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Comparison of PFGE, ribotyping and phage-typing in the epidemiological analysis of *Campylobacter jejuni* serotype HS2 infections

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SUMMARY

In this study we have evaluated the ability of three typing methods, pulsed field gel electrophoresis (PFGE), phage-typing and ribotyping, to discriminate not only between strains of differing serotypes but also between strains within a single serotype, heat stable serotype 2 (HS2). Forty-five isolates derived from cases of campylobacter enteritis occurring in the Cardiff area were examined. These included 18, mostly HS2, strains associated with an outbreak. The typing results for these and a further 39 epidemiologically unrelated strains of serotype HS2 were compared. This is the first report documenting the use of PFGE in an epidemiological investigation of *Campylobacter jejuni* in the UK. The results presented suggest that this technique is the most discriminatory of the three subtyping methods examined.

INTRODUCTION

Campylobacter jejuni is one of the most common causes of acute bacterial gastroenteritis in the United Kingdom [1]. Although most cases are sporadic there have been outbreaks of infections mainly associated with the consumption of undercooked poultry and unpasteurized milk [1]. The routes of transmission in the sporadic cases of *C. jejuni* infection are not fully understood, so there is a need to develop and to evaluate subtyping methods for the species. At present the majority of 'thermophilic' campylobacters isolated in clinical laboratories in the UK are neither routinely speciated nor subtyped.

In this study we have used two genotypic methods, ribotyping and pulsed field gel electrophoretic profiling (PFGE), in combination with phage-typing to characterize strains within the Penner heat stable serotype 2 (HS2) [2] of *C. jejuni*. The HS2 serotype is one of the three most common *C. jejuni* serotypes from human infections in the developed world including the UK [3]. We also examined 17 HS2 strains isolated from cases either directly or indirectly linked to an outbreak associated with a school visit to a farm where raw milk was consumed. Each method was assessed for its ability to identify outbreak isolates and to

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discriminate between outbreak and sporadic strains arising in the same geographical area (Cardiff) at around the same time.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The 77 strains of *C. jejuni* examined in this study comprised: (*a*) 27 sporadic and 18 outbreak-associated (from 15 primary and 3 secondary cases) clinical isolates received from the Cardiff Public Health Laboratory (CPHL) over a 1-month period during April and May 1994; (*b*) 30 epidemiologically unrelated strains of heat stable serotype 2 (HS2) originating from other locations in the UK during 1993 and 1994 that included 13 animal strains (10 bovine and 3 ovine) and 17 sporadic human isolates; (*c*) 2 HS2 reference strains (NCTC 11168 and NCTC 12501) obtained from the National Collection of Type Cultures (NCTC).

All strains were grown at 37 °C for 48 h on 5% v/v defibrinated horse blood agar under microaerobic conditions (5% O_2, 5% CO_2, 2% H_2, 88% N_2) in a variable atmosphere incubator (Don Whitely Scientific Ltd, Shipley, UK) except where indicated otherwise. Stocks of strains were preserved in 10% v/v glycerol in Nutrient Broth No. 2 (Oxoid; CM67) over liquid nitrogen.

Identification and biotyping

The extended biotyping scheme of Lior [4] was used to speciate and biotype the strains. Identification was based on Gram strain, growth at 42 °C, hippurate hydrolysis, rapid H_2S production and DNA hydrolysis.

Serotyping

Strains were serotyped (heat-stable antigens) according to the passive haemagglutination method of Penner and Hennessy [2] using a panel of 45 antisera provided by Dr A. Lastovica (Red Cross Memorial Children's Hospital, Cape Town, RSA).

Bacteriophage typing

The bacteriophage type of each strain was determined according to the Preston phage-typing scheme [5]. The scheme employed 16 phages, 6 of which originated from Illinois, USA and were part of the Grajewski [6] phage-typing system. The remaining 10 phages were isolated in the UK by Salama and colleagues [5].

Ribotyping

Genomic DNA was extracted using cetyltrimethyl-ammonium bromide (CTAB) [7]. *Hae* III and *Pst* I digestion, agarose gel electrophoresis, Southern blotting and hybridization with biotinylated probe DNA were carried out as previously described [8] except the 1500 bp 16S rRNA gene specific probe was generated by PCR amplification of genomic DNA from *C. jejuni* NCTC 11168. The primer sequences used were 5′-AAGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTT-GTTACGACTT-3′ which represent nucleotides 7–27 and 1492–1510 respectively of the *Escherichia coli rrn*B sequence [9]. The primers were commercially synthesized (Pharmacia Biosystems, UK).

Pulsed field gel electrophoresis

The preparation of bacterial DNA for PFGE and the separation of *Sma* I restriction fragments using a CHEF-DR II system (Bio Rad Laboratories, USA) were as previously described [10]. In brief, the bacterial cells were incorporated into 1% w/v agarose plugs and lysed for 48 h at 56 °C in two changes of solution containing 1% w/v *N*-Lauroyl sarcosine, 0·5 mM-EDTA pH 9·5 and 0·5 mg/ml proteinase K. The plugs were washed and stored at 4 °C in Tris-EDTA buffer (10 mM-Tris, 10 mM-EDTA, pH 7·5). *Sma* I (Gibco-BR Ltd, Paisley, UK) and *Kpn* I (Gibco-BRL) digestion of 3 mm agarose gel slices using the manufacturer's buffer was for 6 h at 25 °C and 37 °C respectively. The blocks were loaded onto a 1% w/v agarose gel prepared in 0·5 × TBE (45 mM-Tris, 45 mM boric acid, 1 mM-EDTA). Electrophoretic separation of *Sma* I generated fragments was at 200 V, 14 °C, for 22 h with ramped pulse times from 10 to 35 s; of *Kpn* I generated fragments, 200 V, 14 °C, 23 h with ramped pulse times from 4 to 20 s. DNA degradation in Lior biotype II (DNase positive) strains was prevented by formaldehyde fixation of the bacterial cells prior to their incorporation into agarose [10].

RESULTS

The results are given in Tables 1 and 2.

Serotyping

Nine different HS serotypes were identified among 42 isolates of *C. jejuni* from the Cardiff area (Table 1). Three further strains were non-typable. Seventeen of the 18 primary or secondary outbreak-associated strains and 7 strains associated with sporadic infection in the Cardiff set were serotype HS2. The remaining epidemiologically related isolate (C637/94) was serogroup HS4. Seven of the HS2 strains, including three from the outbreak, also cross-agglutinated with the HS1 antisera although the titres were lower than with the HS2 antisera. NCTC 11168 reacted equally with both HS1 and HS2 antisera. The serotyping results on the remaining 20 Cardiff strains which caused sporadic infection are listed in Table 1.

Ribotyping

The 16S rRNA gene probe detected three well-separated bands on Southern blots of the *Pst* I digested DNAs, and in all but one of the *Hae* III digests. The *Pst* I bands ranged from approx. 6·5–30 kbp and most *Hae* III bands from approx. 1·0–13·5 kbp (Fig. 1). Seventeen of the outbreak-associated strains, from 14 primary cases and 3 secondary cases (all HS2), gave the same *Pst* I pattern (designated P1) and the same *Hae* III pattern (H1) as did most (6/7) of the sporadic HS2 strains from Cardiff. Seventy-seven per cent (23/30) of the HS2 set of strains from locations other than Cardiff gave the same combined ribotype (P1 H1) as did the reference strains NCTC 11168 and NCTC 12501. Five other isolates in the HS2 set of strains showed the H1 *Hae* III ribopattern, but had a different *Pst* I ribopattern (P2) representing a small shift in one of the three bands from 11·5 to 12·4 kbp. This pattern (P2 H1) was seen in the seventh sporadic HS2 isolate from Cardiff. The remaining 20 sporadic Cardiff isolates, which represented

Table 1. *Typing results for human isolates of* C. jejuni *from the Cardiff area*

Strain identity	HS serotype	16S ribotype		Preston phage group	*Sma* I profile	Lior biotype
		Pst I	*Hae* III			
Outbreak strains						
Main Group ($n = 15$) (12 primary cases) (3 secondary cases)	2*	P1	H1	52	A‡	I
Minor Group ($n = 2$)	2, 1	P1	H1	NT	B	II
C637/94	4 complex†	P5	H1	146	I	I
Other isolates						
C587/94	2	P1	H1	52	A	I
C548/94	2	P1	H1	NT	B	II
C616/94	2	P1	H1	52	C	I
C617/94	2	P1	H1	52	C	I
C618/94	2, 1	P1	H1	52	C	I
C639/94	2, 1	P1	H1	52	H	II
C640/94	2	P2	H1	69	U	I
C658/94	4 complex	P2	H3	44	U	I
C590/94	4 complex	P4	H1	44	U	II
C655/94	4 complex	P4	H1	121	I	I
C546/94	4 complex	P4	H6	90	U	II
C606/94	4 complex	P4	H6	NG	U	II
C550/94	4 complex	P5	H1	69	U	II
C589/94	4 complex	P5	H1	121	NC	II
C584/94	53	P4	H6	NG	U	I
C614/94	53	P5	H4	90	U	II
C586/94	1	P13	H17	NT	U	II
C594/94	1	P1	H1	76	U	II
C641/94	11	P4	H15	NT	U	II
C656/94	11	P4	H15	NT	U	II
C659/94	44, 1	P4	H1	NG	U	I
C661/94	5	P4	H5	84	U	I
C613/94	31, 5	P4	H5	102	U	II
C634/94	7, 6, 27	P6	H5	NT	U	II
C610/94	NT	P4	H5	NT	U	II
C657/94	NT	P4	H5	NT	U	I
C662/94	NT	P15	H1	52	H	II

* Three strains cross-reacted with 01 antisera.

† Four complex includes strains expressing any combination of antigens 4, 13, 16, 43, 50. NT, Non-typable; NG, New group; NC, No cuts; U, Unique profile.

‡ A, B, C, H, I, *Sma* I banding patterns that were shared by two or more isolates.

8 different serotypes and included 3 serologically non-typable strains, exhibited a further 7 *Pst* I patterns and 6 *Hae* III patterns, giving 12 different combined ribotypes (Table 1, Fig. 1). The final outbreak associated strain, C637/94, serogroup HS4, had the combined ribotype P5, H1.

PFGE

PFGE analysis of *Sma* I digested DNA yielded between 6 and 9 fragments ranging in size from 40–480 kbp (Fig. 2). The majority (12/15) of outbreak-associated strains from primary cases and all three isolates from secondary cases

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Table 2. *Typing results for* C. jejuni *Penner serotype 2 (HS2) strains from locations other than Cardiff*

Strain identity	Host	*Sma* I profile	16S ribotype		Preston phage group	Lior biotype
			Pst I	*Hae* III		
C113/92	Human	A	P2	H1	42	I
C149/92	Human	A	P2	H1	52	I
C156/92	Human	A	P2	H1	52	I
C579/93	Human	A	P1	H1	44	I
C180/93	Bovine	A	P1	H1	52	I
C181/93	Bovine	A	P1	H1	55	I
C185/93	Bovine	A	P1	H1	52	I
C403/93	Ovine	A	P1	H1	52	I
C407/93	Ovine	A	P1	H1	52	I
C258/94	Human	B	P14	H1	NT	II
C483/94	Human	B	P1	H1	NT	II
C667/93	Human	C	P1	H1	52	I
C25/92	Bovine	C	P1	H1	52	I
C210/92	Human	D	P1	H1	52	II
C11/92	Bovine	D	P1	H1	52	II
C229/93	Bovine	D	P1	H1	52	II
C230/93	Bovine	D	P1	H1	52	II
C706/93	Human	E	P1	H1	NT	II
C32/92	Bovine	E	P2	H1	NG	I
C177/93	Bovine	E	P2	H1	52	I
C816/93	Human	F	P1	H1	52	II
C30/92	Bovine	F	P1	H1	52	II
C455/93	Human	G	P1	H1	63	II
C490/93	Human	G	P1	H1	NG	II
C629/93	Ovine	U	P1	H1	52	II
C572/93	Human	U	P1	H1	NT	II
C707/93	Human	U	P1	H1	NT	II
C617/93	Human	U	P1	H1	69	I
C40/93	Human	U	P28	H6	84	I
C37/92	Human	U	P1	H1	NT	I
NCTC 11168	Human	A	P1	H1	52	I
NCTC 12501	Human	E	P1	H1	52	II

Legend as for Table 1.

gave a *Sma* I pattern identical to that of NCTC 11168 (Pattern A). This banding pattern (A) was observed in one further HS2 strain from Cardiff although the 10 year-old child from whom it was isolated had not participated in the school visit to the implicated farm but lived just two houses away. Two further strains isolated from individuals, who participated in the farm visit, both yielded a second *Sma* I pattern (B) which differed from the main group in the size of 1 of the 9 bands (Fig. 2). Pattern B was also seen in 1 of the 7 strains associated with cases of sporadic infection in the area and 2 of the 30 strains in the HS2 group from other areas. The final outbreak strain, which differed from the others in the results of all tests, produced a quite different *Sma* I PFGE pattern (Pattern I, Fig. 3).

Thirteen *Sma* I banding patterns were observed amongst the remaining 39 non-

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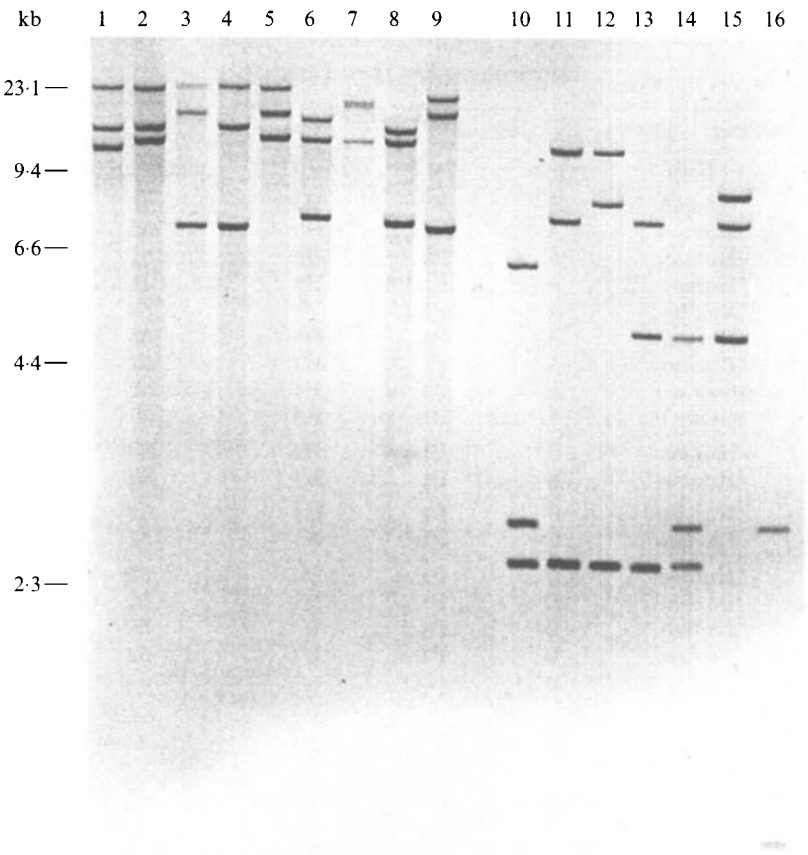

Fig. 1. 16S ribotypes of *C. jejuni*. Representative *Pst* I digests are shown in lanes 1–9. Lanes 10–16 show representative *Hae* III digests. Lanes contain designated ribotype patterns as follows: 1, P1; 2, P2; 3, P4; 4, P5; 5, P6; 6, P13; 7, P14; 8, P15; 9, P28; 10, H1; 11, H3; 12, H4; 13, H5; 14, H6; 15, H15; 16, H17. The numbers on the left side indicate molecular sizes (kilobases).

outbreak HS2 strains tested (Fig. 2). Pattern A was the most common PFGE profile obtained in the HS2 set, occurring in 9/30 strains including three bovine and two ovine isolates (Table 2). Two of the 13 patterns closely resembled that of NCTC 11168 (pattern A) and differed by one or two minor band shifts at the 141 and 156 kbp positions. These slight band shifts separated pattern A from pattern C, and pattern A from that of strain C617/93 (Fig. 2). The altered band sizes were more evident when a 4–18 s ramp was used over 23 h rather than the standard 10–35 s over 22 h; the latter PFGE conditions were usually employed to separate *Sma* I generated fragments. The 21 non-HS2 isolates from Cardiff yielded 19 different *Sma* I PFGE patterns as seen in Figure 3. The DNA of one further strain (C589/94) appeared to be refractory to digestion with *Sma* I.

Kpn I digestion of the DNA samples produced up to 13 easily visualized fragments and most of these ranged in size from approximately 40–200 kbp. Smaller bands of lower intensity could be detected but were not included in the analysis. Isolates separated by only minor shifts in band positions following *Sma* I digestion showed two or three clear differences when *Kpn* I was used. With the latter enzyme, a difference of three bands between the main group of HS2

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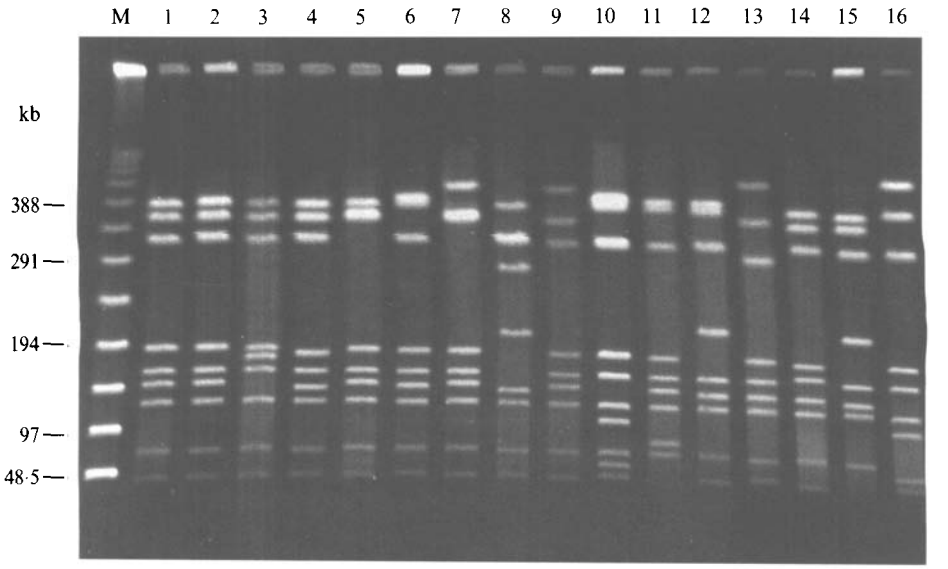

Fig. 2. *Sma* I generated PFGE patterns of HS2 serotype strains of *C. jejuni*. Patterns A to H (Lanes 1–9) were demonstrated in more than one isolate. Those shown in lanes 10–16 were found only in single HS2 strains. Lane M, molecular size markers. Lanes 1–16. 1, NCTC 11168 (Pattern A); 2, C608/94 (outbreak strain, Pattern A); 3, C607/94 (Pattern B); 4, C617/94 (Pattern C); 5, C210/92 (Pattern D); 6, C177/93 (Pattern E); 7, C30/92 (Pattern F); 8, C455/93 (Pattern G); 9 C639/94 (Pattern H); 10, C640/94; 11, C629/93; 12, C572/93; 13, C707/93; 14, C617/93; 15, C40/92; 16, C37/92.

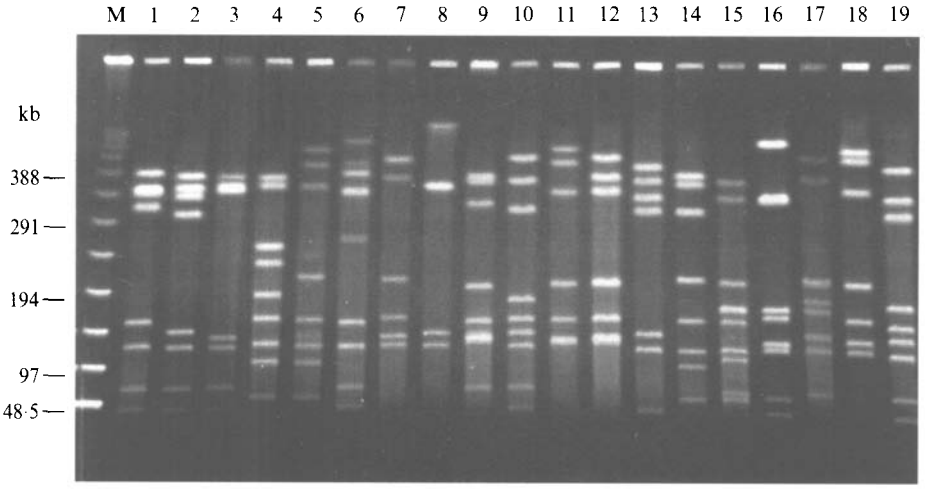

Fig. 3. *Sma* I generated PFGE patterns of *C. jejuni* non-HS2 strains isolated from sporadic cases in the Cardiff area. The pattern shown in lane 1 is Pattern I, i.e. the result obtained for the atypical outbreak strain C637/94. Serotypes of strains in lanes: 1–6, HS4; lanes 7 and 8, HS53; lanes 9 and 10, HS1; lanes 11 and 12, HS11; lane 13, HS44, 1; lane 14, HS55; lane 15, HS31, 5; lane 16, HS6, 7, 27; lanes 17–19 contain serologically non-typable strains. Lane M, molecular size markers.

outbreak strains and NCTC 11168 could be seen (Fig. 4), even though they had identical *Sma* I patterns.

The increased discriminatory power resulting from *Kpn* I digestion was demonstrated with a further three HS2 isolates from Cardiff. All three gave

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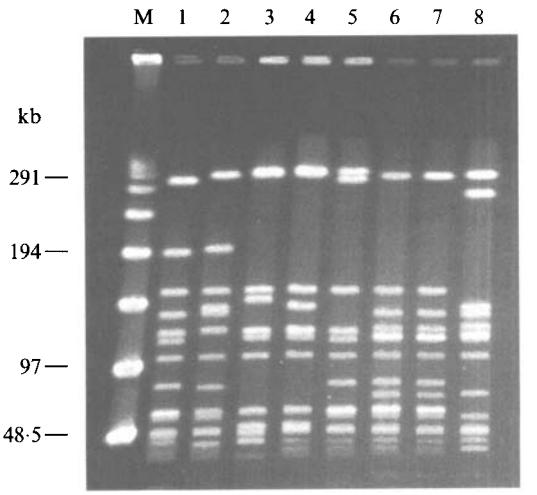

Fig. 4. *Kpn* I generated PFGE patterns of *C. jejuni* strains demonstrating differences between isolates which appeared to be identical following *Sma* I digestion. Isolates in lanes 1–5 were indistinguishable after *Sma* I digestion (*Sma* I Pattern A) as were isolates in lanes 6, 7 and 8 (*Sma* I Pattern C). The strains in lanes 6 and 7 were isolated from two members of the same family. Lane 1, C185/93; lane 2, C407/93; lane 3, C149/92; lane 4, C615/94 (representing outbreak strains); lane 5, NCTC 11168; lane 6, C616/94; lane 7, C617/94; lane 8, C618/94. Lane M, molecular size markers.

identical ribotyping and phage-typing results, and identical *Sma* I PFGE patterns (Pattern C, Table 1). Two of these three isolates were from members of the same family. *Kpn* I digestion of the DNA showed that these two strains were identical but different from the third isolate in the sizes of five bands (Fig. 4).

Phage-typing

The 15 outbreak or outbreak-associated strains, which shared the same *Sma* I and *Kpn* I PFGE profiles, belonged to one phage group (Preston phage group 52). Fifty-nine per cent (23/39) of all the HS2 strains, which were not implicated in this outbreak, also belonged to this phage group 52. The two HS2 outbreak associated strains, which shared a different PFGE pattern to the main group, were both non-typable with the phages as were a further 18% (7/39) of all the sporadic HS2 isolates. In total, eight phage-typing groups occurred in the HS2 strains, three of which (groups 44, 52 and 69) were also seen in the non-HS2 isolates from Cardiff (Tables 1 and 2). Twenty-eight per cent (6/21) of the latter set of strains were not phage-typable.

DISCUSSION

The aim of the present study was to evaluate the usefulness of three methods (ribotyping, phage-typing and PFGE) in the identification of enteritis outbreak-associated strains of *C. jejuni*. The ability of each method to separate these strains from others of the same serotype (HS2), as well as from sporadic isolates of different serotypes occurring in the same locality at around the same time as the outbreak, was assessed. It was of interest to note that the typing results obtained from one of the outbreak strains were consistently different from those of the other epidemiologically linked isolates. However, the specimen from the mother of this

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primary case yielded identical results to the main outbreak group. As we were unable to explain this anomaly, the results from the primary case strain were not included in our analysis.

Ribotyping using *Pst* I and *Hae* III digested DNA and a *C. jejuni* rRNA gene probe did not differentiate epidemiologically unrelated isolates of the same serotype (HS2). Although every strain was typable using this method, all but one HS2 strain gave the H1 (*Hae* III) pattern and 95% of all HS2 strains tested exhibited just two combined ribotypes (P1 H1 and P2 H1). The combined profile, P1 H1, which was evident in approximately 80% of the HS2 serotype isolates examined, was also the most common profile found in a previous study of serotype HS1 strains where it occurred in 73/80 (91%) of HS1 isolates [11]. It would appear that the three copies of the 16S rRNA gene detected in the genome of *C. jejuni* are too few to establish individual strain identity by ribotyping. Single enzyme ribotypes were not limited to one serotype; e.g. the *Pst* I pattern, P4, occurred in strains of six different HS serogroups (4; 53; 11; 44, 1; 5; 31, 5) as well as in two serologically non-typable strains. The *Hae* III pattern, H5, was seen in three different serogroups (5; 31, 5; 7, 6, 27). It is clear that the restriction sites for *Hae* III and *Pst* I are conserved amongst strains of different serotype and that the use of both enzymes to give a combined ribotype is necessary to obtain a basic level of discrimination amongst *C. jejuni* isolates.

If a typing method is to be judged on the criteria of typability, reproducibility and discriminatory power, as suggested by Maslow [12] then we found phage-typing was less satisfactory than either ribotyping or PFGE according to the first two criteria. Overall, approximately 22% of all strains were non-typable by phage-typing. The reproducibility problems associated with this method result in part from the sensitivity of the phage reactions to changes in the culture conditions; e.g. the moisture content of the agar medium. Also, as phage-typing is a phenotypic method, it relies on the presence of surface antigens which may or may not be expressed in a stable manner. In a review of campylobacter typing schemes, Patton and Wachsmuth [3] concluded that the Preston phage-typing scheme showed a 'moderate' power of discrimination. However, we found that phage-typing failed to discriminate effectively between the sporadic HS2 strains and the outbreak HS2 isolates, with 59% of all HS2 strains belonging to one phage group (Group 52) and a further 18% being non-typable.

PFGE was the most discriminatory of the subtyping methods investigated and genetic heterogeneity could be detected in epidemiologically unrelated isolates which had shown the same combined ribotypes. *Sma* I digestion produced a small number of fragments (less than ten) which were generally well separated and relatively simple to compare between strains. Many of the *Sma* I fragments were conserved within the HS2 group, pointing to a general lack of genetic diversity between members of that serotype. Epidemiologically unrelated strains generally showed few band shifts in PFGE profiles and a single band difference separated outbreak strains from several other sporadic HS2 isolates from in the Cardiff area during the same period. It was necessary to use a second enzyme, *Kpn* I, to establish that the majority of outbreak strains were all the same, and to distinguish between them and other isolates with the same *Sma* I generated PFGE profile. It has been previously suggested that isolates with a difference of only one

or two bands can generally be regarded as variants of each other, and a difference of three or more bands is required to demonstrate independent strains [13]. In this study, two isolates which produced identical *Sma* I patterns were then shown to have a five band difference following *Kpn* I digestion. We would therefore suggest that PFGE profiles require careful interpretation as this appears to be dependent on, and specific to, the restriction enzyme used and the bacterial species under investigation.

In terms of the typability criterion, PFGE gave highly reproducible results, and the relative stability of the *Sma* I restriction sites within the HS2 serotype over 2 years covering both differing geographical locations and host species were evident. It is of interest to note that many of the conserved *Sma* I fragments within the HS2 serotype were also conserved within the HS1 serotype [11] indicating that these serotypes are probably clonally related. Four *Sma* I PFGE profiles, which occurred in the HS2 isolates were previously observed in HS1 strains (Patterns A and E, and those of C639/94 and C572/93) [11]. The patterns seen in the other HS serotypes studied did not closely resemble those of the HS2 serotype, which suggested that the other serotypes belonged to more distantly related lineages.

PFGE has been used successfully in the epidemiological investigations of a variety of bacterial genera and species; e.g. *Salmonella typhi* [14], *Escherichia coli* [15], and *Vibrio cholera* [16]. This study is the first in which PFGE has been used in an epidemiological investigation of a *C. jejuni* outbreak in the UK, and the results validate the method for HS2 isolates. When compared to ribotyping it is more rapid to perform, less labour intensive and more discriminatory. In our previous study on HS1 and HS4 sporadic strains, PFGE likewise provided a high level of discrimination [11]. In conclusion, we would suggest Penner (HS) serotyping as the first line method of investigation in the epidemiological typing of *C. jejuni*, with the use of PFGE for intra-serotype strain differentiation.

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