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Flagellin gene polymorphism analysis of *Campylobacter jejuni* infecting man and other hosts and comparison with biotyping and somatic antigen serotyping

R. J. OWEN, C. FITZGERALD, K. SUTHERLAND AND P. BORMAN

National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

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SUMMARY

Flagellin gene sequence polymorphisms were used to discriminate amongst 77 strains of *Campylobacter jejuni* from sporadic and outbreak-associated human enteric infections, and from chickens, sheep and calves. The results were assessed in relation to Lior biotyping and serotyping (Penner somatic antigens). Eight DNA PCR-RFLP patterns (genotypes) were identified by analysis of *Hinf*I fragment length polymorphisms in flagellin gene (*fla*A) polymerase chain reaction (PCR) products. One genotype (F-1) was a feature of 55% of strains. Strains within the genotypes were heterogeneous with respect to somatic antigens with 12 serogroups represented amongst the *C. jejuni* isolates of *fla*A type F-1. Serogroups Pen 1, 2 and 23 were the commonest (45%) amongst the 20 different serogroups represented. Several unique clusters of isolates with diverse biotypes were defined, and one cluster (F-7/Pen 23) contained epidemiologically implicated outbreak strains as well as sheep and calf isolates. We conclude that *Hinf*I *fla*A typing is reproducible and offers high typability, and its combination with serogrouping provides a novel approach to characterizing isolates of *C. jejuni* with improved discrimination.

INTRODUCTION

Campylobacter jejuni is a major cause of acute diarrhoeal disease in man throughout the world [1]. In 1993, the total of laboratory reports for campylobacter gastrointestinal tract infections for England and Wales, was about 39000 [2]. Infections were mainly sporadic but a number of family outbreaks and two major community outbreaks associated with consumption of contaminated milk were reported [3]. Unlike salmonella and most other foodborne pathogens, isolates of campylobacter are rarely referred to reference centres to be typed so there is a paucity of information on the distribution and frequency of different types causing infections in the United Kingdom.

Many phenotyping methods have been described over the past 15 years for identifying strains of *C. jejuni* in epidemiological investigations but biotyping and serotyping are the most widely used [4, 5]. DNA-based typing (genotyping) methods such as restriction endonuclease digest analysis and ribosomal RNA gene

Table 1. *Bacterial strains studied*

Strain No.	Host	Laboratory received from*	Lior Biotype	Penner Serotype†	*Fla*-type (*Hinf*I)
Isolates					
C3/92	Human	Barnstable (1)	I	2	1
C37/92	Human	Lancaster (2)	I	2	1
C38/92	Human	Lancaster	I	18	3
C44/92	Human	Lancaster	II	44	1
C45/92	Human	Lancaster	I	1	1
C46/92	Human	Lancaster	II	44	1
C47/92	Human	Lancaster	I	11	6
C49/92	Human	Lancaster	IV	21	6
C51/92	Human	Lancaster	I	8	3
C53/92	Human	Lancaster	II	2	1
C56/92	Human	Lancaster	II	18	6
C57/92	Human	Lancaster	II	1	1
C80/92	Human	Lincoln (3)	I	18 (1)	1
C84/92	Human	Lincoln	II	2	1
C95/92	Human	Lancaster	II	5	2
C96/92	Human	Lancaster	II	2	1
C107/92	Human	Lincoln	III	10	1
C115/92	Human	Lancaster	II	8 (1)	1
C117/92	Human	Lancaster	II	1	1
C119/92	Human	Lancaster	III	27 (6, 7, 1)	7
C162/92‡	Human	Manchester (4)	I	8 (17)	3
C166/92‡	Human	Manchester	II	23	7
C178/92‡	Human	Manchester	II	23 (36, 17)	7
C187/92‡	Human	Manchester	II	23	7
C188/92‡	Human	Manchester	II	23	7
C80/93	Human	Lancaster	II	5	1
C82/93	Human	Ashford (5)	II	44	1
C117/93	Human	Ashford	IV	6, 7 (27)	7
C123/93	Human	Ashford	II	11	6
C124/93	Human	Ashford	II	1	1
C125/93	Human	Ashford	I	18	3
C129/93	Human	Lancaster	III	6, 7 (27)	7
A1047/92	Human	Cape Town (6)	I	23 (36)	1
A1061/92	Human	Cape Town	II	8 (17)	1
C85/93	Chicken	Exeter (7)	I	1	1
C86/93	Chicken	Exeter	I	1	1
C89/93	Chicken	Exeter	II	2	3
C92/93	Chicken	Exeter	IV	27	4
C93/93	Chicken	Exeter	IV	27	4
C95/93	Chicken	Exeter	I	9	1
C96/93	Chicken	Exeter	I	9	1
C98/93	Chicken	Exeter	III	9	1
C106/93	Chicken	Exeter	I	57	5
C107/93	Chicken	Exeter	I	57	5
C108/93	Chicken	Exeter	II	57	5
C60/92	Sheep	Bristol (8)	II	23 (36, 17)	7
C61/92	Sheep	Bristol	II	2	1
C62/92	Sheep	Bristol	II	23 (36)	7
C72/92	Sheep	Bristol	I	1	1
C73/92	Sheep	Bristol	I	1	1
C75/92	Sheep	Bristol	I	1	1
C4/92	Bovine	Weybridge (9)	II	2	1
C16/92	Bovine	Weybridge	I	1	1
C17/92	Bovine	Weybridge	I	1	1

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Table 1. (*cont.*)

Strain No.	Host	Laboratory received from*	Lior Biotype	Penner Serotype†	*Fla*-type (*Hinf*I)
C36/92	Bovine	Weybridge	I	23 (36, 17)	7
Reference and collection strains					
NCTC 11168	Human	NCTC	I	1	1
NCTC 11828	Human	NCTC	III	7 (6, 27)	7
NCTC 12500	Human	CCUG (10)	I	1	1
NCTC 12501	Human	CCUG	II	2	1
NCTC 12502	Human	CCUG	I	3	3
NCTC 12504	Human	CCUG	I	5	1
NCTC 12505	Human	CCUG	III	6 (7)	7
NCTC 12506	Human	CCUG	I	7	8
NCTC 12507	Human	CCUG	I	8 (17, 1)	1
NCTC 12510	Human	CCUG	II	11	6
NCTC 12515	Human	CCUG	II	17 (8, 1)	3
NCTC 12516	Human	CCUG	II	18	3
NCTC 12520	Human	CCUG	I	23	1
NCTC 12521	Human	CCUG	IV	27 (1)	4
NCTC 12549	Human	CCUG	I	44 (1)	1
NCTC 11351	Bovine	NCTC	I	23	1
NCTC 11827	Human	NCTC	III	7 (6, 27)	7
NCTC 12104	Human	CCUG	I	64, 16	1
NCTC 12106	Human	CCUG	II	1	3
NCTC 12107	Human	CCUG	II	1	1
NCTC 12109	Not known	CCUG	IV	33	1
NCTC 12275	Chicken	CCUG	III	55	1

* Details of sending laboratory: (1) North Devon District Hospital, Barnstable; (2) Dr D. Telford, Lancaster Moor Hospital, Lancaster Pathology Laboratory, Lancaster, Lancs LA1 3JR; (3) Mr D. Bruce, Lincoln PHL, St Anne's Road, Lincoln LN2 5RF; (4) Dr D. M. Jones, Manchester PHL, Withington Hospital, Manchester M20 8LR; (5) Dr T. Donovan, Ashford PHL, The William Harvey Hospital, Kennington Road, Ashford, Kent TN24 0LZ; (6) Dr A. Lastovica, Department of Microbiology, Red Cross War Memorial Children's Hospital, Cape Town, Republic of South Africa; (7) Dr. T. J. Humphrey, Exeter PHL, Church Lane, Heavytree, Exeter EX2 5AD; (8) Dr K. L. Morgan, University of Bristol, Department of Veterinary Medicine, Langford House, Langford, Bristol BS18 7DU; (9) Dr R. Ayling, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB; (10) Dr E. Falsen, CCUG, Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Guldhedsg. 10, S-413 46 Göteborg, Sweden.

† Numbers in parenthesis refer to other weaker cross-reacting antisera (< 1/160).

‡ Isolates that originated from the Kettering outbreak [3].

profiling (ribotyping) have been applied to campylobacters and were shown to provide a high level of discrimination but, because of their complexity and laboriousness, have been used to study only relatively small sets of strains [4–10]. A more simple method of DNA-typing based on arbitrary primer PCR profiling was recently evaluated for *C. jejuni* and sufficient discrimination was found for epidemiological purposes [10].

Study of the flagellin genes of *C. jejuni* provides an alternative genotyping approach. Flagellin is the immunodominant antigen in *C. jejuni* infections, and the genetics of flagella production has been extensively investigated [11]. Nucleotide sequence data indicate significant divergence between strains within some regions of the flagellin gene (*fla* A) of *C. jejuni* [12–15]. Differences between strains

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detected by restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) amplified *fla* A gene product have demonstrated the potential of the technique for epidemiological typing [16–18]. In this study, we present a detailed assessment of flagellin gene typing combined with Lior biotyping and serogrouping by the Penner somatic (O) antigen scheme [19] as an approach to typing a set of contemporary isolates of *C. jejuni*. The strains were selected to represent a geographically diverse range of available serogroups from recent *C. jejuni* infections in the United Kingdom, including several epidemiologically implicated strains, as well as isolates from several animal hosts (chicken, sheep and cows) that form part of the human food chain.

MATERIALS AND METHODS

Bacterial strains

The 77 strains of *C. jejuni* used in this study are listed in Table 1 with their sources and alternative strain numbers. Most human strains were isolated between September 1992 and August 1993 from patients with diarrhoeal illness. Clinical history, where available, suggested they were all sporadic cases of infection except for strains from the Kettering outbreak of milk-associated campylobacteriosis in 1992 [3] which were provided by Dr D. M. Jones (Manchester Public Health Laboratory).

All bacteria were cultivated at 37 °C for 48 h on 5% (v/v) defibrinated sheep blood agar under microaerophilic conditions (5% CO_2, 5% O_2, 2% H_2 and 88% N_2) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, Yorks). Strains were maintained as part of the campylobacter reference collection, and were preserved at −70 °C on glass beads in Nutrient Broth No. 2 (Oxoid CM67) containing 10% (v/v) glycerol, and some were also lyophilized in 5% (w/v) inositol serum.

Biotyping and serotyping

The bacteria were examined using the following conventional bacteriological tests: Gram staining, growth at 42 °C, hippurate hydrolysis, H_2S production (rapid test) and DNA hydrolysis. The methods used were as described previously [20, 21]. Isolates were biotyped according to the extended scheme of Lior [22]. Serotyping was performed according to the somatic O (formerly heat-stable) antigenic scheme of Penner and Hennessy [19] using a panel of 45 O-antisera provided by Dr A. Lastovica (Red Cross War Memorial Children's Hospital, Cape Town, South Africa). Specificity of each antiserum was checked against the respective serogroup reference strain obtained from the NCTC.

Synthetic oligonucleotides

Oligonucleotide PCR primers described by King and Clayton [23] were synthesized in the Virus Reference Laboratory (Central Public Health Laboratory) by standard procedures on an automated DNA synthesizer (Applied Biosystems Model 318A). The primers were based on the conserved N- and C-terminal flagellin sequence of *fla* A of *C. jejuni* 81116 wild type (NCTC 11828), and

had the sequences 5′-ATGGGATTTCGTATTAACAC-3′ for the forward primer (nucleotide positions 83–102 [13]), and 5′-CTATTGTAATAATCTTAAAA-3′ for the reverse primer (nucleotide positions 1794–1813 [13]). The primers were predicted to amplify a 1·723 kbp fragment representing *fla*A of *C. jejuni*. A 20% mismatch between the primers and target sequences in *C. jejuni fla*B [13] was estimated when nucleotide sequences were compared using LaserGene 2000 software (DNASTAR Ltd. London). After dilution in TE (10 mM Tris and 1 mM EDTA pH 8·0) to a concentration of 10 μM, aliquots of the primers were stored at −30 °C.

Extraction of DNA

Bacterial suspensions were made in sterile distilled water (200 μl), and 10 μl was added to 100 μl of the standard PCR reaction mixture listed below. DNA was released by heat lysis in the first stage of the PCR amplification cycle (see below). For the Lior biotype II (DNAase producing) strains, bacterial genomic DNA for PCR amplification was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method according to the DNA miniprep protocol of Wilson [24]. The pellet of DNA was redissolved in 60 μl of TE.

PCR amplification

The template DNA for each strain was added to 100 μl of reaction mixture containing 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·01% (w/v) gelatin, 200 ng each primer and 100 μM each deoxynucleotide. *Thermus aquaticus* (*Taq*) polymerase (2·5 U; Boehringer Mannheim, Poole, UK) was added, and the reaction mixture was overlaid with 60 μl of mineral oil to prevent evaporation. One microlitre of *C. jejuni* NCTC 11168 DNA solution was used as the control target DNA for the PCR. PCR was performed with an OmniGene automatic thermal cycler (Hybaid, Teddington, Surrey). The amplification cycle consisted of an initial denaturation of target DNA at 94 °C for 5 min followed by denaturation at 94 °C for 1 min, primer annealing at 37 °C for 1 min, and extension at 72 °C for 1 min. Samples were amplified through 30 consecutive cycles. The completed reactions were analysed by electrophoresis of a 10 μl aliquot through 1·2% (w/v) agarose gels and the bands were visualized by staining with ethidium bromide and excitation under UV light on a transilluminator.

Restriction endonuclease digestion of PCR product

Ninety microlitres of the *fla* gene PCR product was transferred to a fresh tube. DNA was precipitated by adding 2 volumes (approximately 180 μl) of ethanol 95% (v/v). After mixing gently for 10 min, the samples were centrifuged at 12000 g for 5 min. The pellet was dried under vacuum for 10 min and redissolved in 5 μl of TE. The concentrated DNA was added to 11 μl of sterilized distilled water, 1 μl of spermidine, 1 μl of *Hinf*I (recognition sequence GANTC) and 2 μl of *Hinf*I buffer were added consecutively, giving a final volume of 20 μl. This mixture was incubated at 37 °C for 4 h according to the manufacturer's instructions (Northumbria Biologicals Ltd, Cramlington, Northumberland). The digestion was stopped with 10 μl of stop mix solution. The digested DNA (15 μl) was analysed by submarine gel electrophoresis using at 3% (w/v) NuSieve 3:1

Table 2. *Results on size and frequency of* Hinf*I fragments of* fla *gene PCR amplification product*

Fragment no.	Size (kbp)	Frequency
1	1·23	0·01 (1/78)
2	1·18	0·72 (56/78)
3	0·90	0·06 (5/78)
4	0·83	0·04 (3/78)
5	0·75	0·05 (4/78)
6	0·65	0·11 (9/78)
7	0·52	0·81 (63/78)
8	0·41	0·11 (9/78)
9	0·32	0·18 (14/78)
10	0·23	0·09 (7/78)
11	0·20	0·16 (13/78)
12	0·18	0·08 (6/78)
13	0·14	0·99 (77/78)
14	0·12	0·74 (58/78)
15	0·08	0·06 (5/78)

Agarose (FMC Bioproducts, Flowgen Instruments Ltd, Sittingbourne, Kent) at 25 V for 18 h in a buffer containing 89 mM/l Tris-HCl, 89 mM/l boric acid, 2 mM/l disodium EDTA (pH 8·3). The DNA fragments were then stained with ethidium bromide after electrophoresis and visualized using a UV transilluminator. A photograph of the DNA electrophoresis band pattern was taken as a permanent record.

To establish identity of the *fla* gene PCR product, the *C. jejuni* NCTC 11828 amplicon (5 μg) was digested with *Hae*III under standard conditions (4 h at 37 °C) and the digested DNA was electrophoresed at 23 V for 16 h in an agarose gel. Undigested *fla* gene PCR product of the same strain was analysed simultaneously. Published sequence data for *fla* A of this strain contained no *Hae*III site whereas *fla* B contained a single *Hae*III site (nt 2801) [13].

Fragment-size estimation

DNA fragment sizes in the digest patterns of the PCR products were calculated from migration distances by the MolMatch automated gel reader and analysis system (UV Products, Cambridge). A 123 bp DNA ladder (Gibco-BRL Ltd) was used as size markers for the *Hinf*I digests of the PCR products. Two lanes (outside left and right) containing the markers were included on each gel.

Computation of strain similarities

To compare *fla* patterns from different gels, the bands were coded according to size to minimize errors when determining similarities by computer-assisted methods of analysis. The patterns were screened for 15 bands with different sizes up to 1·23 kbp (Table 2) and positive (presence) and negative (absence) results were recorded. Secondary bands of faint intensity, attributed to partial digests or star activity by the endonuclease, were excluded. Double bands falling within a given range were scored as a single band. Computed similarities among strains were estimated by means of the Dice coefficient (negative matches excluded) and

clustering of strains was based on the unweighted pair group method (UPGMA) to facilitate the generation and the plotting of a dendrogram [25]. All computations were performed using the DNAGE program, a modification of previously described pattern analysis software [26].

RESULTS

Biotypes and their distribution by host

The Lior biotypes of the 77 strains of *C. jejuni* are listed in Table 1. The frequency of occurrence of each biotype amongst the 55 recent clinical isolates (excluding the reference strains) were as follows: biotype-I, 21 strains (40%); biotype-II, 25 strains (46%); biotype-III, 4 strains (7%) and biotype-IV, 4 strains (7%).

Serogroups and their distribution by host

Table 1 lists the Penner serotyping results for each strain and Figure 1 shows the frequency distribution of all the *C. jejuni* strains by serogroup in each host set. Overall in this study, the Pen 1 strains were the commonest (14%), as found in a recent general survey of clinical isolates (R. J. Owen, unpublished results), and they represented all source categories. For the 21 strains (27%) that were agglutinated by several Penner antisera, the serogroups were recorded on the basis of the antiserum giving the highest titre. Some low titre cross-reactions, which are a recognized feature of the Penner scheme [5] were detected between antigens 6 and 7 (referred to as serogroup 6, 7); between antigens 1, 8 and 17; between antigens 1, 6, 7 and 21; and between antigens 17, 23 and 36 (Table 1). All the strains included in the present study were typeable by one or other of the available antisera.

fla *RFLP patterns and designation of genotypes*

The 1·72 kpb PCR-generated *fla* gene product was obtained from each of the 77 strains of *C. jejuni*. Identity of this product as *fla* A was indicated by the fact that the PCR product from NCTC 11828 DNA was not cut after *Hae* III digest analysis (data not shown). However, no other strains were compared, and because the previously established inter-strain heterogeneity in these genes [17] and the possibility that recombination between them occurs *in vivo* [27], we have viewed the PCR product as potentially being derived from either *fla* A or both *fla* A and *fla* B. In Figure 2, some typical *Hinf* I restriction digest patterns of the *fla* gene PCR products from selected strains of *C. jejuni* are presented to illustrate each of the *fla* patterns, apart from F-2 which was represented by a single strain. Each strain pattern comprised between 2 and 6 fragments with sizes in the range 80–1230 bp (Table 2). Summation of the sizes of the various fragments in each profile totalled 1·72 kbp, with relatively minor errors of 14% or less attributable to inaccuracies in sizing.

The *fla* RFLP-PCR patterns of the 77 strains of *C. jejuni* were coded according to the 15 fragment sizes defined in Table 2, and similarities between patterns were determined by numerical methods. At the 95% similarity level, selected to give

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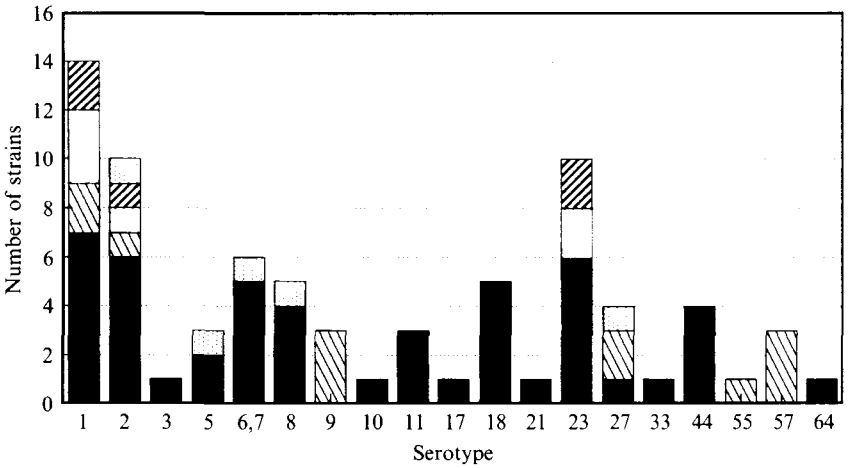

Fig. 1. Distribution of strains from *C. jejuni* from different hosts according to serotype. ■, Human; ▨, Chicken; □, Sheep; ▨, Bovine; ⬚, Unknown.

maximum discrimination between patterns, 8 different *fla* patterns (designated 1–8 were identified (Table 3). Several of the fragment profiles differed only in a single band, and Table 3 shows the basis on which the types were defined.

Distribution of fla*-types by host*

Figure 3 shows the frequency of occurrence of strains within the sets of *C. jejuni* from the four different hosts (human, chicken, sheep and bovine) according to *fla* type. Type F-1 strains, which were the most common overall (55%), were found in all host categories. The human isolates exhibited a diversity of *fla* types except none were type F-5, which was unique to the chicken isolates. The bovine isolates were either types F-1 or F-7.

Association between fla*-types and serogroup*

The associations amongst the strains of *C. jejuni* with respect to serogroup and *fla*-type are shown in Table 4. Strains of each serogroup were comprised of up to three different *fla* types; e.g. the serogroup 1 strains were either type F-1 or F-3. In contrast, several *fla* types were more heterogenous with respect to serogroup: e.g. type F-1 contained strains of 12 different serogroups. By using the combination of both phenotypic and genotypic characters, 6 distinct clusters of three or more strains of *C. jejuni* could be discerned, and their composition is described below in order of size. Groups of two or three strains that were unique in both serogroup and *fla*-type were not included. All isolates were from cases of human diarrhoea unless indicated otherwise [(C), chicken; (S), sheep; and (B), bovine sources].

Cluster 1 (F-1/Pen 1) contained 14 strains of which 11 were recent isolates [C45/92 Lancaster; C57/92 Lancaster; C117/92 Lancaster; C124/93 Ashford; C16/92 Weybridge (B); C17/92 Weybridge (B); C85/93 Exeter (C); C86/93 Exeter (C); C72/92 Bristol (S); C73/92 Bristol (S); C75/92 Bristol (S)], and NCTC 11168, NCTC 12107 and NCTC 12500 (Pen 1 reference strain).

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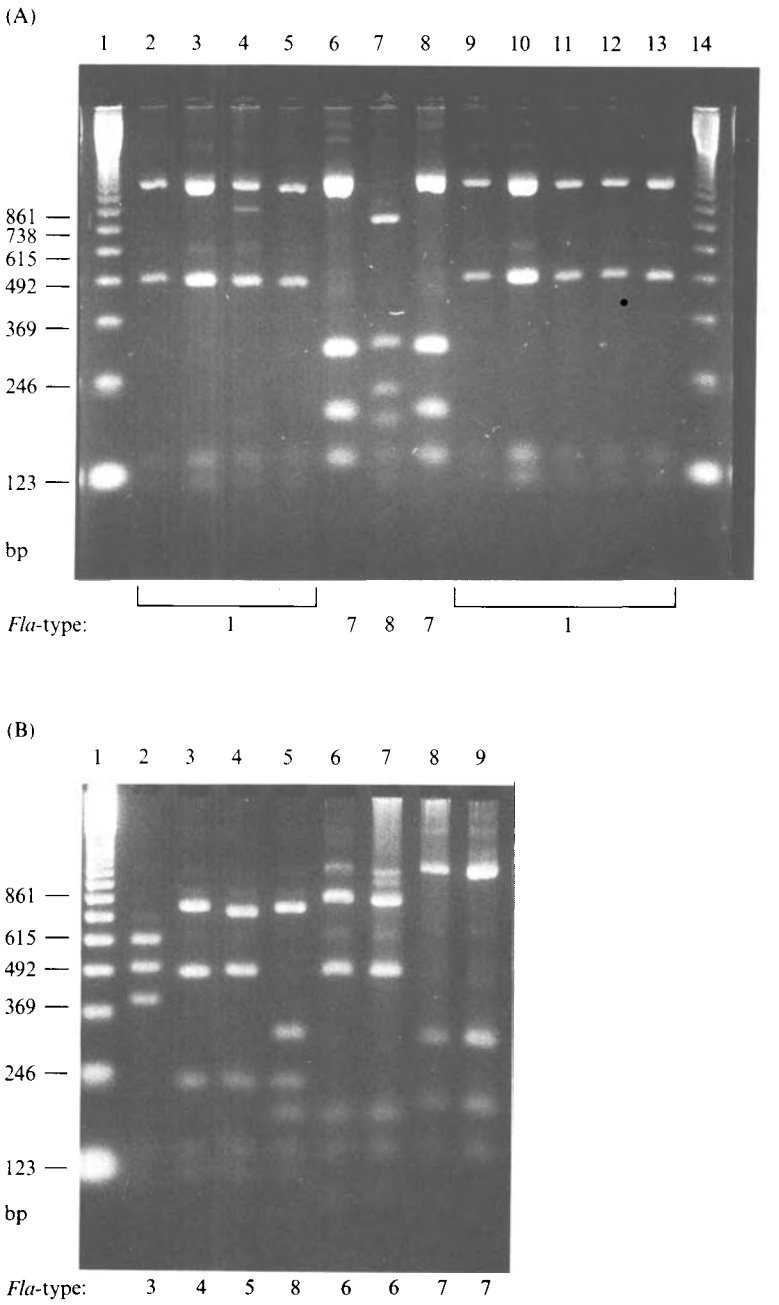

Fig. 2. *Hin*fI restriction digest patterns of the 1·73 kb PCR product from the amplified *fla* genes of representative clinical isolates of *C. jejuni*. (A) Lanes: 2, NCTC 12500; 3, C16/92; 4, C17/92; 5, C45/92; 6, NCTC 12505; 7, NCTC 12506; 8, C129/93; 9, C3/92; 10, C37/92; 11, NCTC 12507; 12, NCTC 12504; 13, C96/92. Lanes 1 and 14, 123 bp ladder marker. (B) Lanes: 2, C125/93; 3, C92/93; 4, C106/93; 5, NCTC 12506; 6, C47/92; 7, C49/92; 8, NCTC 12505; 9, NCTC 11827; Lane 1, 123 bp ladder marker, sizes are as indicated.

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Table 3. C. jejuni *flagellin gene PCR-RFLP patterns* and definition of* HinfI *fla-types*†

RFLP patterns (*Fla*-type)	Number of strains	Mean fragment size (kbp)
F-1	42	1·18. 0·52, 0·14, 0·12
F-2	1	1·23, 0·52, 0·14
F-3	9	0·65, 0·52, 0·41, 0·14, 0·12
F-4	3	0·83, 0·52, 0·23, 0·14, 0·12
F-5	3	0·75, 0·52, 0·23, 0·14, 0·12
F-6	5	0·90, 0·52, 0·18, 0·14, 0·08
F-7	13	1·18, 0·32, 0·20, 0·14
F-8	1	0·75, 0·32, 0·23, 0·18, 0·14, 0·12

* Defined on the basis of a numerical analysis (results not shown).

† Correspondence between the *fla*-types defined above and those described previously [21] were as follows: F-1 = F-1; F-3 = F-5; F7 = F-8; the other types were unique to each strain set.

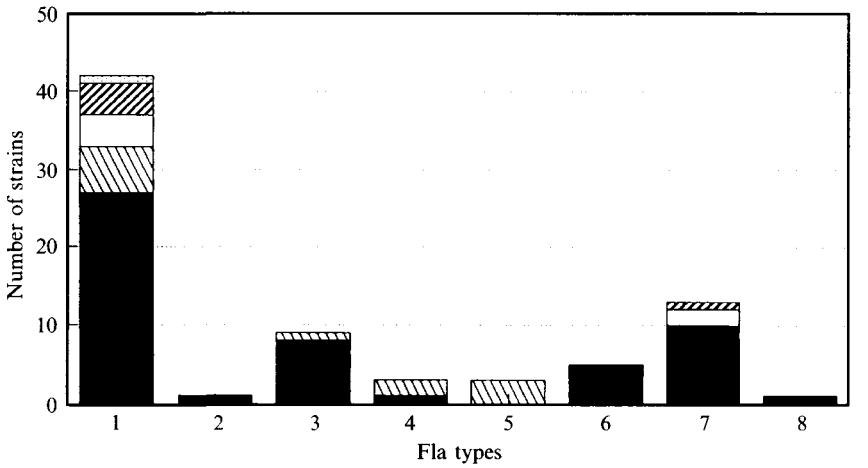

Fig. 3. Distribution of strains of *C. jejuni* from different hosts according to *Hinf*I flagellin gene typing. ■, Human; ▧, Chicken; □, Sheep; ▨, Bovine; ⬚, Unknown.

Cluster 2 (F-1/Pen 2) contained 8 strains of which 7 were recent isolates [C3/92 Barnstaple; C37/92 Lancaster; C53/92 Lancaster; C84/92 Lincoln; C96/92 Lancaster; C61/92 Bristol (S); C4/92 Weybridge (B)], and NCTC 12501 (Pen 2 reference strain).

Cluster 3 (F-7/Pen 23) contained 7 isolates [C166/92 Manchester; C178/92 Manchester; C187/92 Manchester; C188/92 Manchester; C80/92 Bristol (S); C62/92 Bristol (S); C34/92 Weybridge (B)].

Cluster 4 (F-7/Pen 6, 7) contained 5 strains comprising 2 isolates (C117/93 Ashford; C129/93 Lancaster), and NCTC 12505, NCTC 11827 and NCTC 11828 (*Fla*-variant).

Cluster 5 (F-1/Pen 44) contained 4 strains comprising 3 isolates [C44/92 and C46/92 Lancaster; C82/93 Ashford] and NCTC 12549.

Cluster 6 (F-1/Pen 23) contained 3 strains that comprised 1 isolate (A1047/92 RSA), NCTC 11351, and NCTC 12520 (Pen 23 reference strain).

Table 4. *Summary of associations between flagellin genotypes and serotypes of the strains of* C. jejuni

Serotype (Penner)	*Fla*-type (*Hinf*I) 1	2	3	4	5	6	7	8
1	14*	—	1	—	—	—	—	—
2	8*	—	1	—	—	—	—	—
3	—	—	1	—	—	—	—	—
5	2	1	—	—	—	—	—	—
6, 7	—	—	—	—	—	—	5*	1
8	3	—	2	—	—	—	—	—
9	3		—	—	—	—	—	—
10	1	—	—	—	—	—	—	—
11		—	—	—	—	3	—	—
17		—	1	—	—	—	—	—
18	1	—	3	—	—	1	—	—
21	—	—	—	—	—	1	—	—
23	3*	—	—	—	—	—	7*	—
27	—	—	—	3	—	—	1	—
33	1	—	—	—	—	—	—	—
44	4*	—	—	—	—	—	—	—
55	1	—	—	—	—	—	—	—
57	—	—	—	—	3	—	—	—
64	1	—	—	—	—	—	—	—

* See text for further details on these clusters of strains.

DISCUSSION

Previous studies demonstrated that strains of *C. jejuni* have a significant degree of diversity within some regions of the flagellin gene sequences, [13, 14] and it has been suggested that these variations could provide a novel basis for discriminating and grouping isolates in epidemiological investigations [16–18]. The results of the present study provide further evidence of diversity within the *fla* gene sequences of *C. jejuni* with 77 strains giving 8 patterns representing 8 genotypes. However, the majority of strains (55%) were characterized by having the F-1 genotype. In a previous study [17], we described the genotypes of a set of 28 strains of *C. jejuni* including 15 South African clinical isolates. The correspondence between those types and types defined in the present study is shown in Table 3. Combination of the data from the two studies shows that most strains of *C. jejuni* have one principal *Hinf*I *fla* type with minor variants. As suggested previously [17], that DNA type may constitute a major clonal line within *C. jejuni* but was heterogeneous with respect to serogroup and host association. Nachamkin and colleagues [12] recognized 18 *fla*A types amongst 43 non-outbreak strains of 6 common serotypes of *C. jejuni* that were generated with another restriction endonuclease (*Dde*I). They did not give any precise molecular details of the profiles obtained so it was not possible to make direct comparisons with the *Hinf*I profiles described in the present study.

It is widely accepted that there are significant limitations to serotyping as a technique for the epidemiological investigation of *C. jejuni*, mainly because of the difficulties in obtaining and maintaining full panels of suitable antisera, and

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because of non-typable strains and the degree of cross-reactivity of some strains, notably in antisera of the Penner scheme [5, 28]. Also there is molecular evidence that some strains within the same serogroup are genotypically diverse [7, 29] so the discriminatory power of serotyping alone does not meet the requirements of a modern typing scheme. The aim of the present study was to develop a novel genotypic approach to categorizing isolates of *C. jejuni* that could be determined with relative simplicity and reproducibility. Other well documented DNA-based typing techniques, such as DNA digest analysis and ribotyping, are highly discriminatory but are also time consuming and difficult to apply to large numbers of strains [9, 10]. PCR-RFLP analysis of the *fla* gene was selected because previous studies showed it to be suitable for typing purposes because of its relative simplicity and potential for discrimination [16, 17].

We found in the present set of recent clinical and animal isolates, and reference strains of *C. jejuni*, that the technique, if used in combination with Penner serotyping, enabled strain discrimination within *C. jejuni* to be increased from 20 types based on serotyping alone to 28 unique clusters based on a combination of both methods. Lior biotyping by contrast offered a much lower degree of discrimination. Significantly, the results demonstrated that *fla* profiling enabled strains to be further discriminated within 7 of the serogroups examined including common serogroups such as Pen 1 and 2, although conversely, several strains with the same *fla* type mostly had different serotypes. Such results reflected the complexity and diversity of strains within *C. jejuni*.

An interesting result of using a combination of typing techniques based on independently expressed features was that several clusters of isolates of *C. jejuni* with common features could be identified. Three of these clusters (Table 4) contained between 7 and 9 similar strains yet were diverse in host and geographical location. Cluster 3 (F-7/Pen 23) was of particular interest because it contained representative isolates from a community outbreak associated with contaminated milk in Kettering in 1992 [3] as well as unrelated isolates from sheep and bovine sources. However, the Pen 23 sero-reference strain (NCTC 12520) had a different *fla* type and was grouped separately in cluster 6 (F-1/Pen 23) with two other epidemiological unrelated strains. These results indicated the value of using genomically based features to provide an additional level of subtyping within certain serogroups of *C. jejuni*. However, the degree of association between such isolates, which were diverse with respect to biotype, also needs to be determined on other typing criteria, such as phagetype and ribotype.

We conclude that PCR-RFLP analysis of the *fla* A gene with *Hinf* I provides a novel approach to discriminating between strains of *C. jejuni*. Our results based on a selection of contemporary strains of different Penner serogroups suggest that *Hinf* I *fla* gene profiles offer a high degree of typability but alone do not provide significantly improved discrimination over other available DNA-typing methods. However, when used in combination with serotyping, *fla* typing offers a moderate degree of enhanced discrimination and could be considered valuable as an adjunct technique. Secondary (weaker intensity) fragments were evident in some of our *Hinf* I RFLP profiles, but were not problematic to interpret provided the main high intensity bands were used as the basis for defining the types. Further investigation of a wider range of *C. jejuni* serostrains, particularly those of the Pen

4 complex and non-typable strains which were not included in the present study, are needed. Also restriction endonucleases such as *Dde* I [12] or *Alu* I [18] that were reported to give a higher frequency of cutting than *Hinf* I, need to be fully assessed with respect to discriminatory potential and reproducibility. As a general purpose method, we suggest that *fla* A gene PCR-RFLP profiling using multiple enzyme digests may be of value for future typing of *C. jejuni* from different hosts in well defined and highly focused epidemiological contexts.

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