *Campylobacter* spp. in Danish broilers (26).

The 94 isolates originated from broiler farms where at least five flocks in most or all houses were positive for *Campylobacter* spp. in 1998. Nine farms in total (A, B, C, D, E, F, H, J, and K) met that criterion; eight were located on the peninsula of Jutland, and one was located on the island of Bornholm (Fig. 1). From each farm in Jutland one house that was infected by *C. jejuni* in at least five rotations was selected at random. If more than one house met this criterion, a house where flocks were slaughtered in a low number of batches was selected. Broiler house numbers are used in combination with farm designations below (e.g., A2 stands for farm A, house 2).

One additional farm (G) on the island of Bornholm was included. The Born-

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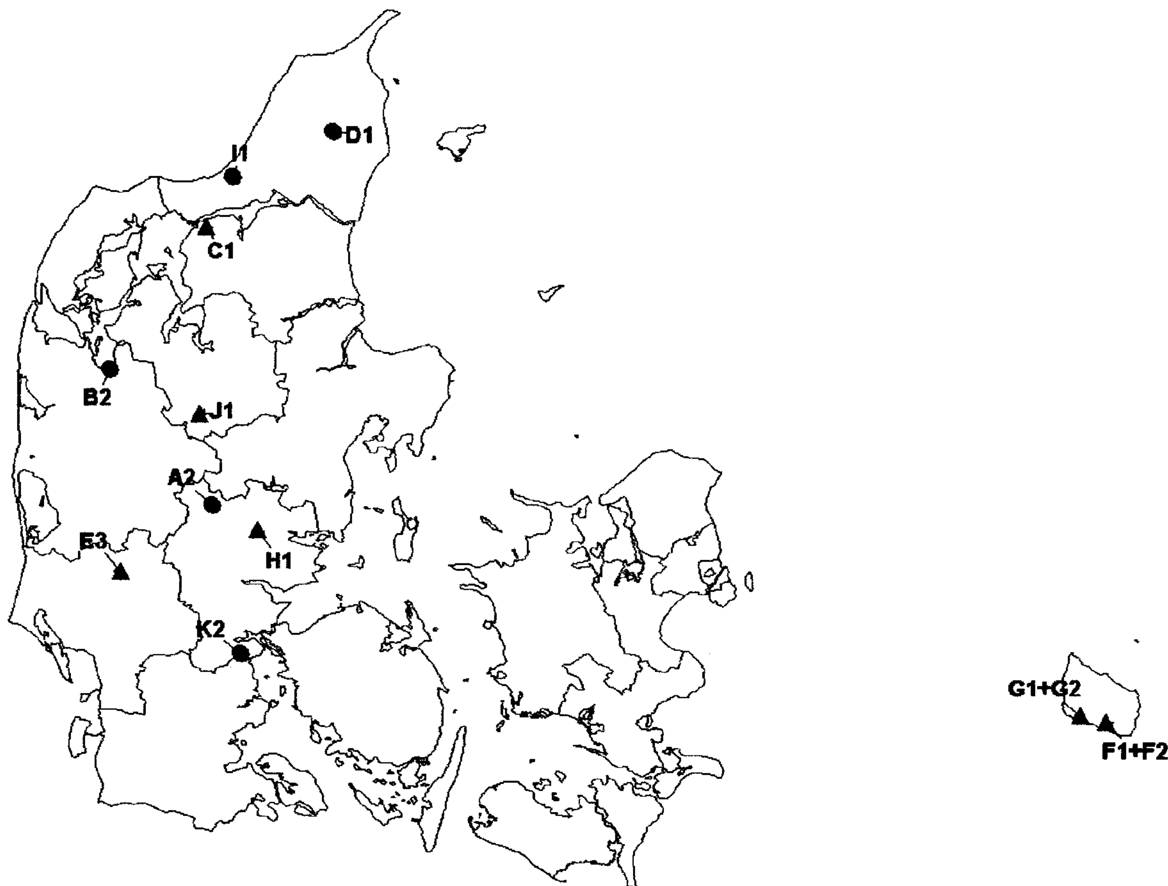

FIG. 1. Geographical distribution of the broiler farms under study. ▲, farms contaminated by *C. jejuni* clones with *fla* type 1/1 or 1/1b; ●, other farms.

holm farms raised older chickens up to 49 days of age, produced five flocks each year, and slaughtered in several batches. Both farms consisted of two houses, and both houses were under study.

From all flocks (75 flocks in total, of which *C. jejuni* was not isolated from 10 flocks), one sample, consisting of a pool of 10 cloacal swabs taken at the abattoir, was collected from each batch at slaughter (Table 1). However, on farms F and G up to eight batches were slaughtered from each flock. In most cases one or two isolates were selected from the first batches that were slaughtered from each flock. From J1, one isolate from rotation 2 and one isolate from rotation 4 were lost during storage, and the same was the case with one isolate from H1 rotation 7.

Bacteriological analysis was done as described previously (26). In brief, swabs were left for approximately 2 min in a tube containing 3 ml of sterile water, whereafter 10 μl was streaked onto modified CCDA plates (Oxoid CM 739/SR 155). The plates were incubated in a microaerobic atmosphere for 48 h. Isolates were identified to species level by standard procedures: oxidase (positive), catalase (positive), the ability to hydrolyze hippurate (positive), the production of indoxyl acetase (positive), and resistance to nalidixic acid (negative) and cephalothin (positive) (15).

MapInfo Professional (version 5.5; MapInfo Corporation, Troy, N.Y.) was used for topographic visualization of the farms and *fla* type results.

Typing methods. (i) Serotyping. Serotyping was performed according to the Penner serotyping scheme as previously described (14) but with the use of all 66 antisera of the system (47 *C. jejuni* antisera and 19 *Campylobacter coli* antisera).

(ii) PCR-RFLP. PCR-RFLP profiles of the *flaA* gene were produced as previously described using restriction endonucleases *Dde*I and *Alu*I (19). Computer-assisted identification using GelCompar (Applied Maths, Kortrijk, Belgium) was used for identification of RFLP profiles, as previously described (13).

(iii) PFGE. PFGE analysis was performed as previously described (19) using restriction endonucleases *Sma*I (Gibco), *Kpn*I (Gibco), and *Bam*HI (Gibco), with the following ramping parameters for *Bam*HI digests: 2 to 5 s, 11 h; 6 to 12 s, 6 h; and 15 to 20 s, 5 h. MRPs were compared visually and assigned to arbitrarily defined profile types. Repeated gel runs were done to confirm profile identity or similarity. Isolates that could not be distinguished by MRPs using *Sma*I, *Kpn*I, or *Bam*HI were considered to belong to the same clone (16). Epidemiologically related isolates (isolated on the same farm) that could be distinguished by minor differences in *Bam*HI MRPs but not by other MRPs were considered to belong to the same clone (22, 23). Epidemiologically related isolates (isolated on the same farm) that could be distinguished by not more than two band differences in each of the *Sma*I and *Kpn*I MRPs and shared significant similarity in the *Bam*HI MRPs were presumed to be related (22).

RESULTS

Twenty-three *fla* types, 37 *Sma*I MRP types, and 42 *Kpn*I MRP types were found in this study (Table 1). Representative *fla* profiles are shown in Fig. 2, and MRPs are shown in Fig. 3. General features of *fla* types (13, 19) and of MRPs (16, 19) were as previously described.

Thirteen clones that occurred in more than one broiler flock were identified (Table 1). Minor differences in *Bam*HI MRPs discriminated within two such clones; both were restricted to one broiler farm each. In clone 5, isolates P0193, P0194, P0195, and P0196 could be distinguished from isolates P0190, P0191, and P0192 by an upward band shift at around 155 kb, and in addition isolate P0190 could be distinguished by a difference in the intensity in one band and the presence of one extra band at

TABLE 1. Serotypes and genotypes of *C. jejuni* isolates from broiler flocks

Farm and house no.	Rotation	Isolate no.	*fla* types (*Dde*I/*Alu*I)	MRP type			Clonal group[a]	Serotype
				*Sma*I	*Kpn*I	*Bam*HI		
A2	1	P0181	48/48	22	27	1	12	O:4 complex
	2	P0182	1/1	10	9	ND[b]		O:5
		P0183	1/1	10	10	ND		O:5
	3	P0184	48/48	22	28	1	(12)	ND
	5	P0185	48/48	22	27	1	12	ND
	6	P0186	5/5	14	31	ND		O:12
B2	1	P0173	11/11a	12	14	21		ND
	2	P0174	33/33	19	23	2	8	O:4 complex
	3	P0175	11/11a	12	15	21		ND
	4	P0176	33/33	19	23	2	8	ND
		P0177	33/33	19	23	2	8	ND
	5	P0178	9/9	34	41	ND		ND
	6	P0179	66/66	19	23	2	8	O:4 complex
	7	P0180	33/33	19	23	2	8	ND
C1	1	P0187	7/7	30	38	ND		ND
	1	P0188	51/51a	25	32	ND		ND
	2	P0189	67/67	27	34	ND		ND
	3	P0190	1/1	8	8	6	5	O:53
		P0191	1/1	8	8	7	5	ND
	4	P0192	1/1	8	8	7	5	ND
	5	P0193	1/1	8	8	8	5	ND
		P0194	1/1	8	8	8	5	ND
	6	P0195	1/1	8	8	8	5	ND
	7	P0196	1/1	8	8	8	5	ND
D1	1	P0158	65/65	26	33	ND		ND
	2	P0159	5/5	23	29	ND		O:12
		P0160	5/5	23	29	22		ND
	3	P0161	2/2	14	17	9	**13**	ND
		P0162	31/31b	17	21	ND		O:29(42)
	4	P0163	1/1	9	13	ND		O:4 complex
	6	P0164	8/8	32	39	ND		O:42
E3	1	P0204	1/1b	5	4	10	1	O:2
		P0205	1/1b	5	4	10	1	ND
	2	P0206	1/1b	5	4	10	1	ND
	3	P0207	1/1b	5	4	10	1	ND
	4	P0208	1/1b	5	4	10	1	ND
		P0209	33/33	18	22	3	9	ND
	5	P0210	66/68	20	25	4	(9)	O:4 complex
	6	P0211	66/66	18	22	5	9	ND
	7	P0212	1/1b	6	5	11	(1)	O:2
		P0213	1/1b	6	5	12	(1)	ND
F1	1	P0103	1/1	4	7	13	**6**	O:2
	2	P0102	1/1	4	7	13	**6**	ND
		P0166	30/30	15	19	14	11	O:11
		P0117	30/30	15	19	14	11	ND
	3	P0100	2/2	14	17	9	**13**	O:1,44
		P0118	2/2	14	17	9	**13**	ND
		P0120	30/30	15	19	14	11	ND
		P0121	30/30	15	19	14	11	ND
	4	P0105	1/1	7	6	15	7	ND
		P0106	1/1	37	12	20		O:1,44
	5	P0104	1/1	7	6	15	7	O:2
F2	1	P0112	1/1	4	7	13	**6**	ND
	2	P0110	1/1	4	7	13	**6**	ND
		P0111	1/1	4	7	13	**6**	O:2
	3	P0122	2/2	14	17	9	**13**	O:1,44
		P0123	2/2	14	17	9	**13**	ND
		P0124	30/30	15	19	14	11	ND
		P0125	30/30	15	19	14	11	ND
		P0108	1/1	4	7	13	**6**	ND

Continued on following page

TABLE 1—*Continued*

Farm and house no.	Rotation	Isolate no.	*fla* types (*Dde*I/*Alu*I)	MRP type			Clonal group[a]	Serotype
				*Sma*I	*Kpn*I	*Bam*HI		
	4	P0115	39/14a	13	16	ND		ND
	5	P0113	1/1	7	6	15	7	ND
		P0114	1/1	7	6	15	7	ND
G1	3	P0141	2/2	14	17	9	**13**	O:1,44
	4	P0132	8/8	31	40	ND		O:42
		P0133	1/1	1	1	16	**2**	ND
	5	P0134	2/2	14	17	9	**13**	ND
		P0135	2/2	14	17	9	**13**	ND
G2	3	P0140	1/1	4	7	13	**6**	O:2
	4	P0136	2/2	14	17	9	**13**	ND
	5	P0137	5/5	24	30	ND		O:12
		P0138	1/1	11	11	ND		O:4 complex
		P0139	2/2	14	17	9	**13**	ND
H1	1	P0165	1/1	1	1	16	**2**	0:2
	2	P0166	1/1	2	1	16	**(2)**	ND
	3	P0167	1/1	1	1	16	**2**	ND
		P0168	9/9	35	42	ND		O:31
	4	P0169	1/1	1	1	16	**2**	ND
	5	P0170	1/1	1	1	16	**2**	ND
	6	P0171	1/1	1	1	16	**2**	ND
	7	P0172	69/69	28	36	ND		O:21
J1	1	P0142	1/1	3	2	17	3	O:2
	4	P0147	1/1	3	2	17	3	ND
	5	P0149	31/31	16	20	ND		NT[c]
	6	P0153	44/44	21	26	23		ND
		P0154	1/1	4	3	18	4	ND
	7	P0156	1/1	4	3	18	4	ND
K2	1	P0197	7/7	29	37	19	10	O:4 complex
	2	P0198	68/68	36	35	ND		O:31
	3	P0199	35/35	38	18	ND		O:1,44
	5	P0200	7/7	29	37	19	10	ND
	6	P0201	7/7	29	37	19	10	ND
	7	P0202	7/7	29	37	19	10	ND
		P0203	7/7	29	37	19	10	ND

[a] Parentheses indicate that the isolate showed a high degree of similarity to the clonal group. Clonal groups that were found on more than one farm are indicated in boldface.
[b] ND, not done.
[c] NT, nontypeable.

around 48 kb. In clone 9, isolate P0209 could be distinguished from P0211 by a minor band shift at 130 kb (Fig. 3, lanes 19 and 28).

In addition, there were five isolates from four different flocks that had slightly different MRPs using *Sma*I and/or *Kpn*I (not more than two band differences in each set of MRPs) than clones from the same farm (Table 1).

Clones 2 (and isolate P0166), 3, 4, 6, and 7 all had *fla* type 1/1, whereas clone 1 (and isolates P0212 and P0213) had the slightly different *fla* type 1/1b, which differed from 1/1 by a minor band shift in the *Alu*I profile. Representative isolates from each of those clones all had serotype O:2. Furthermore, a high proportion of bands in the MRPs obtained using either restriction endonuclease were shared by all or several clones (Fig. 3).

Isolates from clones 8 and 9 were distinguished from each other by single band shifts in each set of MRPs. The positions of the upper bands in the *Bam*HI MRPs (240 and 200 kb, respectively), the positions of two bands in the 350- to 400-kb range in the *Sma*I MRPs, and the positions of the second-largest band in the *Kpn*I MRPs (165 and 190 kb, respectively) distinguished isolates from the two farms (Fig. 3, lanes 18, 19, 27, and 28). Among these isolates, *fla* types 33/33 and 66/66 were found on both farms, and one additional *fla* type, 66/68, was found on farm E3. *fla* profiles 33 and 66 differed by two bands using *Dde*I and by one band using *Alu*I. *Alu*I profile 68 resembled profile 66 (two band differences) more than it resembled profile 33 (Fig. 2).

Ten clones were restricted to one farm each, two clones (clones 2 and 6) were both found on two different farms, and one clone (clone 13) was found on three farms. One or more isolates from each clone were serotyped. Representatives from six clones were serotype O:2, three were serotype O:4 complex, and the remaining belonged to serotypes O:1,44; O:11, and O:53.

Isolates that were identical or highly similar to isolates from

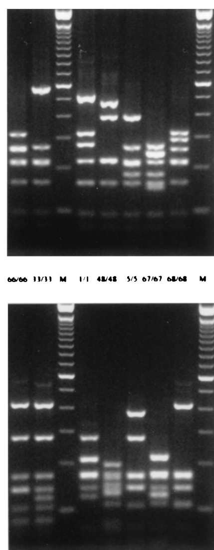

FIG. 2. *fla* profiles of representative isolates using restriction endonucleases *Dde*I (upper panel) and *Alu*I (lower panel). The numbers between the photographs indicate the *Dde*I or *Alu*I profile type.

other rotations in the same farm were found in 47 flocks, or 72% of the positive flocks (63% of all flocks). A unique isolate was found in 21 flocks, or 32% of the positive flocks (28% of all flocks).

fla type 1/1 or 1/1b was represented by 41 isolates (44%), of which 33 were distributed on seven clones, including the six O:2 clones and O:53. The remaining clones had *fla* types 2/2 (O:1,44), 7/7 (O:4 complex), 30/30 (O:11), and 48/48 (O:4 complex), and two clones (O:4 complex) had *fla* type 33/33 or 66/66.

DISCUSSION

Farm-specific as well as unspecific clones were found in this study. The finding of farm-specific clones that occur in successive rotations strongly indicates that those clones persist on the individual farms, either inside the broiler house or in the near environment. In some cases, stretches of flocks where a clone was isolated were interrupted by flocks where a different isolate or no isolate was found. This can be due to shortcomings of the cultivation method or to the sampling size. Some flocks may be infected by two different strains, but only one isolate from each batch is obtained by the method used. Therefore, the importance of persistent clones, as well as of sporadic isolates, can be underestimated here. Evidently, farm-specific sources are of primary importance, as they can account for the majority of the positive flocks in this study, at least the 63% of all flocks where a persistent clone was actually isolated. In contrast, sporadic or unique isolates were found in 28% of flocks, strongly indicating that sporadic sources were of minor importance in the farms under study.

No obvious contamination source could explain the finding of clones 2 and 13 on farms located in different parts of the country. The Bornholm farms but not the remaining farms used a feed mill and abattoir located on the island of Bornholm and a Swedish hatchery. Therefore, feed, hatchery, or abattoir is not likely to be the source of common clones. This finding can be explained by a ubiquitous occurrence of the highly stable clones 2 and 13. The occurrence of two different clones on both of the Bornholm farms could be explained by the fact that the broiler flocks were divided into a high number of batches at slaughter, thereby increasing the risk of introducing contamination by new clones to the farms, in combination with the ability of those clones to persist on the farm after introduction.

Studies in several European countries (3, 5, 8) have identified risk factors such as the lack of appropriate hygiene barriers around broiler houses and infestation with insects or rodents. A possible explanation for the persistence or survival of *Campylobacter* clones on farms could be that local populations of rodents or beetles, etc., that are able to evacuate the house during cleaning and disinfection serve as reservoirs (3, 8, 9). It is also possible that this problem may be related to the handling of used litter. Used litter from broiler houses presumably contains large numbers of campylobacteria when removed from the house. Experiments have shown that *C. jejuni* has a good ability to survive during anaerobic digestion of animal waste (10, 11). An earlier study showed that houseflies can function as a vector for *C. jejuni* when a reservoir is located near the broiler house (20). If litter is not handled properly on the farm, it therefore has the potential to function as a continuous source of campylobacteria for broiler flocks housed on the farm. Further epidemiological investigations may clarify the role of litter handling in campylobacter colonization of broiler flocks.

Genotypic variation in *C. jejuni* and its impact on the applicability of genotyping methods for epidemiological studies have been extensively debated in recent years (6, 7, 24, 25). Minor differences in MRPs obtained using the frequently cutting enzyme *Bam*HI were observed in epidemiologically related isolates, but according to generally accepted criteria for the interpretation of genotyping data, this is consistent with a clonal relationship (22, 23). Single isolates that were distinguished from clones 1, 2, 9, and 12 by minor differences in the MRPs obtained using *Sma*I and/or *Kpn*I were found. The epidemiological information supports the argument that isolates P0166, P0184, P0210, P0212, and P0213 originated from clones 2, 12, 9, and 1, respectively. Assuming that those five isolates were genotypic variants of the clones, the occurrence of genotypic variants in this study was sporadic. We speculate that the identified clones may be representatives of *C. jejuni* strains that are well adapted for the colonization of broiler flocks and for survival around broiler houses. Successful as they are, their survival skills may not easily be improved by random genetic events, and genetically modified variants will remain outnumbered by the mother strains.

Clones 1 to 4 and clones 6 and 7 shared a high degree of similarity in MRPs. Furthermore, representative isolates from each clone all had serotype O:2, and all isolates had *fla* type1/1 or 1/1b. The results indicated that this group of clones may be representatives of a subgroup of *C. jejuni* that is largely clonal. Further investigations may clarify the importance of the identified clones in human infections.

The variable *fla* type identified in clones 8 and 9 showed an interesting pattern in that both clones, isolated from two different broiler farms (E3 and B2), expressed the same two *fla*

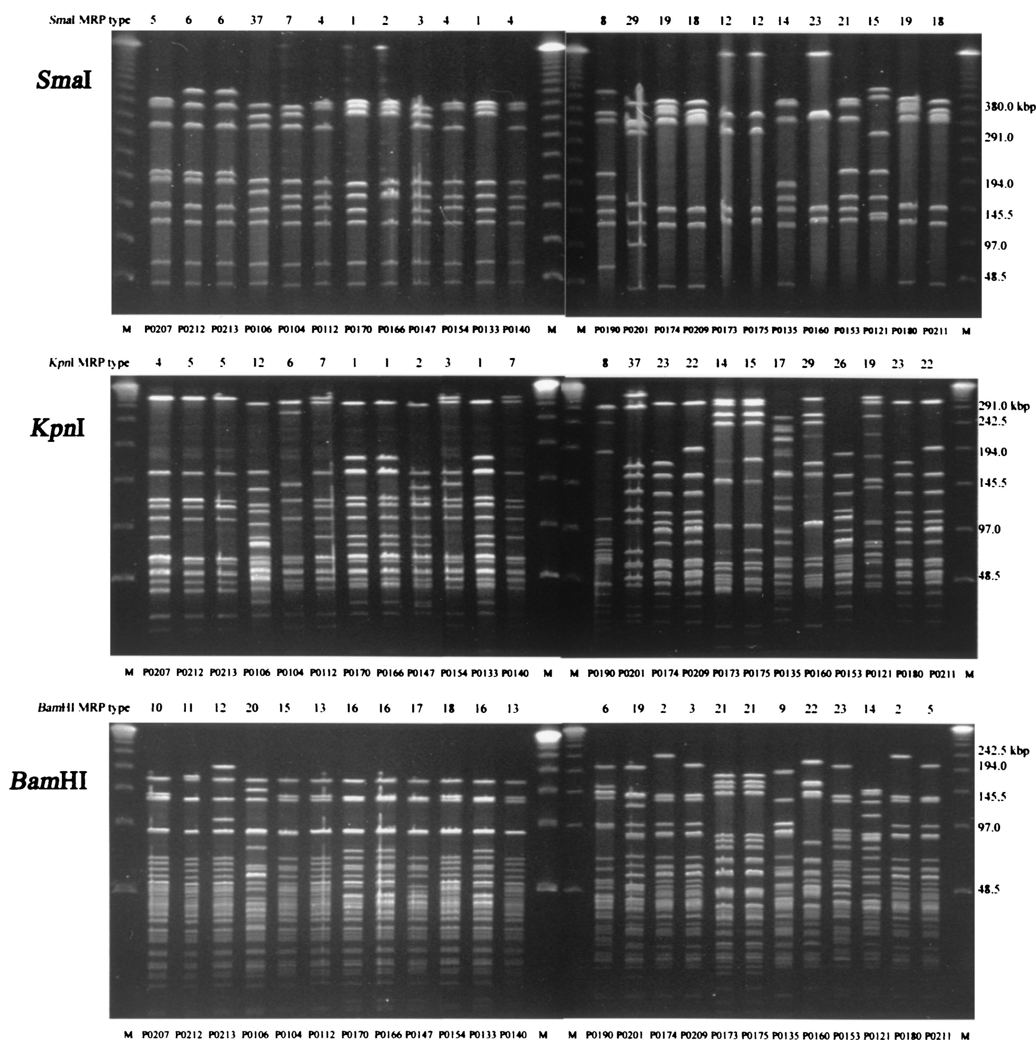

FIG. 3. Macrorestriction profiles of representative isolates of *C. jejuni* using restriction endonucleases *Sma*I, *Kpn*I, and *Bam*HI. The numbers below each photograph indicate the isolate numbers as listed in Table 1. The numbers above the photographs indicate MRP types, as shown in Table 1.

types, which are highly similar. Harrington and colleagues (7) described intergenomic recombination events in the *fla* genes of some strains, and similar events may explain our finding. It is possible that *fla* type variation is a feature of some clonal lineages, and this should be kept in mind during epidemiological studies.

In conclusion, we have shown that local contamination reservoirs are of importance for the colonization of broiler farms with *C. jejuni*. Colonization by sporadic isolates was of lesser importance here but may be more significant in farms where persistent clones do not propagate. The possible existence of common contamination reservoirs is also indicated but in this study accounted for a minor proportion of infected flocks. Highly stable clones of *C. jejuni* were identified, of which a subgroup belonging to serotype O:2 appear to be interrelated. Further typing studies involving human and broiler isolates might help in elucidating the importance of the identified clones in relation to human infections.

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