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Flagellin Gene Typing of *Campylobacter jejuni* by Restriction Fragment Length Polymorphism Analysis

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We developed and studied a molecular typing approach for *Campylobacter* spp. with restriction fragment length polymorphism (RFLP) analysis of the flagellin gene *flaA* in *C. jejuni*. Using polymerase chain reaction, we amplified the *flaA* gene from strains comprising different HL:O serotypes by using a primer set directed at the conserved 5′ and 3′ *flaA* gene sequence to generate a 1.7-kb amplicon. The amplicon was further digested with the restriction enzyme *Dde*I, and the fragments generated were analyzed by agarose gel electrophoresis. In 43 non-outbreak strains of six common HL serotypes (HL 1, 2, 4, 5, 9, and 36) in the United States, 18 RFLP patterns were observed. In U.S. outbreak strains previously studied by 10 other typing methods, *flaA* typing correlated with the HL serotype within each outbreak, and six additional *flaA* types were identified. Our results suggest that RFLP analysis of the *flaA* gene from *Campylobacter* spp. has sufficient discrimination to be useful as a practical typing method for clinical and epidemiologic investigations.

Campylobacter jejuni is one of the most common causes of bacterial gastroenteritis in the United States and worldwide (19), with an estimated annual incidence of 2 million cases in the United States. Although most of the cases in the United States occur sporadically, common-source outbreaks do occur. *Campylobacter* infections are acquired by ingestion of contaminated food, water, or milk products, and although numerous vehicles for transmission of *Campylobacter* infections have been described, poultry appear to be the single most important vehicle for sporadic cases in the United States and other developed nations (18, 19). Numerous typing schemes have been developed for studying the epidemiology of *Campylobacter* infections, and the usefulness of these methods was recently reviewed by Patton and Wachsmuth (13). The most commonly used methods today include serotyping to detect heat-labile (HL) antigens (9) or O (formerly called heat-stable) antigens (15). These methods have been applied widely in epidemiologic investigations (13), but they require appropriate serotyping reagents that are expensive and time-consuming to produce. Therefore, only a few reference laboratories can provide serotyping studies. An alternative method that would be simple to perform, would not require specialized reagents (i.e., antisera), and could be used by many investigators for clinical and/or epidemiologic studies is highly desirable. The flagellin gene *flaA* in *Campylobacter* spp. appears to have significant sequence heterogeneity (4, 20), such that molecular analysis of the flagellin genes might serve as a good epidemiologic marker. We developed a polymerase chain reaction (PCR) method to amplify the *flaA* gene of *C. jejuni* and used restriction fragment length polymorphism (RFLP) analysis of this gene to determine whether such a system would provide an alternative to serotyping.

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MATERIALS AND METHODS

Bacterial strains. A total of 65 *C. jejuni* isolates from the collection maintained in the Campylobacter Reference Laboratory, Centers for Disease Control and Prevention, were studied. Forty-three strains comprising six of the common HL serotypes in United States are listed in Table 1. These strains were referred to as non-outbreak strains; most strains were isolated from sporadic cases of infection. In a few instances, one or two strains isolated from an outbreak were included, but in no instance was more than one HL serotype from a particular outbreak used. For additional studies, we examined 22 well-defined strains from four *Campylobacter* outbreaks (Table 2) that had been typed by 10 different methods (14).

Bacterial extracts. Bacteria were grown on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) overnight at 42°C in a Tri-Gas incubator (Forma Scientific) with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2. A loopful of bacteria was mixed with 0.2 ml of sterile double-distilled water, vortexed, and heated for 5 min in a boiling water bath. The bacteria were sedimented by centrifugation in a microcentrifuge (model 235 A; Fisher Scientific) at 13,000 × *g* for 5 min, and the supernatant was removed for testing.

PCR. A 5-μl aliquot of the bacterial extract was used as template for PCR. Primers were synthesized by using an Applied Biosystems PCR Mate (Forster City, Calif.). The forward primer, 5′-GGATTTCGTATTAACACAAATGGT GC-3′, corresponds to nucleotides 1 through 26 in the *flaA* gene, and the reverse primer, 5′-CTGTAGTAATCTTAAA ACATTTTG-3′ corresponds to nucleotides 1705 through 1728 of *flaA*, on the basis of previous sequence data (4). PCR was performed with an MJ Research MiniCycler, model PTC-150 (Watertown, Mass.). Samples first were incubated for 1 min at 94°C and then were cycled 35 times at 92°C for 30 s, at 55°C for 1.5 min, and at 72°C for 2.5 min. The samples were then incubated at 72°C for 5 min and were maintained at 4°C until processed. For some strains that did not produce a PCR product when the water extract was

TABLE 1. Source of strains and *flaA* type of common HL serotypes isolated in the United States[a]

HL type	Strain	Source	O type[b]	*flaA* type
1	D3302	Colorado	4	1
	D3534	Illinois	8	1
	D3774[c]	HL 1 Reference strain	4	1
	D3481	Minnesota	16,50,13	1
	D3173	Washington	4	1
2	D3775[c]	HL 2 Reference strain	1	3
	D3115	Washington	4,43,50w	4
	D3195	Oregon	8	1
	D3044	Minnesota	1,8	5
	D3306	New York	1,8	1
4	A5721	Arizona	2	1
	D3448	New York	2	6
	D1090	Georgia	4	7
	D3391	California	2	8
	D3140	Oklahoma	16,50	9
	D3016	Missouri	8,44	7
	D2772	Maine	2	7
	D2692	Kansas, milk	2	10
	D1130[d]	Georgia	3,13,8w	7
	D1094	Georgia	2	7
	D3269	Oregon	2	7
	D3497	Missouri	2	7
	D3776[e]	England	2	7
	D3109	California	2	7
	D3533	Illinois	2	10
5	D3510	New York	23,36	11
	SSU6391	Vermont	36,23	11
	A6571	Illinois	36,23	12
	D3552	New York	23,36	13
	D3172	Washington	23,36,15w	13
	D3170	Washington	21,18,29	13
	D2289	Vermont, cow feces	22,36,23	13
	D2677	Kansas, cow feces	36,23,15w	13
	D1148	Georgia	1	1
9	D3102	Wisconsin	5^+	6
	D3329	Alabama	5^-,5^+w	14
	D3242	Massachusetts	5^+w	14
	D3573	New York	5^-,5^+	14
36	D3392	Minnesota	3	15
	D3150	Alabama	3	16
	D3778[c,f]	Reference strain	3,13	17
	D3162	Colorado	3,43	18
	D3138	Minnesota	16,50w	2

[a] All are human isolates except where noted.
[b] Formerly the heat-stable serotype.
[c] Country of origin not indicated.
[d] Hippurate negative.
[e] NCTC11168; HL 4 reference strain.
[f] HL 36 reference strain.

used, the DNA from the water extract was further extracted (twice) with phenol-chloroform according to the method of Sambrook et al. (17) and recovered by ethanol precipitation. The pellet was resuspended in double-distilled water, the DNA concentration was measured by optical density at 260 and 280 nm, and 1 μg of DNA was used as template for repeat PCR. After amplification, 5 μl of the PCR product was electrophoresed with 0.8% Agarose (ultraPure Agarose, electrophoresis grade; Bethesda Research Laboratories, Inc. [BRL], Gaithersburg, Md. [catalog no. 5510UA]) in 1× Tris-borate-EDTA (TBE) buffer (Gel-Mix Running Mate, BRL) containing 1 μg of ethidium bromide per ml to determine the presence or absence of the *flaA* amplicon.

TABLE 2. *flaA* type and serotype of *C. jejuni* outbreak strains

Outbreak	Strain[a]	Source	EI[b]	HL	O[c]	*flaA* type
1	EDL18	Human	Y	5	36,23,22	13
	EDL22	Human	Y	5	23,36,22	13
	SSU9892	Cow, dairy A	Y	5	23,36,22	13
	SSU9894	Cow, dairy A	Y	5	23,36,22	13
	SSU9896	Cow, dairy A	Y	4	2	7
	EDL2	Cow, dairy B	N	5	36,23	13
	EDL3	Cow, dairy B	N	5	36,23	13
	EDL4	Cow, dairy B	N	5	36,23	13
2	D226	Human	Y	4	2	7
	D224	Cow, feces	Y	4	2	7
3	D1117	Child	Y	4	2	7
	D1118	Milk	Y	4	2	7
	D1114	Calf	Y	4	2	7
	D1108	Cow, feces	Y	1	4	19
	D1159	Pig (*C. coli*)	N	44	46,15	20
4	D445	Human	Y	77	19	21
	D450	Human	Y	77	19	21
	D452	Human	Y	77	19	21
	D462	Bird	N	5	19,23,34	22
	D467	Bird	N	5	23,19,34	22
	D472	Bird	N	NT[d]	19,13	23
	D473	Bird	N	NT	NT	24

[a] Strains were previously described in reference 14.
[b] EI, epidemiologically implicated in outbreak (Y, yes; N, no).
[c] Formerly the heat-stable serotype.
[d] NT, nontypeable.

RFLP analysis. A 5-μl sample of the PCR product was digested with the restriction enzyme *Dde*I ([catalog no. 835293] Boehringer Mannheim) by using incubation buffer H (Boehringer Mannheim) (50 mmol of Tris-HCl per liter, 100 mmol of NaCl, 10 mmol of $MgCl_2$, 1 mmol of dithioerythritol, pH 7.5) according to manufacturer's instructions overnight at 37°C. The digest (17 μl) was mixed with 1.1 μl of H_2O–1.9 μl of 10× loading buffer, and was analyzed by electrophoresis by using 4% NuSieve GTG Agarose (FMC BioProducts, Rockland, Me. [catalog no. 50081]) containing 1 μg of ethidium bromide per ml. A 123-bp DNA ladder was used as standard for molecular size determinations (Gibco BRL [catalog no. 5613SA]). Gels were run at 120 V for 5 h in 0.5× TBE buffer (BRL). The gels were then examined by transillumination, and photographs were taken. In some instances, the PCR product was first concentrated by ethanol precipitation before digestion (n = 22) and then was analyzed as described above.

Serotyping. All isolates used in this study were serotyped by the HL method of Lior et al. (9) and the O method of Penner and Hennessey (15) with reagents prepared at the Campylobacter Reference Laboratory, Centers for Disease Control and Prevention (12).

RESULTS

Of the 65 isolates studied, we found that 83% could be successfully amplified by a simple water extraction of the

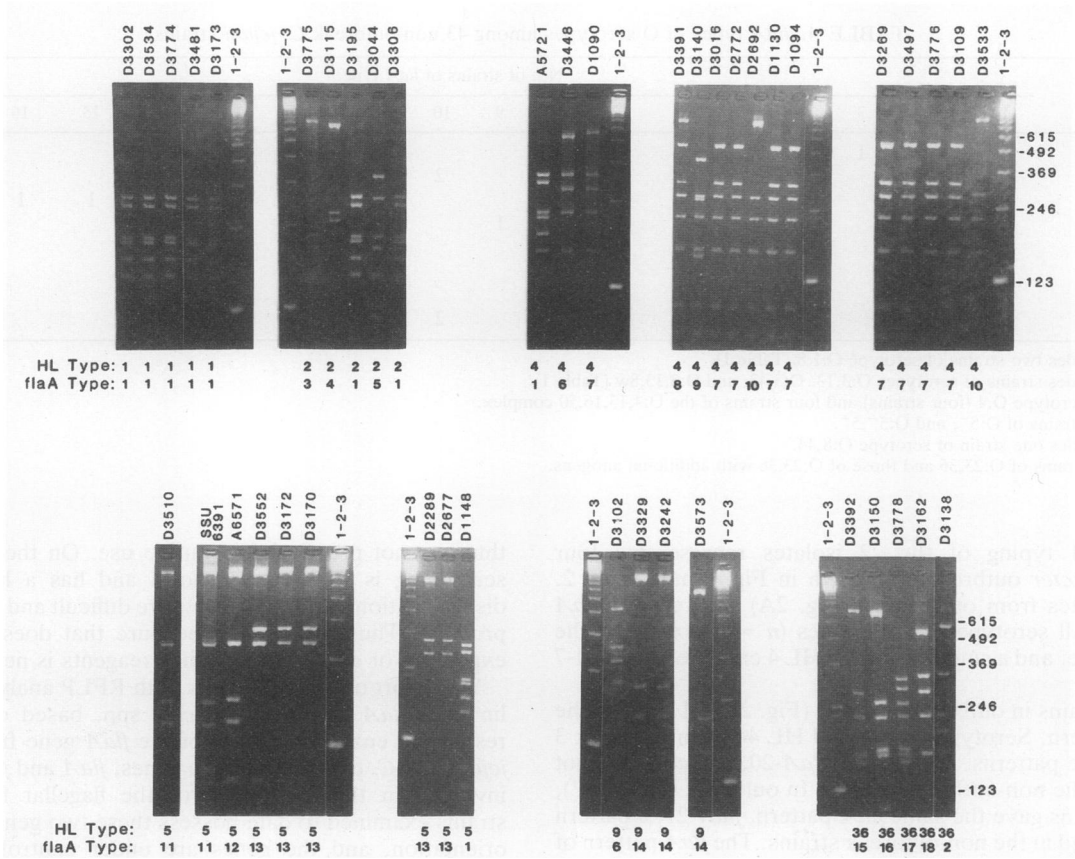

FIG. 1. RFLP patterns among 43 non-outbreak strains of *C. jejuni*. Strains are grouped according to HL type. Below each photograph is the HL type and designated *flaA* type for each strain. Each set of gels has a 123-bp marker ladder, and the size for the ladder is indicated on the far right.

chromosomal DNA. We substituted *Taq* buffer for water with the remaining strains (n = 11), 4 of which could be amplified. The remaining seven isolates required additional purification by phenol-chloroform extraction.

We observed 18 distinct *flaA* RFLP patterns when representative isolates of different HL serotypes were studied (Fig. 1 and Table 3). All five strains of serotype HL 1 exhibited the same *flaA* type, designated *flaA*-1. Multiple *flaA* types were identified among strains of the five additional serotypes tested. There were four distinct patterns observed in HL 2 strains, *flaA*-1, *flaA*-3, *flaA*-4, and *flaA*-5. Two of the five HL 2 strains examined also showed the *flaA*-1 type. Six patterns were observed in the 15 HL 4 strains, *flaA*-1, *flaA*-6, *flaA*-7, *flaA*-8, *flaA*-9, and *flaA*-10. Nine of the 15 strains showed the *flaA*-7 type. Nine isolates of serotype HL 5 exhibited four *flaA* types, *flaA*-1, *flaA*-11, *flaA*-12, and *flaA*-13, five isolates of which showed *flaA*-13. Four HL 9 strains exhibited two *flaA* patterns, *flaA*-6 and *flaA*-14, with three of the four strains being *flaA*-14. Finally, five HL 36 strains exhibited five different *flaA* types, *flaA*-2, *flaA*-15, *flaA*-16, *flaA*-17, and *flaA*-18, which were different from all other *flaA* types. Sixteen of 18 *flaA* types were restricted to a single HL type. *flaA*-1 and *flaA*-6 types were distributed among four and two HL types, respectively.

We also analyzed the correlation between *flaA* type and O antigen type, as shown in Table 4. The 18 *flaA* types identified in non-outbreak strains comprised eight major O groups. Similar to the distribution of *flaA* types among common HL types, there were multiple *flaA* types within a particular O group. Most of the *flaA* types were restricted to a single O type; however, *flaA*-1, *flaA*-6, *flaA*-7, and *flaA*-13 were present in several O types.

TABLE 3. *flaA* types of common HL serotypes among 43 non-outbreak *C. jejuni* strains

HL type (no. of strains)	No. of strains of *flaA* type:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 (5)	5																	
2 (5)	2		1	1	1													
4 (15)	1					1	9	1	1	2								
5 (9)	1										2	1	5					
9 (4)						1								3				
36 (5)		1													1	1	1	1

TABLE 4. *flaA* types of O serotypes among 43 non-outbreak *C. jejuni* strains

O type (no. tested)	No. of strains of *flaA* type:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1[a] (4)	2		1		1													
2 (11)	1					1	6	1		2								
3[b] (5)							1								1	1	1	1
4[c] (8)	4	1		1			1		1									
5[d] (4)						1								3				
8[e] (3)	2						1											
21,18,29 (1)													1					
23,36[f] (7)										2	1	4						

[a] Also includes two strains of serotype O:1,8 (Table 1).
[b] Also includes strains of serotypes O:3,13, O:3,43; and O:3,13,8w (Table 1).
[c] Includes serotype O:4 (four strains) and four strains of the O:4,13,16,50 complex.
[d] Includes strains of O:5^+, and O:5^-,5^+.
[e] Also includes one strain of serotype O:8,44.
[f] Includes strains of O:23,36 and those of O:23,36 with additional antigens.

The *flaA* typing of the 22 isolates representing four *Campylobacter* outbreaks is shown in Fig. 2 and Table 2. Eight isolates from outbreak 1 (Fig. 2A) showed two *flaA* patterns. All serotype HL 5 isolates ($n = 7$) exhibited the *flaA*-13 type, and a single isolate of HL 4 exhibited the *flaA*-7 type.

HL 4 strains in outbreaks 2 and 3 (Fig. 2B) all showed the *flaA*-7 pattern. Serotypes HL 1 and HL 44 from outbreak 3 gave unique patterns, *flaA*-19 and *flaA*-20, respectively, not seen with the non-outbreak strains. In outbreak 4 (Fig. 2C), HL 77 strains gave the same *flaA* pattern, *flaA*-21, a pattern not observed in the non-outbreak strains. The *flaA* pattern of two HL 5 strains, *flaA*-22, differed from other non-outbreak strains and from other HL 5 strains identified in outbreak 1. Two HL nontypeable strains in outbreak 4 also exhibited unique *flaA* patterns, *flaA*-23 and *flaA*-24. Overall, six *flaA* types not identified in the non-outbreak strains were seen among the outbreak isolates.

Typing results were compared with epidemiologic data (14), and within each epidemic, HL serotyping and *flaA* typing correctly identified the relationship among isolates. Both methods distinguished the epidemiologically implicated and nonimplicated strains within each outbreak.

Among the 22 outbreak strains, the *flaA* typing method was more discriminating than HL typing and identified eight types, versus the seven types identified by HL typing. *flaA* typing also distinguished strains of the same serotype; for example, serotype HL 5 strains in outbreaks 1 and 4 had different *flaA* types.

DISCUSSION

Numerous typing schemes have been used to study the epidemiology of *Campylobacter* spp. (13), including both phenotypic and genotypic methods. Phenotypic methods include serotyping (9, 15), biotyping (2, 8), and bacteriophage typing (5, 16). Genotyping methods have included restriction endonuclease analysis (11, 13, 14), analysis with genes coding for rRNA (7, 14), plasmid analysis (3), and multilocus enzyme electrophoretic typing (1). Patton and Wachsmuth (13) categorized typing methods by difficulty of performance (simple to complex) and discrimination (none to high). A good typing system should not be too complex to perform, yet should have high discrimination power. Methods such as multilocus enzyme electrophoretic typing have a high level of discrimination but are complex to perform and thus are not practical for routine use. On the other hand, serotyping is simple to perform and has a high level of discrimination, but the reagents are difficult and expensive to produce. Thus, a simple procedure that does not require expensive or difficult-to-produce reagents is needed.

We report our initial results with RFLP analysis of flagellin gene *flaA* in *Campylobacter* spp. based on PCR and restriction enzyme analysis of the *flaA* gene fragments. *C. jejuni* and *C. coli* possess two genes, *flaA* and *flaB*, that are involved in the expression of the flagellar filament. All strains examined to date possess these two genes in tandem orientation, and the genes are under control by distinct promoters (4, 6, 10). The *flaA* gene contains three regions; C1 and C2 represent the N-terminal and C-terminal conserved region, respectively, while the internal V1 region is variable (4). Using the observation that the N- and C-terminal regions of *flaA* are conserved, we used a primer set in a PCR that could amplify the *flaA* gene. We surmised that restriction analysis of the *flaA* gene with an appropriate restriction enzyme would give different types on the basis of the fact that the internal regions of these genes are variable (4, 20).

Using a limited number of strains from six commonly isolated HL serotypes in the United States, we were able to distinguish 18 *flaA* types. A predominant *flaA* type was apparent in several of the serotypes. In serotypes HL 3, 5, and 9, the predominant *flaA* types were *flaA*-7, *flaA*-13, and *flaA*-14, respectively (Table 1). In contrast, other serotypes had no dominant *flaA* type. For example, all five HL 36 strains had a distinct *flaA* pattern. Additional HL 36 strains and additional HL serotypes need to be examined to determine whether variability in *flaA* type is characteristic of all strains of a given serotype. We also observed overlap between HL types and *flaA* types. For example, the *flaA*-1 type was found in strains of serotypes HL 1, 2, 4, and 5 (Table 1). Similar findings were observed when *flaA* types were compared with O types of the non-outbreak isolates.

These results suggest extensive heterogeneity among the *flaA* genes in *C. jejuni*. Thornton et al. (20) also showed significant heterogeneity within the internal regions of the flagellin genes, as demonstrated by DNA hybridization. They suggested, however, that *Campylobacter* flagellins are more conserved than is indicated by the HL serotyping scheme. Our data would suggest, however, that, at least at the nucleotide level, there is much more heterogeneity than is suggested by HL serotyping.

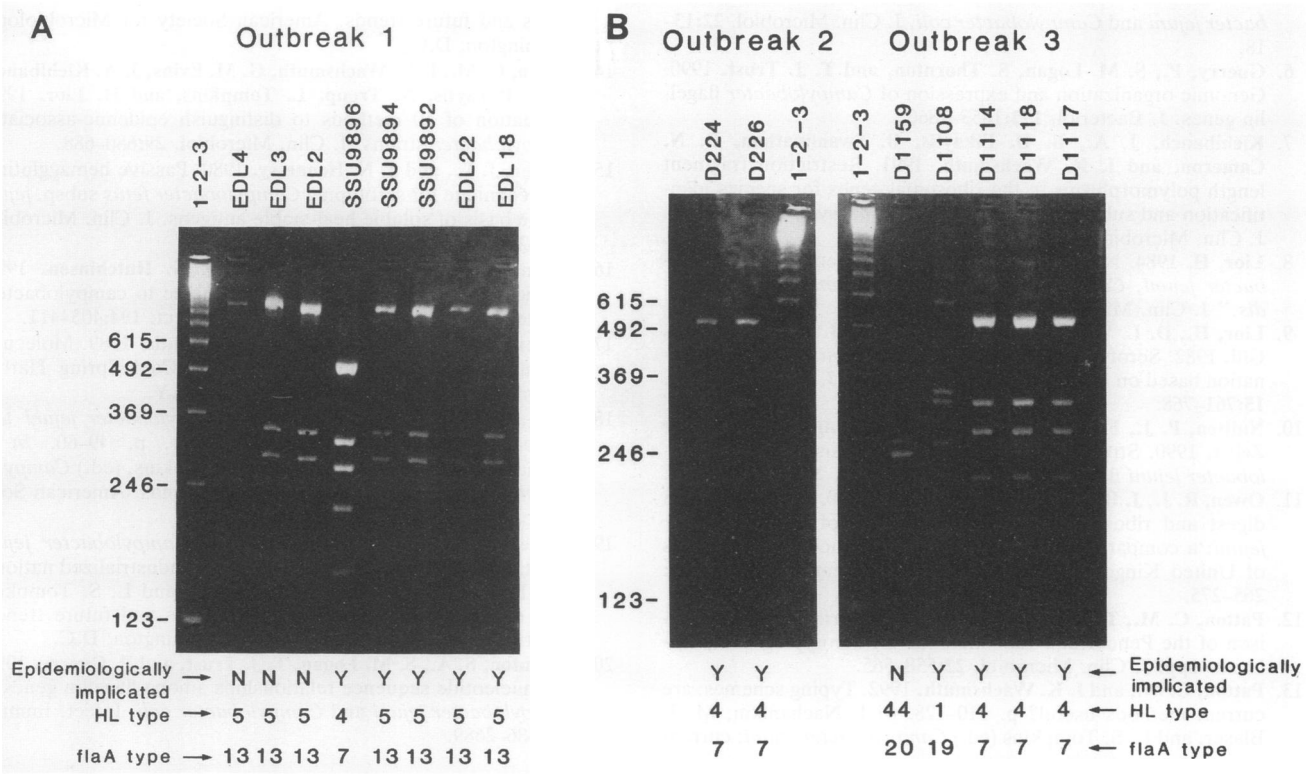

FIG. 2. RFLP patterns among strains associated with outbreaks 1 (A), 2 and 3 (B), and 4 (C) as described in reference 14. A 123-bp marker ladder is located in lane one. Notes at bottom of figures indicate whether the strain was epidemiologically linked in theoutbreak, the HL type, and the designated *flaA* type.

The value of *flaA* typing for epidemiologic studies was demonstrated in the examination of the outbreak strains. Within each epidemic, there was 100% correlation between the HL and *flaA* typing methods. These results provide strong support for *flaA* typing as an alternative to serotyping. Before *flaA* typing can be used on a broader scale for investigations, further studies of more strains of the serotypes studied and strains of additional serotypes are needed. Although we examined only *C. jejuni* strains, *flaA* typing should also be applicable to *C. coli*; extended studies should include this species also. Whether this method is applicable to other *Campylobacter* spp. is not known because the flagellar genes in other species have not been characterized. Future studies including additional serotypes are needed to truly assess the utility of this method for epidemiologic investigations.

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