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Evaluation of Methods for Subtyping *Campylobacter jejuni* during an Outbreak Involving a Food Handler

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In October 1998, the Centers for Disease Control and Prevention (CDC) assisted in an investigation of an outbreak of campylobacteriosis at a school in Salina, Kansas. Twenty-two isolates were submitted from the Kansas state public health laboratory to CDC, 9 associated with the outbreak and 13 epidemiologically unrelated sporadic isolates. Pulsed-field gel electrophoresis (PFGE) using *Sma*I and *Sal*I was initially used to validate the epidemiologic data. We then tested the ability of other subtyping techniques to distinguish the outbreak-associated isolates from unrelated sporadic isolates. The methods employed were somatic O serotyping, PCR-restriction fragment length polymorphism (RFLP) analysis of *flaA*, DNA sequence analysis of 582 bp of *flaA* that included the short variable region (SVR), and sequencing of the entire *flaA* gene. PFGE was the most discriminatory technique, yielding 11 *Sma*I and 10 *Sal*I restriction profiles. All outbreak isolates were indistinguishable by PFGE, somatic O serotyping, and sequencing of the 582-bp region of the *flaA* gene. *fla* typing by PCR-RFLP grouped one sporadic isolate with the outbreak strain. Analysis of the DNA sequence of a 582-bp segment of *flaA* produced strain groupings similar to that generated by PCR-RFLP but further differentiated two *flaA* PCR-RFLP types (with a 1-bp difference in the 582-bp region). Two sporadic strains were distinct by *flaA* PCR-RFLP but differed only by a single base substitution in the 582-bp region. The entire *flaA* gene was sequenced from strains differing by a single base pair in the 582-bp region, and the data revealed that additional discrimination may in some cases be obtained by sequencing outside the SVR. PFGE was superior to all other typing methods tested for strain discrimination; it was crucial for understanding the Kansas outbreak and, when *Sma*I was used, provided adequate discrimination between unrelated isolates.

The significance of campylobacters as important human pathogens is now well established. In the United States, *Campylobacter jejuni* is the most common cause of bacterial enteritis; an estimated 2.5 million cases of human *Campylobacter* infection occur each year (17). The rise in the number of *Campylobacter* infections is most likely the result of increased case ascertainment (34) and a growing awareness of the organism among the public, physicians, and the public health community. In 1998, 44% of laboratory-confirmed cases of bacterial gastroenteritis reported to the Centers for Disease Control and Prevention (CDC)-U.S. Department of Agriculture-Food and Drug Administration collaborating sites, foodborne disease active surveillance network (FoodNet) were caused by *Campylobacter* species (4).

Although outbreaks of *Campylobacter* infection occur (27), the majority of infections are sporadic. The control of *Campylobacter* infection will ultimately depend on a more thorough understanding of sources, transmission routes, and pathogen-host interactions (1). Our current understanding of the epidemiology of *Campylobacter* infection remains incomplete. To gain more insight, laboratory methods are needed that differentiate epidemiologically related isolates from unrelated isolates. Over the last 20 years, a large number of phenotypic and genotypic typing methods have been applied to *Campylobacter* isolates (25). Phenotypic techniques, such as biotyping (3, 16), serotyping (15, 28), and phage typing (7, 32), are useful for strain characterization and are still in widespread use, but they do have limitations. These include the considerable time and labor investment required for maintenance of reagents; cross-reactivity between antigens, notably in the somatic O (Penner) serotyping scheme; and the occurrence of nontypeable isolates (13, 25, 29). Genotyping offers greater capacity for differentiating strains and can be useful in making phylogenetic as well as epidemiologic inferences. Restriction endonuclease analysis, ribotyping, PCR-based methods, pulsed-field gel electrophoresis (PFGE), and, more recently, DNA sequencing-based typing of the *flaA* gene have all been used to subtype *Campylobacter* (38). However, there are questions regarding the stability of the *Campylobacter* genome (8, 37); at this time, no technique has been solely identified as the "gold standard" for typing *Campylobacter*.

In October 1998, CDC assisted the Kansas Department of Health and Environment in an investigation of an outbreak of campylobacteriosis at a school in Salina, Kansas, involving students, staff, and visitors. During the same period, additional persons with *Campylobacter* infection were identified in the surrounding community. It was unclear if they too were associated with the school outbreak, and PFGE was used to help

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TABLE 1. Typing results for Kansas *C. jejuni* isolates

Strain no.	Somatic O serotype	Flagellin gene type			PFGE type	
		PCR-RFLP (*Dde*I)	Sequencing		*Sma*I	*Sal*I
			SVR	Full		
Outbreak associated						
D5475	19	D1	1	NT[b]	A	1
D5477	19	D1	1	1	A	1
D5479	19	D1	1	NT	A	1
D5482	19	D1	1	NT	A	1
D5483	19	D1	1	NT	A	1
D5484	19	D1	1	NT	A	1
D5486	19	D1	1	NT	A	1
D5476	19	D1	1	NT	A	1
D5480	19	D1	1	NT	A	1
Sporadic infection						
D5478	4 complex[a]	D2	2	2	B	2
D5487	4 complex	D2	2	NT	B	2
D5498	4 complex	D2	2	NT	B	2
D5493	4 complex	D2	2b	2b	H	7
D5494	4 complex	D2	2	NT	I	8
D5497	4 complex	D1	1b	1b	K	10
D5492	4 complex	D6	5b	4	G	5
D5488	38, 29	D4	4	NT	C	3
D5489	38	D4	4	NT	C	3
D5490	8	D5	5	3	E	4
D5491	1, 8	D5	5	NT	F	5
D5495	2	D7	6	NT	J	9
D5481	5	D3	3	NT	D	3

[a] 4 complex consists of strains expressing any combinations of antigens 4, 13, 16, 43, and 50.
[b] NT, not tested.

determine that community cases were not linked to the outbreak (21). In this study, we sought to assess the abilities of additional subtyping techniques to correctly characterize this epidemiologically well-defined collection of *Campylobacter* isolates in terms of their abilities to distinguish between outbreak-associated isolates and non-outbreak-associated isolates causing sporadic infection in the Kansas community. In addition, a range of criteria, including the ease of use, cost, and rapidity of each method, was considered. The methods evaluated were somatic O serotyping, PCR-restriction fragment length polymorphism (RFLP) analysis of *flaA*, DNA sequence analysis of 585 bp of *flaA* that included the short variable region (SVR), sequencing of the entire *flaA* gene, and PFGE using *Sma*I and *Sal*I. Our findings show that PFGE is the most discriminatory subtyping method for molecular epidemiologic studies of *Campylobacter*.

MATERIALS AND METHODS

We tested 22 isolates from stool specimens submitted by the Kansas Department of Health and Environment. They were nine outbreak-associated isolates from Saline County, five others from the same county, and eight epidemiologically unrelated isolates from other counties in the same state isolated during the same period. All isolates were cultivated at 37°C for 48 h on heart infusion agar with 5% (vol/vol) defibrinated rabbit blood (Becton Dickinson Biosciences, Franklin Lakes, N.J.) under microaerobic conditions. Isolates were speciated by standard procedures (2).

Somatic O serotyping was performed as described by Penner and Hennessy (28) using a panel of 24 antisera which represent common serotypes in the United States (26). Isolates that were nontypeable underwent passage on blood agar an additional eight times until an antigen was detected. Flagellin PCR-RFLP analysis was done as previously described (19). Sequencing of 582 bp of the *flaA* gene that included the SVR and the entire coding sequence was performed by the method of Meinersmann et al. (18).

Preparation of *C. jejuni* DNA, macrorestriction analysis using the restriction enzymes *Sma*I and *Sal*I, and PFGE were performed previously (21). Electrophoresis was carried out for 22 h at 200 V and 14°C constant temperature in a CHEF-DRIII system (Bio-Rad, Richmond, Calif.) with pulse times ramped from 10 to 35 s for *Sma*I and 4 to 50 s for *Sal*I. Simpson's index of diversity was calculated as described previously (9).

Nucleotide sequence accession numbers. The sequences obtained in this study have been deposited in GenBank under accession numbers AF369577 to AF369587.

RESULTS

A summary of the typing results is given in Table 1.

Serotyping. Seven different somatic O (heat-stable) serotypes were identified among the 22 *C. jejuni* isolates (Table 1). Seven of nine isolates associated with the outbreak were serotype O:19, and the remaining two were initially nontypeable by standard techniques. Testing of the nontypeable isolates after eight transfers resulted in detection of the O:19 antigen. The isolates associated with sporadic infection exhibited a range of serotypes; none were serotype O:19 (Table 1).

Flagellin gene typing. All isolates produced an *flaA* PCR amplicon of the expected 1.7-kb size. Amplicons were digested with *Dde*I, and *flaA* types were assigned based on the different fragment patterns. Restriction analysis by *Dde*I gave seven different *flaA* types among the 22 *C. jejuni* isolates (Fig. 1). All outbreak-associated isolates had an indistinguishable *flaA* type, designated D1 (Table 1). One sporadic strain (D5497; serotype O:4 complex) also had this *flaA* type. The second most common *flaA* type (D2) was seen in five of the seven O:4 complex isolates. The remaining strain with this serotype had a unique *flaA* type. Of the four remaining *flaA* types, two (D4 and D5) were each seen in two isolates, and two (D3 and D7) were unique.

Flagellin gene sequencing. A 582-bp segment including the SVR of *flaA* was sequenced and analyzed. A numerical designation was assigned to each unique sequence (Table 1). The 582-bp sequences from all nine of the outbreak-associated isolates were indistinguishable. Strain D5497, which had a *flaA* PCR-RFLP pattern indistinguishable from that of the outbreak strain, differed in its SVR sequence from the outbreak

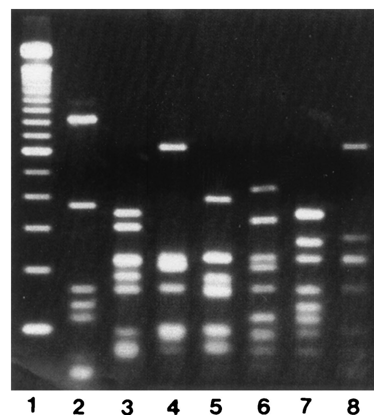

FIG. 1. *Dde*I *flaA* PCR-RFLP patterns of Kansas *C. jejuni* strains. Lanes: 1, 100-bp ladder marker; 2, D5475; 3, D5478; 4, D5481; 5, D5488; 6, D5490; 7, D5492; 8, D5495.

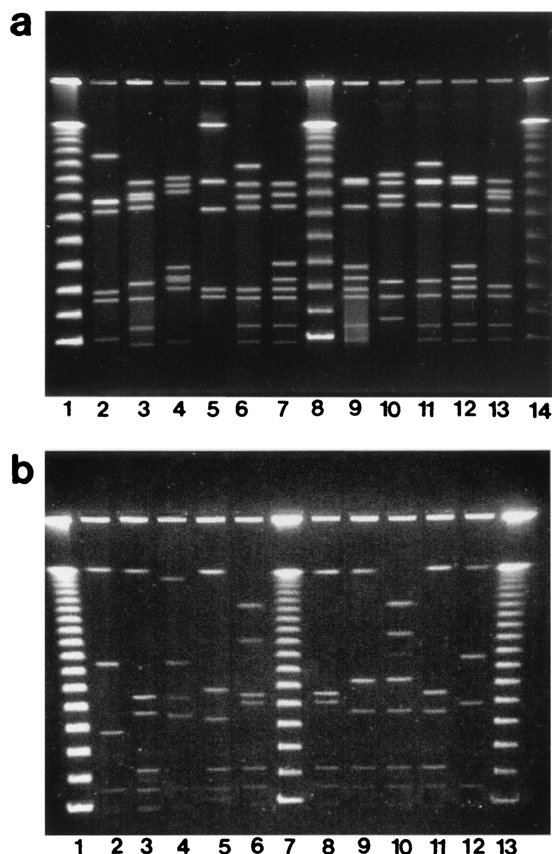

FIG. 2. (a) PFGE restriction profiles of *Sma*I-digested DNA of Kansas *C. jejuni* strains. Lanes: 2, D5482; 3, D5487; 4, D5488; 5, D5481; 6, D5490; 7, D5491; 9, D5492; 10, D5493; 11, D5494; 12, D5495; 13, D5497; 1, 8, and 14, 48.5-kb DNA ladder. (b) PFGE restriction profiles of *Sal*I-digested DNA of Kansas *C. jejuni* strains. Lanes: 2, D5482; 3, D5487; 4, D5481; 5, D5488; 6, D5490; 8, D5491; 9, D5493; 10, D5494; 11, D5495; 12, D5497; 1, 7, and 13, 48.5-kb DNA ladder.

strain by a single base pair. *flaA* PCR-RFLP type D2 was also further differentiated by SVR sequencing; one strain (D5493) differed from the other four isolates with the same *flaA* type by a single base pair in the 582-bp region sequenced, which was outside the SVR. The SVR sequence of strain D5492 (*flaA* pattern D6) differed from the SVR sequence of strains D5490 and D5491 (*flaA* pattern D5) by a single base pair, though their RFLP patterns were related but different (Fig. 1, lane 6 [D6] versus lane 7 [D7]).

The entire coding region of *flaA* was sequenced from one isolate from each of the three groups of strains that differed by a single base pair in the 582-bp sequence. The outbreak strain and one sporadic strain, D5497, had indistinguishable *flaA* PCR-RFLP patterns and a 1-bp mismatch in the SVR. Two additional mismatches were found in the rest of *flaA*. Strains D5478 (as well as D5487, D5494, and D5498) and D5493 had identical *flaA* PCR-RFLP patterns and a 1-bp mismatch in the 582-bp sequence that was outside the SVR. There were no additional base substitutions in the rest of the gene in these strains. Strains D5490 (and D5491) and D5492 were distinct by *flaA DdeI* RFLP but differed by 1-bp in the SVR. The *flaA* sequences from these strains were 99.9% identical for the first approximately 800 bp of *flaA* (where the SVR is located) but more divergent at the 3′ end of the gene (94% identity).

PFGE analysis. PFGE analysis of *Sma*I-digested DNA from all isolates yielded between 5 and 10 fragments ranging in size from approximately 40 to 480 kb (Fig. 2a). All outbreak-associated isolates had indistinguishable macrorestriction profiles (pattern A). Ten different macrorestriction profiles were seen among the remaining 13 isolates from sporadic infections; none of them was identical to pattern A. Two macrorestriction profiles were observed among multiple strains, with all isolates of a given profile having identical serotypes and *flaA* types. The remaining eight strains all had unique macrorestriction profiles. *Sal*I digestion of DNA yielded up to eight fragments ranging in size from approximately 40 to 560 kb (Fig. 2b). The strains were grouped almost identically to those assigned using *Sma*I, except for two strains, D5491 and D5492. With *Sma*I, each strain had a unique profile, with the two profiles differing from each other by three bands; with *Sal*I, they were indistinguishable.

Discrimination potentials of the different typing methods. A summary of the discrimination among the *C. jejuni* strains by the different techniques used is shown in Table 2. The numerical index of discrimination (9) ranged from 0.749 for serotyping up to 0.827 for PFGE using *Sma*I.

DISCUSSION

We studied a collection of outbreak-associated and sporadic isolates from patients in Kansas who were all ill during the same time period in order to compare the relative usefulness of current subtyping techniques. The evaluation of the practical utility of these methods included both correct discrimination in this well-characterized event and the ability to perform the method rapidly and economically. Correct separation of outbreak cases from sporadic cases was achieved by several of the methods: PFGE, serotyping, and sequencing of the SVR. PFGE was the most discriminatory technique used in this study, with a numerical index of discrimination of 0.827 for *Sma*I and 0.823 for *Sal*I.

Although serotyping is a practical and valid phenotypic method for epidemiologic typing of *Campylobacter* and has been useful in both clinical (12) and outbreak (23, 36) investigations, it can produce ambiguous results. This can be due to the occurrence of nontypeable strains, transient antigen expression, and cross-reactivity between certain antigens (25, 29). The method requires a panel of antisera that is costly to maintain; all these factors limit the use of this technique in surveil-

TABLE 2. Discrimination indices for methods used to type *C. jejuni*

Typing method	No. of types	Isolates in main type (%)	Discrimination index
Serotyping	7	41	0.749
PCR-RFLP of *flaA* gene	7	45	0.753
Sequencing of *flaA* gene			
SVR	9	41	0.804
PFGE			
*Sma*I	11	41	0.827
*Sal*I	10	41	0.823

lance studies. In practical terms, serotyping is laborious and requires at least 5 to 7 days to complete, considering the need to repeatedly subculture isolates before testing. A number of studies have reported that repeated subculturing resulted in nontypeable strains becoming typeable by serotyping (10, 23). In this study, initial serotyping results identified two of nine outbreak isolates (D5476 and D5480) as nontypeable in the panel of 24 antisera used, yet epidemiologic data supported the fact that these isolates were part of the school outbreak. Only after repeated subculturing (eight transfers) were the two nontypeable isolates identified as serotype O:19.

Somatic serotype O:19 has been reported as the cause of a number of *Campylobacter* outbreaks (11, 24, 31). The prevalence of serotype O:19 in sporadic cases of uncomplicated campylobacteriosis has been reported to be between 1 and 6% (20). Several studies suggest that Penner O:19 is overrepresented in Guillain-Barré syndrome-associated *C. jejuni* isolates (S. Fujimoto, N. Yuki, T. Itoh, and K. Amako, Letter, J. Infect. Dis. **165:**183, 1992). No cases of Guillain-Barré syndrome were recognized in this outbreak.

Several subtyping methods based on *flaA* have been reported, including PCR-RFLP (19, 22) and *flaA* sequencing (18). They are generally simple, cost-effective, and relatively rapid (2 days). However, the use of a single genetic locus as an epidemiologic tool requires caution, especially when making inferences about clonal ancestry, since one gene may not be representative of the entire genome (38). Indeed, this has been reported previously for the *flaA* locus (8) and is also demonstrated in our study. One sporadic strain (D5497) had a *flaA* PCR-RFLP profile indistinguishable from that of the outbreak pattern (D1), yet it had a different serotype (O:4) and different PFGE profiles. The occurrence of strains with different serotypes having identical *flaA* types has been shown previously (22, 33). Thus, relying solely on *flaA* PCR-RFLP analysis can lead to misinterpretation of the data.

Sequence-based subtyping of the SVR, a 267-bp sequence located near the 5′ end of *flaA* that provides a level of discrimination similar to that detected in the entire *flaA* sequence (18), was more discriminatory than PCR-RFLP analysis (Table 2) and correctly differentiated the outbreak strain from the sporadic strains. Among the unrelated strains, sequencing of the SVR, as well as PFGE, further differentiated the O:4 complex strains. In our study, sequences outside the SVR provided additional discrimination not seen in the SVR. Although it is advantageous to sequence only a small region of the *flaA* gene, further evaluation may be necessary to clarify whether it is representive of the entire gene. Despite these limitations, molecular characterization of *flaA* via PCR-RFLP analysis or sequencing may be useful for rapid, preliminary characterization of strains when the aim is to establish an epidemiologic link in a well-defined setting. Furthermore, sequencing of *flaA* provides a precise measure of genetic variability, as it is based on the DNA sequence, not band matching. However, an initial investment in an automated DNA sequencer is necessary to carry out this method, making it less accessible for smaller laboratories. Recent advances in the development of high-throughput sequence capabilities, microarray technologies, and powerful bioinformatics tools means that sequence-based techniques are valuable and should be investigated further.

While PFGE is also somewhat labor-intensive, we regard it as the current gold standard because it examines polymorphisms throughout the genome and it has the highest discriminatory power of the typing methods tested. Recently CDC, in collaboration with state health departments and the Food and Drug Administration, established PulseNet, a computer network to rapidly analyze and compare PFGE patterns from different sources of several important food-borne pathogens (35). Until recently, one disadvantage of PFGE was the length of time required to perform the technique, which for *Campylobacter* was typically 3 to 4 days. The development of a rapid PFGE protocol (24 to 30 h) for *Campylobacter* (30) and the addition of this important enteric organism to the PulseNet system, which is currently under way, will make this a rapid and standardized technique more amenable to routine use. Together with traditional epidemiologic methods, this genotypic database should enable us to make more accurate and relevant epidemiologic conclusions.

Interestingly, analysis of our study data revealed two small clusters of related isolates among the sporadic cases, one containing three and the other two isolates. This suggests that the *Campylobacter* isolates in both cases came from a common source, although we have no epidemiologic evidence to support this. Epidemiologic and microbiological analysis of a larger panel of sporadic infections may be helpful in determining whether these related isolates may have had a common source.

The observations described in this study provide an insight into the usefulness of some of the currently available subtyping methods. Two additional high-resolution genotyping techniques have recently been described that may have utility for the subtyping of *Campylobacter*: amplified fragment length polymorphism (AFLP) analysis (5) and multilocus sequence typing (MLST) (6). AFLP has the advantage of whole-genome analysis, as does PFGE, and provides automated data acquisition and analysis. MLST, which involves comparative DNA sequencing of several genetic loci, provides precise information regarding strain relationships and simplifies interlaboratory comparisons, both of which have proven difficult with PFGE. Further investigation into the utility of these methods is needed, and to address this, we have initiated a project to assess the application of DNA sequence-based subtyping methods for national and global epidemiologic studies of *Campylobacter*.

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