Flagellin Gene Polymorphism Analysis of *Campylobacter* Compared with Antigen Serotyping

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Flagellin gene sequence polymorphisms were used to discrimate amongst 53 strains of *Campylobacter jejuni* and *C. coli*. The *Campylobacter* strains were made up of forty- three strains of *Campylobacter jejuni* and 10 strains of *Campylobacter coli.* The results were analysed in relation to Penner serotyping. Twenty DNA PCR-RFLP patterns (genotypes) were identified by analysis of Dde I fragment length polymorphisms in flagellin gene (fla A and fla B) polymerase chain reaction (PCR) products. Flagellin gene 13 genotype was a feature of 15% of strains, followed by flagellin gene 8 (9%). Differences in fragment patterns were observed not only between members of two species, but also between individual strains of the same species. The strains that were non-typable by the Penner serotype were distributed into 6 flagellin gene types.

In conclusion, Dde I fla typing is reproducible and offers high typability. However, when the scheme is used in combination with the Penner serotype it provides improved discrimination for the characterizing and subtyping of isolates.

Introduction

Campylobacter jejuni and to a lesser extent *C. coli* are a major cause of acute diarrhoeal disease in man throughout the world (Healing *et al.,* 1992). The majority of infections are sporadic, but large outbreaks often occur from milk and environmental sources such as water. For over 15 years, many phenotypic methods have been described for identifying strains of *C. jejuni* and *C. coli* in epidemiological investigations. However, biotyping and serotyping are the most widely used (Patton and Wachsmuth, 1992). Such schemes when used even in conjunction have several disadvantages which include, lack of reproduciblity, low typability, the requirement of a battery of antisera, which might not be available and poor resolution. DNA based typing (genotyping) methods such as restriction endonuclease digest analysis and ribosomal RNA gene profiling have been applied to campylobacters with high discrimination but these are often complex and laborious procedures. The study of flagellin genes of *Campylobacter jejuni* and *C. coli* provides an alternative genotyping approach. Flagella antigens have been shown to be immunodorminant during infection (Taylor, 1992). Both *C. jejuni* and *C. coli* possess two flagellin genes, fla A and fla B (Guerry *et al.,* 1990; Nuijten *et al.,* 1990). Nucleotide sequence data indicate significant divergence between strains within some regions of the flagellin gene (fla A) of *C. jejuni* (Nuijten *et al.,* 1990; Fischer and Nachamkin, 1991). The PCR – RFLP patterns obtained using amplified fla A gene product have demonstrated the potential of the technique for epidemiological typing (Owen *et al.,* 1994; Nachamkin *et al.,* 1993).

The aim of the work is to compute differences between strains detected by restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) of the amplified fla A and fla B gene product as a demonstration of the potential of this technique for epidemiological typing of local strains.

Methods

Bacterial strains

The fifty-three strains of *Campylobacter* were made up of twenty-nine human strains and twenty-

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four chicken strains. Also included in this study were 12 Penner sero- reference strains corresponding to the Penner strains used in this study. All bacteria were cultivated at 37 °C for 48 h on 5% (v/v) defibrinated sheep blood agar {Columbia agar, CM 331(Oxoid) + sheep blood}under microaerophilic conditions (5% $C0_2$, 5% 0_2, 2% H_2 and 88% N_2) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd. Shipley, Yorks, U. K.).

Serotyping

Serotyping was performed according to the somatic (formerly heat-stable) antigenic scheme of Penner and Hennessy (1980) using a panel of 60 O-antisera. The serotyping procedures was as described by Jones *et al.* (1985). Briefly, the serotype of each strain of *C. jejuni* and *C. coli* was determined by testing each, in the 64 immune antisera diluted 1/40 in microtitration plates. Positive reactions showed agglutination, while those that did not agglutinate with any of the 64 antisera were regarded as non-typable. The serotype was indicated by listing all reactive antisera in order of strength of titre, with the antiserum giving the strongest reaction listed first.

Synthetic oligonucleotides

Oligonucleotide PCR primers described by Cruachem Ltd. (U. K.) was used. The fla A/B primers had the sequences 5' – ATAAACACCAAC ATC GGT GCA – 3' for the forward primer (nucleotide positions 13–33) and 5' – GTTACG TTG ACTCATAGC ATA – 3' for the reverse primer (nucleotide positions 1664–1684). The primers were predicted to amplify a 1.671 kb product.

Extraction of DNA

Extraction of DNA was done by the SDS lysis method of Marmur (1961) and the isoquick method of Micro Probe Corporation (Bothell, USA).

PCR amplification

The template DNA (5 µg) for each strain was added to 45 µl of reaction mixture. The reaction mixture was overlaid with 50 µl of mineral oil to prevent evaporation. Five microlitres of *C. jejuni* NCTC 11168 DNA solution was used as the control target DNA for the PCR, while 5 µl DNAse free water was used as negative control. PCR was performed with an MJ Research thermal cycler (MA USA). The amplification cycle consisted of an initial denaturation of target DNA at 94 °C for 5 min, followed by primer annealing at 55 °C for 45 s, extension at 72 °C for 5 min then 95 °C for 1 min, samples were amplified through 40 consecutive cycles. A further extension was done at 55 °C for 1 min and 72 °C for 5 min. The completed reactions were analysed by electrophoresis of a 5 µl aliquot through 1% (w/v) agarose gels and bands were visualised by staining with ethidium bromide and excitation under UV light on a transilluminator.

Restriction endonuclease digestion of PCR product

Seventeen microlitres of the fla gene PCR sample was transferred to a fresh tube, to which was added 2 µl of Dra I (New England Biolabs (NEB), U.K) Alu I (NEB) and Dde I (NEB), 10 x buffer and 1 µl of Dde I (NEB) enzyme (these three enzymes were used to find out the most suitable for the typing of the *Campylobacter* species). This mixture was incubated at 37 °C for 4 h. The digestion was stopped by incubating the tube at 65 °C for 10 min. The digested DNA (20 µl) samples were analysed by submarine gel electrophoresis using at 4% (w/v) Nusieve Agarose (FMC Bioproducts, Flowgen Instruments Ltd. Sittingbourne, Kent, U.K) at 120 V for 5 h in TBE (89 mM/ l, Tris (Hydroxymethyl) Aminomethane Hydrochloride, 89 mM/l Boric acid, 2 mM/l disodium EDTA (pH 8.3) containing 1 µg/µl ethidium bromide and visualized using a UV transilluminator and photographs taken.

Fragment size estimation

DNA fragment sizes in the digest patterns of the PCR products were calculated from migration distances by MolMatch automated gel reader and analysis system (UV products, Cambridge, U.K). A 100 bp ladder and Hinf I (NEB) digest marker were used as size markers for the Dde I digests of the PCR products and the markers are represented by two lanes, outside left and right.

Results

Serogroups and their distribution by host

Table I lists the Penner serogrouping and Fla results for each strain. Overall, in this study the Penner 53 strains were the commonest (15%), and they represent the chicken strains. Five strains coagglutinated with several antisera and they represent (9%). The common human strains were Penner 27 and they represent (9%). Penner 4 and Penner 4 complex represent strains from both human and chicken. Nineteen percent of the strains were non-typable by the Penner scheme.

Table I. Bacterial strains studied and their fla types.

Strain Code No	Host	Penner serotype	Fla type
CD 1	human	24	1
CD 2	human	24	1
CD 3	human	24	1
CD 4	human	24	1
CD 5	human	24	1
CD 6	human	24	1
CD 13	human	31	1
CD 10	human	2	2
CD 11	human	2	3
CD 28	chicken	NT	3
CD 39	chicken	NT	3
CD 12	human	4	4
CD 15	human	1	5
CD 16	human	2	6
CD 17	human	4	7
CD 18	human	5+	8
CD 20	human	NT	8
CD 64	human	27	8
CD 65	human	27	8
CD 66	human	27	8
CD 67	human	27	8
CD 68	human	27	8
CD 21	chicken	NT	9
CD 22	chicken	4	10
CD 23	chicken	4,13,16,50	11
CD 24	chicken	4,13,16,50	11
CD 25	chicken	4,13,16,50	11
CD 26	chicken	4,13,16,50	11
CD 27	chicken	4,13,16,50	11
CD 29	chicken	21	12
CD 30	chicken	53	13
CD 33	chicken	53	13
CD 42	chicken	53	13
CD 43	chicken	53	13
CD 44	chicken	53	13
CD 45	chicken	53	13
CD 46	chicken	53	13
CD 48	chicken	53	13
CD 38	chicken	NT	13
CD 40	chicken	NT	13
CD 31	chicken	38	14
CD 37	chicken	NT	15
CD 41	chicken	53	15
CD 55	human	23	16
CD 61	human	1	17
CD 62	human	2	18
CD 63	human	13,16,50	19
CD 69	human	NT	20
CD 70	human	NT	20
CD 71	human	NT	20
CD 72	human	NT	20

fla RFLP patterns and designation of genotypes

The 1.671 kb PCR-generated fla gene product was obtained from each of the 53 strains of *C. jejuni* (43) and *C. coli* (10). Each strain pattern comprised 2 fragments for Dra I, 3–6 fragments for Alu I and 4 to 7 fragments for Dde I with sizes in the range of 29.75 kb to 832.93 kb. Dde I and Alu I gave fragment patterns suitable for typing and subtyping the isolates, however only Dde I was analysed in this study. Figs 1 and 2 show some typical Dde I restriction digest patterns of the fla gene PCR products from selected strains of *C. jejuni* and *C. coli*. Table II lists the common *C. jejuni* and *C. coli* flagellin gene PCR-RFLP patterns of Dde I and their molecular sizes, while Table III lists a summary of the association between flagellin genotypes and serotypes of *C. jejuni* and *C. coli*.

Type F-1 and F-20 were unique for human *C. coli* strains; while type F-13 were unique for *C. jejuni* chicken strains. Type F-8 were unique for *C. jejuni* human strains.

Of the 11 NT strains, 4 (18%) belonged to type F-20, 2 (18%) were type F- 3 and F-13, while one each were type F-8, 9 and 15.

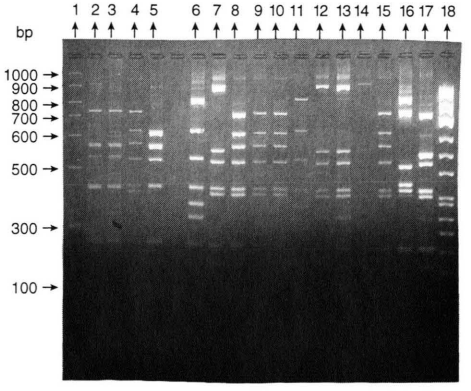

Fig. 1. Dde 1 restriction digest patterns of the 1.6 kb PCR product from the amplified fla genes of *C. jejuni* and *C. coli*. Lanes: 1,100bp ladder marker; 2, CD 46; 3, CD 37; 4, CD 39; 5, CD 20; 6, CD 12; 7, CD 3; 8, CD 1; 9, CD 66; 10, CD 65; 11, Penner 27; 12, CD 19; 13, Penner 13; 14, CD 24; 15, CD 10; 16, CD 16; 17, Penner 2; 18, Hinf 1 digest marker.

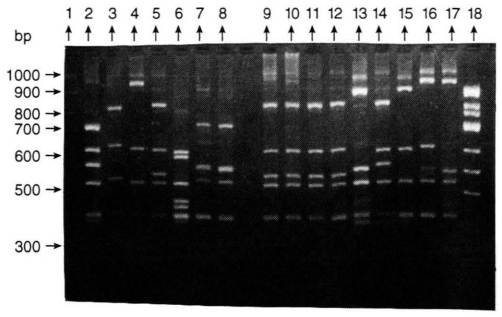

Fig. 2. Dde 1 restriction digest patterns of the 1.671 kb PCR product from the amplified flaA/B genes of *C. jejuni* and *C. coli*. Lanes: 1, 100 bp ladder marker; 2, CD 5; 3, CD 2; 4, CD 40; 5, Penner 4; 6, CD 31; 7, CD 25; 8, CD 44; 9, CD 33; 10, CD 48; 11, CD 30; 12, CD 26; 13, CD 23; 14, CD 24; 15, CD 41; 16, CD 29; 17, CD 22; 18, Hinf 1 digest marker.

Association between fla-type and serogroup

The association amongst the strains of *C. jejuni* and *C. coli* are shown in Table III. Strains of each serogroup comprised of up to four different fla types. For example serogroup 1 strains were either type F-17 or F-5. In contrast several fla types were more heterogenous with respect to serogroup: type F-8 contained strains of three different serogroups, that is 5+, 27 and NT. Serogroup 53 of the chicken strains comprised of two different fla types: F-13 and F-15. Serogroup 2 comprise of 4 fla types, F-2, F-3, F-6 and F-18. Serogroup 4 comprise of fla types 4, 7 and 10.

Discussion

The results of the present study provide evidence of diversity within the fla gene sequences of *C. jejuni* and *C. coli*, with 53 strains giving 20 patterns representing 20 genotypes. This is in agreement with Fischer and Nachamkin (1991), who pointed out a significant degree of diversity within the flagellin gene sequences in *C. jejuni*. Eleven percent of the *C. coli* strains exhibited the F-13 genotype with the F-3 genotype common for both the human and chicken hosts. Twenty Fla types were recognized amongst 12 serotypes of *C.*

Table II. Common *Campylobacter jejuni* and *C. coli* flagellin gene PCR-RFLP patterns of Dde I.

RFLP patterns (fla type)	No. of strains	Mean fragment size (kb)
F-1	7	419.42, 262.20, 227.20, 161.67, 82.95
F-3	3	798.08, 308.66, 216.97, 158.67, 78.66
F-8	7	533.48, 300.21, 234.70, 227.15, 154.25, 78.14
F-11	5	671.66, 244.56, 216.19, 156.79, 147.98, 114.17, 80.03
F-13	10	407.10, 312.30, 264.39, 222.77, 162.76, 153.45, 83.19
F-15	2	471.25, 316.16, 250.28, 222.64, 163.29, 152.67, 79.41
F-20	4	314.03, 236.00, 215.13, 157.40, 105.31, 82.21

Table III. Summary of association between flagellin genotypes and serotypes of the strains of *C. jejuni* and *C. coli*.

Serotype (Penner)	Fla type (Dde I)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1					1												1			
2		1	1															1		
4				1			1			1										
4,13,16,50											5								1	
5+								1												
21												1								
23																1				
24	6																			
27								5												
31	1																			
38														1						
53													8		1					

jejuni and *C. coli* that were generated with the restriction enzyme Dde I. This is similar to a previous report by Nachamkin *et al.* (1993) who recognized 18 fla A types amongst 43 non-outbreak strains of 6 common serotypes of *C. jejuni* generated with this enzyme, while Owen *et al.* (1994) recognized 8 fla types amongst 19 serotypes of *C. jejuni*. Although, serotyping is simple to perform and has a high level of discrimination, the reagents are difficult and expensive to produce. Several reports have also shown that the serotyping technique has significant limitations for the epidemiological investigation of *C. jejuni* and *C. coli*, mainly because of the difficulty in obtaining full panels of suitable antisera with the resultant non-typable strains and the degree of cross-reactivity of some strains notable in the antisera of the Penner scheme (Preston and Penner, 1989). From this study about 19% of the strains were non-typable, while 10.3% of the strains had a degree of cross-reactivity with the antisera. The discriminating power of serotyping alone does not therefore meet the requirement of a modern typing scheme. There is also molecular evidence that some strains within the same serogroup are genotypically diverse (Fayos *et al.*, 1992, 1993). From this study, four strains of Penner serogroup 2 had four different fla types, while several strains had different fla types with their corresponding Penner sero-reference strains e.g Penner, 1, 2, 4, 23, 31 and 38. A contrast to the above is HS 27 strains, which had the same fla type with their corresponding Penner sero-reference strain. It is also interesting to note that several strains with the same fla type, mostly had different serotypes, this reflects the complexity and diversity of strains within *C. jejuni* and *C. coli*. A similar work was reported by Owen *et al.* (1994). Of 11 NT strains, there were 6 fla types, in which 4 had fla type 20, and two others shared the same fla 13 pattern. Of note also, is the fact that a particular strain, identified as hippurate negative (CD 40) shared the same fla type (F-13) with a chicken *C. jejuni* Pen 53 and so it could be said to be a hippurate negative *C. jejuni*, since it has the Fla pattern with a Penner 53. This data suggests that at the nucleotide level, there is more heterogeneity than that of the Penner serotyping. The size of Dde I fragments of flagellin gene PCR amplification product of *C. jejuni* and *C. coli* strains differ. The *C. jejuni* varied between 798 kb to 71 kb, while those of *C. coli* varied between 419 kb and 67 kb, an exception to that of *C. jejuni* is the molecular weight range of the Penner 53 strains which was from 471 kb to 79 kb. This study shows fla typing technique to be relatively simple, highly discriminatory, typable and very useful for the epidemiological studies of *C. jejuni* and *C. coli*.

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Fayos A., Owen R. J., Desai M. and Hernandez J. (1992), Ribosomal RNA gene restriction fragment diversity amongst Lior biotypes and Penner serotypes of *Campylobacter jejuni* and *Campylobacter coli*. FEMS Microbiol. Lett. **95**, 87–94.

Fayos A., Owen R. J., Hernandez J. and Lastovica A. (1993), Molecular sub-typing by genome and plasmid analysis of *Campylobacter jejuni* serogroups 01 and 02 (Penner) from sporadic and outbreak cases of human diarrhoea. Epidemiol. and Infect. **111**, 415–427.

Fischer S. H. and Nachamkin I. (1991), Common and variable domains of the flagellin gene, fla A in *Campylobacter jejuni*. Molecular Microbiol. **5**, 1151 –1158.

Guerry P., Logan S. M., Thornton S. and Trust T. J. (1990), Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172**, 1853–1860.

Healing T. D., Greenwood M. H. and Pearson A. D. (1992), *Campylobacter* and enteritis. Rev. Med. Microbiol. **3**, 159–167.

Marmur J. (1961), A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Molecular Biol. **3**, 208–218.

Nachamkin I., Bohachick K. and Patton C. M. (1993), Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. J. Clin. Microbiol. **31**, 1531–1536.

Nuijten P. J. M., Van-Asten F. J., Gaastra W. and Van der Zeijst B. A. M. (1990), Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265**, 17798–17804.

Owen R. J., Fitzgerald C., Sutherland K. and Borman P. (1994), Flagellin gene polymorphism analysis of *C. jejuni* infecting man and other hosts and comparison with biotyping and somatic antigen serotyping. Epidemiol. Infect. **113**, 221–234.

Patton C. M. and Wachsmuth K. I. (1992), Typing schemes: Are current methods useful? In: *Campylobacter jejuni*: *Current status and future trends* (Nachamkin I., Blaser M. J. and Tompkins L. S. ed.). ASM (U. S. A.), p 110–128.

Penner J. L. and Hennessy J. N. (1980), Passive haemagglutination technique for serotyping *Campylobacter fetus* subsp *jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. **12**, 732–737.

Preston M. A. and Penner J. L. (1989), Characterization of cross reacting serotypes of *Campylobacter jejuni*. Can. J. Microbiol. **35**, 265–273.

Taylor D. E. (1992), Genetics of *Campylobacter* and *Helicobacter*. Annu. Rev. Microbiol. **46**, 35–64.