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Analysis of HL and O Serotypes of *Campylobacter* Strains by the Flagellin Gene Typing System

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We recently developed a molecular typing system for *Campylobacter jejuni* and *Campylobacter coli* based on restriction fragment length polymorphism analysis of the flagellin gene, *flaA* (I. Nachamkin, K. Bohachick, and C. M. Patton, J. Clin. Microbiol. 31:1531–1536, 1993). We extended the typing system to 83 flagellin types (designated *flaA*-1, *flaA*-2, etc.) on the basis of analysis of 404 isolates of *C. jejuni* and *C. coli* including common serotypes isolated in the United States, a selection of less common serotypes, and serotype reference strains. Of the 295 strains previously shown to belong to common HL and O serotypes (C. M. Patton, M. A. Nicholson, S. M. Ostroff, A. A. Ries, I. K. Wachsmuth, and R. V. Tauxe, J. Clin. Microbiol. 31:1525–1530, 1993), six *flaA* types accounted for 53.6% of strains as follows: *flaA*-1, 21.7%; *flaA*-7, 14.9%; *flaA*-27, 5.1%; *flaA*-49, 4.4%; *flaA*-13, 3.7%; and *flaA*-21, 3.7%. Seventy-five percent of the strains were within 15 *flaA* types, 90% were within 30 *flaA* types, and all 295 strains were contained within 52 *flaA* types. Within each HL or O serotype, there usually were multiple *flaA* types. For 12 common HL serotypes and 7 common O serotypes, more than 50% of these isolates were a single *flaA* type. A database was developed by using commercially available restriction fragment length polymorphism analysis software (ProRFLP; DNA ProScan, Inc., Nashville, Tenn.) that should allow other investigators to perform typing with this system.

Campylobacter jejuni subsp. *jejuni* (referred to as *C. jejuni*) is one of the most common causes of bacterial gastroenteritis in the United States and worldwide, with an estimated annual incidence of 2 million cases in the United States (15). *C. jejuni* typically causes an acute enterocolitis accompanied by fever and abdominal cramping lasting 3 to 5 days. Although complications are infrequent, campylobacters may cause reactive arthritis and infections may be confused with acute appendicitis; resulting in unnecessary surgery, and there is increasing evidence that *Campylobacter* infection is associated with the development of Guillain-Barré syndrome (5, 6). Although deaths associated with campylobacter infection are uncommon, the morbidity due to the disease is significant and results in substantial economic costs (13).

Most *C. jejuni* cases in the United States occur sporadically; however, common-source outbreaks do occur. *Campylobacter* infections are acquired by ingestion of contaminated food, water, or milk, and although numerous vehicles for the transmission of *Campylobacter* infection have been described, chicken appears to be the single most important vehicle for sporadic cases in the United States and other developed nations (14, 15). Other reservoirs for the organism include turkeys, cattle, swine, goats, ducks, and sheep (14). Poultry can become contaminated with *Campylobacter* strains at various stages of production by endogenous or exogenous sources (12, 14). A system for tracking specific strains or serotypes of *Campylobacter* strains would be useful for studying the epidemiology of human infections, analyzing modes of transmission, and devising and assessing intervention strategies important in agriculture.

Serotyping and other means of typing *Campylobacter* spp. have played an important role in understanding the epidemiology and identifying the sources of *Campylobacter* infection. Numerous typing schemes have been developed, and the usefulness of these methods has been reviewed (9). The most commonly used methods today include serotyping to detect heat-labile (HL) antigens (4) or O (formerly called heat-stable) antigens (11). These methods have been applied widely in epidemiologic investigations (9, 10), but they require appropriate serotyping reagents that are expensive and extraordinarily time-consuming to produce. Serotyping methods are moderately complex, yet they have an excellent ability to discriminate among unrelated strains (9). However, only a few reference laboratories can provide serotyping, and such services are not readily available to investigators. A widely available alternative method that would be simple to perform, that would not require specialized reagents (i.e., antisera), and that could be used for many applications is highly desirable (3, 9).

Molecular methods have increasingly been used as alternatives to serologic methods to type *Campylobacter* strains. We recently developed a typing system based on restriction fragment length polymorphism (RFLP) analysis of the flagellin gene, *flaA*, in *C. jejuni* and *C. coli* (7). This system is referred to as flagellin gene typing, and each RFLP pattern has a designated *flaA* type (*flaA*-1, *flaA*-2, etc). We showed that flagellin gene typing correlated with the HL and the O serotypes when analyzing outbreak-related strains and also discriminated between strains within a particular serotype (7). The purpose of the current study was (i) to extend the initial flagellin typing system by using a large collection of isolates, (ii) to examine previously analyzed strains to identify the most common serotypes of *C. jejuni* and *C. coli* isolated in the United States, and (iii) to establish the distributions of common *flaA* types in the United States. We further developed a database using commercially available image analysis software that can be used by other investigators to type strains.

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TABLE 1. Distributions of *flaA* types among 295 *Campylobacter* strains isolated in the United States containing common HL and O serotypes

flaA type	Frequency of each type (no. of strains)	% of each type	Cumulative %
1	64	21.7	21.7
7	44	14.9	36.6
27	15	5.1	41.7
49	13	4.4	46.1
13, 21	11	3.7	53.6
16	10	3.4	57.0
40	9	3.1	60.0
48	8	2.7	62.7
9, 30	7	2.4	67.5
44	6	2.0	69.5
2, 15, 18	5	1.7	74.6
6, 10, 25, 33, 43	4	1.4	81.4
4, 5, 14, 39, 53, 62	3	1.0	87.5
8, 26, 31, 37, 41, 42, 52, 54, 70, 76, 80	2	0.7	94.9
3, 11, 19, 23, 29, 34, 46, 51, 57, 59, 63, 72, 73, 83, 85	1	0.3	100

MATERIALS AND METHODS

Bacterial strains. All 404 *Campylobacter* isolates were obtained from the Campylobacter Reference Laboratory at the Centers for Disease Control and Prevention. Isolates included *C. jejuni* ($n = 322$), hippurate-negative *C. jejuni*/*C. coli* ($n = 21$), *C. coli* ($n = 14$), *C. lari* ($n = 3$), and 44 *C. jejuni*, *C. coli*, and *C. lari* HL reference strains. The hippurate-negative group of *C. jejuni*/*C. coli* strains are grouped separately since they could not be differentiated on the basis of phenotypic studies. The majority of strains were from human sources; 32 isolates were from animals, and 1 was from cow's milk. Two-hundred ninety-five of the strains were characterized previously and represented a random sample of strains from humans with sporadic cases of infection in the United States (8). Briefly, these strains were collected between July 1989 and June 1990 and came from individuals with diarrheal illness who resided in 1 of 19 counties representing all nine standard census regions of the United States plus California. Additional strains represented less common HL and O serotypes and serotype reference strains. Sixty-five of the isolates, previously typed with the flagellin typing system (7), were reanalyzed by the modified method described below.

Serotyping. Serotyping was performed by using both O and HL typing systems (4, 11) as described previously (8).

Flagellin gene typing. Flagellin gene typing was performed as described previously, with some modifications (7). Bacteria were grown on Trypticase soy agar with 5% sheep blood (Becton Dickinson) at 42°C overnight under microaerobic conditions in a Tri-Gas incubator (Forma Scientific, Marietta, Ohio). Using a 10-μl loop, a small loopful of bacteria was resuspended in 0.2 ml of sterile water, vortexed, and heated for 5 min in a boiling water bath. After centrifugation at 14,000 × *g* for 5 min (Eppendorf Microfuge, model 5415C), the clear supernatant was used as a template for further testing. If a yellow tinge was observed in the supernatant, presumably from bacterial proteins, this usually represented too much inoculum and a new extract was prepared.

PCR was performed with the following reagents at the indicated final concentrations (total final volume, 100 μl): PCR buffer (1×), $MgCl_2$ (1.5 mM), forward and reverse primers (1 μM), deoxynucleoside triphosphates (200 μM); Ultrapure dNTP set; Pharmacia Biotech), 27-2033-01; and *Taq* polymerase (2.5 U; 5 U/μl; 17038-018; Gibco BRL). For each PCR, 5 μl of template was used.

The forward primer sequence was 5′-GGATTTCGTATTAACACAAAT GGTGC-3′, and the reverse primer sequence was 5′-CTGTAGTAATCTTAA AACATTTTG-3′. PCR was performed with a Gene AMP PCR System 9600 (Perkin-Elmer Cetus) with the following cycling conditions: 94°C for 1 min and then 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min and 45 s for 35 cycles. After the last cycle, the sample was held at 72°C for 5 min. Five microliters from the PCR mixture was first checked for the presence of the expected 1.7-kb amplicon representing the *Campylobacter* flagellin gene by electrophoresis on a 0.8% agarose minigel. If a strong band was obtained, 8 μl of the PCR product (containing ca. 6 μg of DNA based on the basis of the optical density at 260 nm) was digested with the restriction enzyme *Dde*I (175S; New England Biolabs), according to the manufacturer's instructions, with 1.5 μl of 10× *Dde*I buffer, 5 μl of sterile water, and 0.5 of μl of *Dde*I enzyme, and the mixture was incubated at 37°C for 4 to 6 h. The *Dde*I-digested product was analyzed by agarose gel electrophoresis by using 4% Nusieve GTG Agarose (FMC Bioproducts, Rockland, Maine) in 1× Tris-acetate (TAE) buffer (0.04 M Tris-acetate, 0.001 M EDTA). One-hundred milliliters of gel was prepared by the boiling method as described by the manufacturer. After dissolving the gel, 100 μg of ethidium bromide (10 mg/ml of stock) was added and poured into a gel casting tray (10 by 14 cm) with a 16-well comb (slots of 1 by 3 mm). The gel was cooled for at least 30 min at room temperature, and then enough 1× TAE buffer was used to cover the gel and the gel was placed at 4°C for 30 min prior to use. Samples were electrophoresed at 50 V for 16 h at room temperature by using 1× TAE running buffer containing 0.5 μg of ethidium bromide per ml. A 123-bp ladder (5613SA; Gibco BRL) was used as a molecular size standard. Under these conditions, the 123-bp marker ran within 1 cm of the gel bottom. The gels were examined with a UV transilluminator and were photographed with Polaroid type 57 high-speed 4-by-5 sheet film.

Image analysis. Computer analysis of the images was performed by using the software program Pro-RFLP Macintosh, version 2.0 (DNA ProScan, Inc., Nashville, Tenn.). Briefly, the photographs were scanned into the computer (Macintosh Quadra 800) by using a flatbed scanner (HP Scanjet IICX; Hewlett-Packard, Inc., Palo Alto, Calif.) and DeskScan II, version 2.0, software. The images were imported into Adobe Photoshop (LE version 2.5; Adobe Systems), where the images were adjusted for contrast and labeled. The photographs were analyzed by the Pro-RFLP software program and were processed according to the manufacturer's instructions. During this process, lanes containing samples and standards were manually identified and the bands were marked, and band sizes were automatically calculated by the software. Each unique pattern was saved in a Pro-RFLP image database file whereby new images could be compared with existing patterns (Campylobacter RFLP Database version 1.0; © 1994 Trustees of the University of Pennsylvania). Pattern comparisons were performed at the 5% stringency level, with one-to-one matching; i.e., the pattern being searched had to have the same number of bands and each band had to be within ±5% of the band size stored in the database. If a match occurred, the image in the database was recalled to visually compare the two patterns to ensure matching. If a complete match did not occur, the unknown pattern was given a new type designation and was stored in the image database file for future comparisons.

RESULTS

Database. We analyzed 404 *Campylobacter* strains using the flagellin gene typing system and identified 83 distinct flagellin types, designated *flaA*-1, *flaA*-2, etc. Sixty-seven *flaA* types were detected among *C. jejuni* strains, 17 *flaA* types were detected among *C. coli* strains, and 12 *flaA* types were detected among *C. jejuni*/*C. coli* strains. None of the *flaA* types was found to be unique among the different species; however, *flaA*-59 accounted for 15% of *C. coli* strains and 21.1% of *C. jejuni*/*C. coli* strains but was observed in only 1 (0.28%) *C. jejuni* strain.

Identification of common *flaA* types in the United States. We analyzed 295 of the original 298 O and HL serotype strains isolated in the United States to determine the most common *flaA* types (8). Six common *flaA* types emerged from the analysis of these strains; *flaA*-1, *flaA*-7, *flaA*-27, *flaA*-49, *flaA*-13, and *flaA*-21 accounted for 53.6% of the strains (Table 1). Seventy-five percent of the strains fell into 15 *flaA* types, and all 295 strains were typed within 52 *flaA* types. Thirty-seven *flaA* types were infrequently encountered (fewer than five occurrences each) and accounted for approximately 25% of the strains tested. Within the 52 *flaA* types, there were 71 different HL serotypes and 74 different O serotypes.

The distribution of *flaA* types was correlated with the O serotype for each strain (Table 2). Within the O serotypes tested, in nine O serotypes at least 10 strains were represented. Among these strains, a single *flaA* type usually predominated, but other *flaA* types were represented. Some predominant *flaA* types occurred within multiple O serotypes, such as *flaA*-1 (O1, O1,8, O8, O8,17, O4 complex) and *flaA*-7 (O1, O1,8, O2). Within O19 strains, a single *flaA* type, *flaA*-21, occurred in 80% of the isolates. A predominant *flaA* type was not apparent in O:3 and O6,7,25 strains.

The distribution of *flaA* types was also correlated with the HL serotype for each strain (Table 3). *flaA*-1 occurred with several HL serotypes (HL1, H2, HL33, HL49), as did *flaA*-7 (HL4, HL16). *flaA*-27 occurred in both HL8 and HL40 serotypes. A predominant *flaA* type was not observed in the HL36, HL7, HL24, HL17, HL28, and HL32 serotypes.

TABLE 2. Distributions of *flaA* types among *Campylobacter* strains isolated in the United States[a] containing common O serotypes

O type[b]	No. of strains tested	Predominant *flaA* type (%)[c]	Other *flaA* types (% for each *flaA* type)[c]
1 or 1,8	34	**1** (47); **7** (26)	5,**9**,52 (6); 26,**27**,51 (3)
4,13,16,43,50	33	**1** (30.3)	**1,21** (11.8); 2,6,**7**,**15**,**18**,21 (6); 4,**16** (9.1); 8,**9**,39,41,83 (3)
8 or 8,17	29	**1** (58.6)	**7** (17); **9** (7); 3,5,**30**,62,72 (3.5)
4	27	**1** (44)	**2** (11); **18** (7.4); 6,**15**,25,31,33,37,39,42,44,80 (3.7)
5^+5^-	22	**49** (54)	14,53 (13.6); **7** (9); **1**,23 (4.5)
2	20	**7**(65)	43 (15); 33 (10); 8,34 (5)
3	17	None	**7**,**15**,**16**,62 (12); **1**,6,10,**13**,26,33,43,46,63 (6)
6,7,25(29)[d]	14	None	**13** (43); 25 (21); 48 (36)
19	10	**21** (80)	4,70 (10)
15,38	9	**30** (33); **16** (22)	**7**,10,31,76 (11)
11	9	**27** (55.6)	**1**,**7**,10,**16** (11)
10,17	8	**7** (38)	**1**,**30**,37,70,80 (12.5)
21 or 21,29	7	**27** (85.7)	**13** (14.3)
23,36	6	**13** (50)	11,**16**,**48** (16.7)

[a] The strains were previously characterized by Patton et al. (8).
[b] $O:5^-$ and O:25 are *C. coli* serotypes. The other serotypes listed are *C. jejuni* serotypes.
[c] Numbers in boldface type represent the 15 most common *flaA* types in the United States.
[d] Parentheses indicate that the presence of the antigen varies.

We analyzed the distributions of the *flaA* types in the United States from a sample of strains obtained from different U.S. census regions. The most common *flaA* types were seen in every region of the country. Some unique *flaA* types were observed in particular regions; however, the numbers of strains with uncommon *flaA* types were small. In New England (represented by strains from Massachusetts; $n = 17$), *flaA*-3 was observed. *flaA* types 11 and 34 were observed in the Mid-Atlantic region (represented by strains from New York State; $n = 30$), *flaA* types 29 and 46 were seen in the East North-Central region (represented by strains from Illinois and Wisconsin $n = 43$), *flaA*-80 was observed in the Mountain region (represented by strains from Colorado; $n = 33$), *flaA* types 41 and 57 were observed in the Pacific region (represented by strains from Washington and Oregon; $n = 73$), and *flaA*-19 was observed in California ($n = 17$). Several regions did not show any unique types, such as the West North-Central region (Minnesota and Missouri; $n = 32$), the East South-Central region (Alabama; $n = 19$), and the Southeast region (Virginia and Georgia; $n = 7$).

TABLE 3. Distribution of *flaA* types among *Campylobacter* isolates isolated in the United States[a] containing common HL serotypes

HL type[b]	No. of strains tested	Predominant *flaA* type (%)[c]	Other *flaA* types (% for each *flaA* type)[c]
1	34	**1** (68)	**2** (8.8); **15**,**18** (5.9); 8,25,33,37 (3)
4	27	**7** (43.8)	**13**,33,43 (7.4); 5,8,**9**,26,34 (3.7)
9	22	**49** (50)	14,53 (13.6); **7** (9.1); **1**,**9**,23 (4.5)
36	22	None	**15**,**16**,62 (13.6); **1**,6,**7** (9.1); **2**,10,**18**,26,33,46,63 (4.5)
2	19	**1** (53)	**7**,**9** (10.5); 3,4,5,**27**,52 (5.2)
33	16	**1** (56.3)	**7** (31.3); 5,52 (6.3)
8	12	**27** (50)	**16** (16.7); **1**,10,19,29 (8.3)
6	11	**13** (46)	25 (27.3); **27**,48 (9.1)
13	10	**30** (40)	**16** (20); **7**,10,31,76 (10)
7	9	None	**21** (33); 4,**7** (22); 16,**18** (11)
49	9	**1** (89)	72 (11)
11	8	40 (75)	39,54 (12.5)
16	7	**7** (42.8)	**1**,39,41,70 (14.3)
24	7	None	**1**,**16**,39,42,44,80,83 (14.3)
5	6	**13** (50)	11,**16**,48 (16.7)
70	5	**21** (60)	48,70 (20)
41,60	5	**44** (80)	42 (20)
17	4	None	**1**,6,31,43 (25)
28	4	None	**9** (50); **1**,**27** (25)
32	4	None	40 (50); **7**,**48** (25)
38	4	**48** (100)	
40	4	**27** (100)	
Rough	14	**7** (29); **1** (21)	6,**27**,**40**,43,**49**,51,80 (7)

[a] The strains were previously characterized by Patton et al. (8).
[b] The HL serotype includes strains that reacted in two or more antisera; for example, HL1 includes HL1,2; 1,7; 1,24; etc.
[c] Numbers in boldface type represent the 15 most common *flaA* types in the United States.

DISCUSSION

In our initial description of the flagellin gene typing system, 24 *flaA* types were identified among non-outbreak-related and outbreak-related strains (7). In that study, flagellin gene typing showed a complete correlation with conventional serotyping in distinguishing outbreak-related from non-outbreak-related strains obtained from four different outbreaks. We have now identified 83 *flaA* types among a set of 404 isolates that included the originally described strains, additional reference serotype strains, and 295 strains representing the O and HL serotypes isolated in the United States (8). The results of the present study establish the distribution of common *flaA* types among strains of *C. jejuni* and some *C. coli* strains isolated in the United States.

Several important pieces of information emerged from our analysis of U.S. strains with this new typing system. First, a reference point for further epidemiologic studies of *Campylobacter* infections by flagellin gene typing has been established. Among the selection of serotypes in the United States, 52 *flaA* types were identified, and of these types, 15 accounted for 75% of the strains tested. *flaA* types 1 and 7 accounted for approximately one-third of the total number of isolates.

Second, the present study also confirms the heterogeneity of *Campylobacter* strains isolated in the United States (1). Both common and uncommon *flaA* types were observed in all regions of the United States. We did note that certain *flaA* types were restricted to certain regions; however, the number of strains of each of these types was small. Surveys of isolates within particular geographic regions using flagellin gene typing would be of help in defining whether unique types circulate in particular parts of the country. Most O and HL serotypes were represented by multiple *flaA* types, but within a particular O or

HL serotype, there was some strain similarity by *flaA* typing. Among the strains for which a sufficient number of representative serotypes were tested, at least one *flaA* type occurred in 30% of the strains within a particular HL or O serotype.

Of particular interest were the results of *flaA* typing of O:19 strains. Of 10 strains belonging to this serotype, 8 (80%) were *flaA*-21. O:19 strains are strongly associated with the development of Guillain-Barré syndrome (5, 6). O:19 strains appear to have a unique lipopolysaccharide structure that may be involved in the pathogenesis of Guillain-Barré syndrome and suggests a clonal nature to these particular strains (2, 16). The high percentage of *flaA*-21 types among these isolates further suggests such a clonal origin. Analysis of other O:19 strains from patients with and without Guillain-Barré syndrome by flagellin gene typing and other molecular methods will help to further clarify this type association. It is also interesting that in our previous analysis of strains from one water-associated outbreak, epidemiologically linked isolates were all O:19 strains and also had the *flaA*-21 type (7, 10).

With regard to some of the technical issues, the boiling method used to prepare chromosomal templates is simple to perform, and approximately 85% of the strains prepared in this manner could be amplified with the *flaA* primer set. However, inoculum size appears to be important, because too much inoculum inhibits the PCR. We found that when too large an inoculum was used, the supernatant had a yellow appearance after boiling. Thus, this color seems to be a good marker for a poorly prepared sample. Repreparing the template with a smaller amount of organism usually resulted in successful amplification. Further studies to standardize the inoculum are in progress.

We (I.N.) used a commercially available software package to analyze the RFLP patterns and to create an image-based database for comparing unknown strains. The *Campylobacter* strains characterized in the present study had between three and seven bands resulting from digestion with the restriction enzyme *Dde*I. While certain *flaA* types were easily recognizable by visual inspection, manual matching of the patterns would be a daunting task for less common *flaA* types. The band sizes observed for a particular RFLP pattern do vary from one gel to another; however, under the conditions used in our study, the variation was usually less than 5%. Pro-RFLP software is designed to take into account variations in band sizes, and the stringency of matching is controlled by the user. Thus, one can perform matching searches with as little as 1% variation among patterns. For routine purposes, we used a 5% stringency for matching patterns and occasionally used stringencies of between 5 and 10%. The software is relatively easy to use and can be used on either Macintosh or personal computer platforms; our database created in Macintosh format can be converted to the personal computer format. With a modest investment in software and equipment (i.e., flatbed scanner), typing of *Campylobacter* strains by the flagellin gene typing system should be achievable by virtually any laboratory interested in molecular typing. The images for each type, however, can be used in other similarly designed software packages.

Flagellin gene typing is relatively easy to perform, and the ability to type strains is not limited by the availability of specialized antisera. Patton et al. (8) used 57 unabsorbed O antiserum specimens and 55 HL antiserum specimens to serotype the strains used in our study, and some strains were not typeable or rough with the HL antisera. Serotyping by these methods is also complicated by the weak cross-reactions with certain antigens and the variability of detecting certain antigens.

In contrast, there is no limit to the number of strains that can be typed by flagellin gene typing. For example, 14 strains that were characterized as rough and untypeable with the HL system were successfully typed by flagellin gene typing. If a strain does not match a given type in the type database, the pattern is assigned a new designation and is available for future typing. The current study predominantly evaluated *C. jejuni* strains. Although only a small number of *C. coli* serotypes was analyzed, *flaA*-59 occurred frequently in these strains as well as in *C. jejuni*/*C. coli* strains (hippurate-negative strains), but was observed in only 1 (0.28%) of the hippurate-positive *C. jejuni* strains. Whether this *flaA* type is a specific marker for *C. coli* serotypes deserves further investigation. Only three *C. lari* strains were included in the study, and thus, it is not known whether this system will be useful for typing *C. lari* strains. Further studies evaluating *C. lari* and other *Campylobacter* species are planned for the future.

Finally, multicenter studies evaluating the software and interlaboratory accuracy of typing unknown strains are ongoing. With the modification, if needed, and validation of methods, flagellin typing should be a useful adjunct to serotyping *Campylobacter* strains.

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