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Validity of *Sma*I-defined genotypes of *Campylobacter jejuni* examined by *Sal*I, *Kpn*I, and *Bam*HI polymorphisms: evidence of identical clones infecting humans, poultry, and cattle*

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SUMMARY

We describe here an examination of the validity of molecular types of *Campylobacter jejuni* as defined by separation of *Sma*I-digested DNA using pulsed-field gel electrophoresis (PFGE), recently suggested as part of a molecular subtyping scheme. Thirty-four Danish strains from humans, water, poultry and cattle were assigned to one of six *Sma*I 'profile groups' (PGs), with two additional strains included as genotypically distinct controls. The interstrain relationships were reexamined by PFGE of *Sal*I, *Kpn*I and *Bam*HI-digested DNA, and also by serotyping with heat-stable antigens. All outbreak-related strains were indistinguishable by all criteria, as were two sets of two randomly-isolated human strains. Two groups of indistinguishable isolates contained randomly isolated strains from more than one source (poultry, humans and/or cattle), a finding with significant epidemiological connotations. All 'genetically identical' strains belonged to the same serotype, whereas genetic differences were detected between strains assigned to the same *Sma*I PG but differing in serotype. We conclude that PFGE-based genetic fingerprinting can yield invaluable data for epidemiological studies of sporadic *C. jejuni* infection, but that results based on one restriction site polymorphism must be checked with another enzyme.

INTRODUCTION

Campylobacter jejuni subsp. *jejuni* (hereafter *C. jejuni*) is well established as an important cause of gastroenteritis and other illnesses in humans and animals [1]. Estimates of the incidence of diarrhoeal campylobacter infection suggest that 1% of the population per year will be affected by campylobacteriosis [2]. The incidence of this infection in Denmark has recently shown a marked increase [3]. Most cases of campylobacteriosis in humans occur sporadically, with the principal route of infection believed to be foodborne [1, 2]. Accurate identification of the sources of sporadic campylobacter infection is especially difficult, since the organisms are widely distributed in nature. Effective and accurate typing methods are therefore needed to help clarify the complex epidemiology of such infections. However, strain discrimination using phenotypic typing methods such as bio-, phage- and serotyping may be significantly compromised due to poor reproducibility or inadequate discriminatory power [4, 5]. Consequently, there has been an increased interest in the use of genomic typing methods which examine relatively stable chromosomal differences and are generally more sensitive than phenotypic schemata [4–12]. Of

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the genotypic methods presently available, macrorestriction profiling by pulsed-field gel electrophoresis (MRP–PFGE) appears to be the most discriminatory [5, 8–10], possibly a consequence of its ability to examine restriction polymorphisms along the entire bacterial chromosome. However, optimal use of any such genotypic method is clearly related to the restriction enzyme used. There is therefore a need to examine the validity of genotypes defined on the basis of polymorphisms around a single restriction site, if the method is to be considered as potentially definitive, as suggested for *Sma*I MRP–PFGE [9].

It has been shown previously that certain *Sma*I-defined macrorestriction types can be further distinguished by *Kpn*I [8]. Such results have significant implications for any epidemiological hypothesis made on the basis of MRP–PFGE data generated by use of *Sma*I. Moreover, since the interpretation of any typing data is considered particularly demanding in situations where the epidemiologic scenario is not well defined [5, 10], it is essential to establish the validity of *Sma*I genotypes if considering the use of MRP–PFGE for continuous surveillance.

The aim of the present study was to investigate the validity of *Sma*I MRP–PFGE defined 'types' of Danish *C. jejuni* strains isolated from diverse sources by comparing the results of MRP–PFGE typing using additional restriction enzymes *Sal*I, *Kpn*I and *Bam*HI, and additionally by comparing the results obtained with serotyping information. These data also allow for an evaluation of the potential of MRP–PFGE typing for molecular epidemiological investigations performed as part of the continuous surveillance programme of zoonotic diseases in Denmark.

MATERIALS AND METHODS

Bacterial strains and identification procedures

Thirty-six strains of *C. jejuni* from human ($n = 20$), poultry ($n = 10$), and cattle ($n = 5$) faeces, and a single water isolate, were studied (Table 1). The strains under investigation were selected on the basis of an initial study of 108 strains (S. L. W. On, unpublished data), of which 73 were obtained by random sampling within a 6-month period as part of the Danish zoonosis surveillance programme; and 35 strains were associated with a water-borne outbreak of *C. jejuni* on the region of Jutland in Denmark [13]. *Sma*I-derived macrorestriction profiles were obtained for all strains and those with indistinguishable, near-identical (minor shifts noted in the molecular weight of certain band fragments), similar (differing by the presence or absence of one band) or unique profiles were subsequently identified by visual analysis. Consequently, 22 randomly isolated strains could be assigned to one of six *Sma*I 'profile groups' (PGs), of which one (PG 2) included a representative number ($n = 12$) of outbreak-associated isolates. Two single randomly isolated strains were included as genotypically unique study references.

The species identity of all strains was checked by performing several key phenotypic tests (derived from [14]). These were: Gram-stained cell morphology; production of oxidase, catalase and urease; hydrolysis of hippurate, and indoxyl acetate; reduction of nitrate; microaerobic growth at 42 °C, and on minimal, and nalidixic acid- and cephalothin-containing media. All tests were performed using recommended, standardized methods [15, 16].

Serotyping

Strains were serotyped in microtitre plates using passive haemagglutination of heat-stable antigens as described by Penner and Hennessey [17]. Details of antisera production and dilutions, and interpretation of reactions have been described previously [18].

Macrorestriction profiling

DNA-containing agar blocks were prepared from 72 h cultures using the method of Gibson and colleagues [19], modified as described previously [20]. However, the optical densities of the bacterial suspensions used were adjusted to 1·2 at 405 nm and 300 μl aliquots of each gently mixed with 700 μl chromosomal grade agarose (Bio-Rad Laboratories Copenhagen, Denmark, product no. 162-0135).

For macrorestriction profiling, 1·5–2·0 mm thick slices were cut from the DNA-containing blocks and preincubated at room temperature for 1 h with the manufacturer's recommended buffer for the pertinent enzyme. Digestion of DNA was performed subsequently by adding 20 U of the pertinent enzyme (i.e. *Sma*I, *Sal*I, *Kpn*I or *Bam*HI [Amersham Life Sciences, Birkerød, Denmark]) and incubation for 5 h under the manufacturer's recommended conditions. DNA fragments were separated by PFGE in 0·9 % (*Kpn*I digests) or 1·0 % (*Sma*I, *Sal*I and *Bam*HI digests) pulsed field certified agarose (Bio-Rad, product no. 162-0137) using a contour-clamped homogeneous electric field

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Table 1. *Details of strains of* Campylobacter jejuni *used and typing results*

Study no.	Strain no.	Source	*Sma*I	*Sal*I	*Kpn*I	*Bam*HI	Serotype
1	380-827	Poultry	1	A	i	I	55
2	787-657	Poultry	1	B	ii	II	6, 7
3	4039	Cattle	1	B	ii	II	6, 7
4	835-770	Poultry	1	C	iii	III	42
5	328-684	Poultry	U	U	U	U	55
6	4006	Cattle	2	D	iv	IV	1, 44
7	5001*	Human	2	D	v	V	2
8	5003*	Human	2	D	v	V	2
9	5014*	Human	2	D	v	V	2
10	5015	Human	2	D	vi	V	2
11	5016*	Human	2	D	v	V	2
12	5008*	Human	2	D	v	V	2
13	SSI 7405*	Human	2	D	v	V	2
14	SSI 7455*	Human	2	D	v	V	2
15	SSI 7596*	Human	2	D	v	V	2
16	SSI 7674*	Human	2	D	v	V	2
17	SSI 7481*	Human	2	D	v	V	2
18	Vand-B*	Water	2	D	v	V	2
19	SSI 7868*	Human	2	D	v	V	2
20	116/2	Poultry	3	E	vii	VI	1, 44
21	116/20	Poultry	3	E	vii	VI	1, 44
22	5025	Human	3	E	vii	VI	1, 44
23	104-733	Poultry	3†	F	viii	VII	1, 44
24	065-913	Poultry	3	E	vii	VI	1, 44
25	282-690	Poultry	4	D	ix	VIII	2
26	5012	Human	4	D	ix	VIII	2
27	4026	Cattle	4	D	ix	VIII	2
28	5029	Human	4	D	ix	VIII	2
29	4009	Cattle	4	D	x	VIII	2
30	5002	Human	5	G	xi	IX	2
31	5004	Human	5	G	xi	IX	2
32	5024	Human	6	D	xii	X	2
33	5030	Human	6	D	xii	X	2
34	4017	Cattle	6	D	xiii	X	2
35	309-669	Poultry	6	D	xiv	XI	1, 44
36	5040	Human	U	U	U	U	2

* Outbreak isolate. All molecular types are arbitrarily defined. U, unique PFGE profile.

† Defined as similar to other PG 3 strains (see text for details).

(CHEF) apparatus (Bio-Rad model DR-III, Copenhagen, Denmark) using the following ramping parameters for each digest: *Sma*I: 5–10 s, 4 h; 10–40 s, 14 h; 50–60 s, 4 h; *Sal*I: 3–10 s, 4 h; 10–45 s, 18 h; *Kpn*I: 4–20 s, 22 h; *Bam*HI: 2–5 s, 7 h; 6–15 s, 8 h; 18–20 s, 5 h.

Macrorestriction profiles (MRPs) were visualized after gels were stained in ethidium bromide, destained in water and photographed under ultraviolet light.

RESULTS

Phenotypic testing

All strains gave reactions typical of *C. jejuni* subsp. *jejuni* in the tests used, when compared to data on type and reference strains [14].

Serotyping

Strains were assigned to one of the eight serotypes defined by the serotyping scheme used (Table 1). Serotype 2 accounted for 23 strains (including 12

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outbreak strains), with 6 and 2 strains respectively assigned to serotypes 1, 44 and 6, 7. The remaining five strains were of distinct serotypes.

Of the six *Sma*I profile groups, three contained strains which belonged to different serotypes (Table 1). However, all strains which were indistinguishable by MRP–PFGE by all four restriction enzymes used belonged to the same serotype (Table 1).

General features of macrorestriction profiles

The MRPs obtained for each restriction enzyme are shown in Figure 1. *Sma*I MRPs comprised 5–9 fragments ranging from *c*. 30 to >630 kb in size, whilst *Sal*I MRPs were somewhat simpler and contained 5–7 fragments within the same size range. *Bam*HI MRPs were considerably more complex: 3–6 bands could be observed between *c*. 92 and 267 kb, but accurate estimates regarding the number of fragments smaller than 92 kb was not possible due to their high frequency. *Kpn*I MRPs comprised 10–16 fragments $\leqslant 400$ kb; accurate estimations of the lowest fragment size could not be made due to the limitations of the molecular weight standard used (λ ladder).

Comparison of *Sma*I strain groupings with *Sal*I, *Kpn*I and *Bam*HI-derived types

All MRPs were evaluated and assigned to arbitrarily-defined 'profile groups' (PGs). The results are summarized in Table 1 which also lists the serotype information for each strain. All outbreak-related strains belonged to serotype 2 and were indistinguishable, irrespective of the endonuclease used for MRP–PFGE. Of the 6 *Sma*I PGs, 5 contained subsets of strains that were not further differentiated by any of the endonucleases used: most of these strains were isolated from a single source. However, 3 and 4 such strains belonging to *Sma*I PGs 3 and 4 respectively had been isolated from humans, poultry, and (*Sma*I PG 4 only) cattle. Two strains (respectively isolated from poultry and cattle) belonging to *Sma*I PG 1 were differentiated only by a minor difference in the intensity of a single band fragment of their *Bam*HI profiles; both isolates were serotype 6, 7.

DISCUSSION

The results presented here provide important data concerning the optimal use of MRP–PFGE in continuous epidemiological surveillance of *C. jejuni* infection and also demonstrate a clear link between sporadic human infection and two food animal sources, poultry and cattle. Our results show that *Sma*I MRP–PFGE is a generally robust means of accurately determining *C. jejuni* strain relationships. All 12 outbreak-related strains proved indistinguishable by each of the restriction enzymes used, whereas the 2 strains showing unique *Sma*I genotypes were found to be distinct by additional restriction polymorphisms. Of the 22 other strains assigned to 1 of the 6 *Sma*I profile groups, 14 were not differentiated further by the use of other endonucleases. However, the integrity of *Sma*I-defined genotypes cannot be taken for granted. Eight isolates belonging to *Sma*I profile groups 1, 2, 3, 4 and 6 (as defined here) were shown to be distinct from other strains belonging to their respective *Sma*I profile groups, by virtue of MRPs produced by additional restriction endonucleases. These findings are consistent with the results of Gibson and colleagues [8], who demonstrated that certain strains giving the same *Sma*I MRP yielded distinct MRPs when endonuclease *Kpn*I was used. Nonetheless, we note here that our results are not entirely unexpected for some strains (namely strains belonging to *Sma*I PG 1, PG 3 (104–733) and 309–669 (PG 6), since minor differences in the banding patterns can be observed when compared to other membranes of the appropriate *Sma*I profile group (Fig. 1). The assignation of such 'similar' MRPs to the same profile group is, however, consistent with present recommendations concerning the interpretation of such molecular typing data [5, 10]. Furthermore, we also note that two strains identified as Penner 6, 7 (787–657 [poultry] and 4039 [cattle]) differed only in the intensity of a single band where *Bam*HI was employed and such minor variation is in agreement with a clonal relationship [5, 10].

In a recent overview of typing methods, Arbeit [5] stated that 'no typing method confirms that the entire genomes of two organisms are identical'. The general validity of this view is emphasized by our results and those of Gibson and colleagues [8], since the resolution of MRP–PFGE has been shown to depend on the choice of restriction endonuclease. However, in using a range of endonucleases for typing (as in the present study), it is reasonable to presume that strains shown to be indistinguishable in each analysis are, for epidemiological purposes, genetically identical, since this process would effectively have mapped multiple restriction sites within the DNA content of the whole bacterial cell. Although most genetically identical

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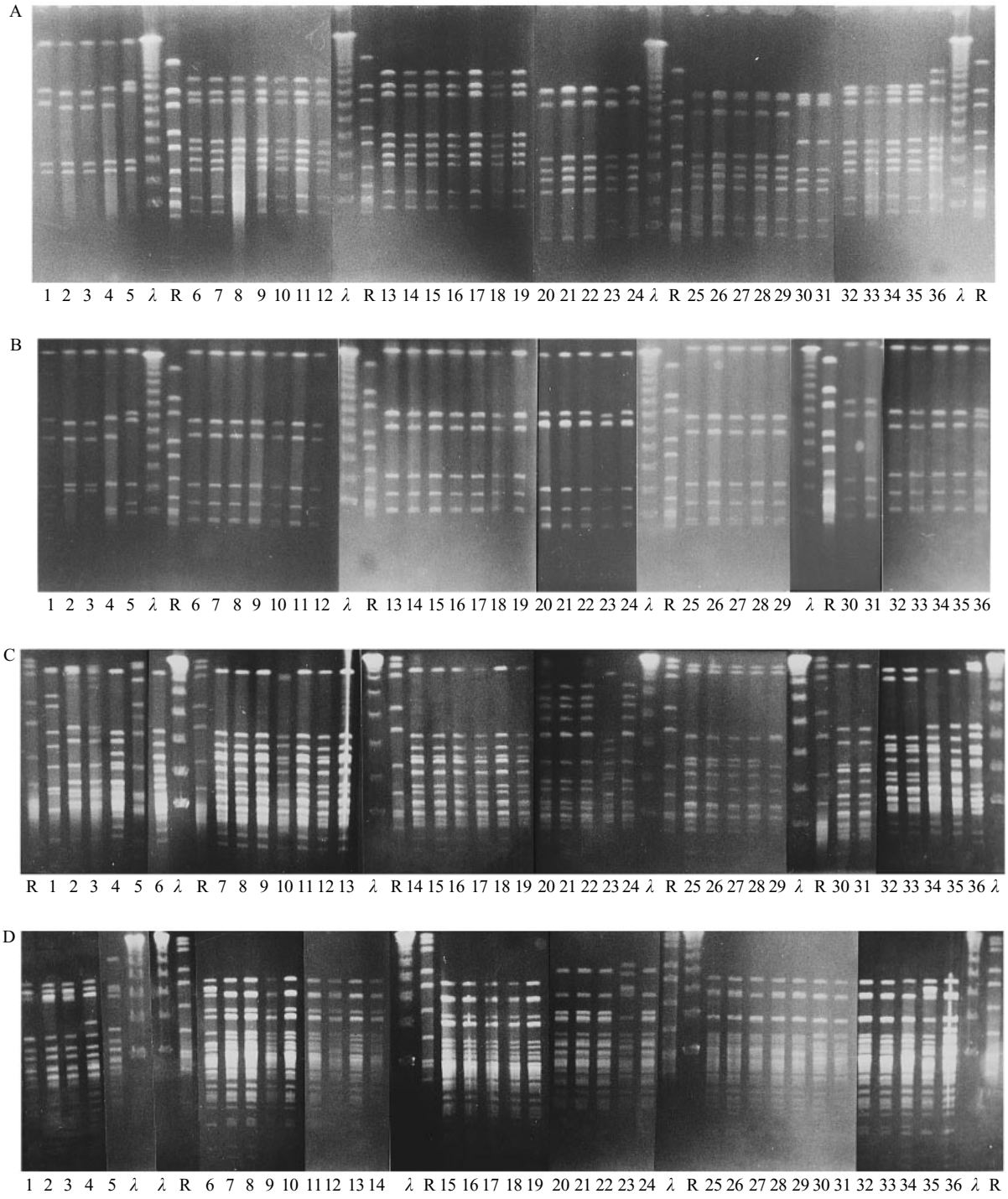

Fig. 1. Macrorestriction profiles of 36 strains of *C. jejuni* using restriction endonucleases *Sma*I (A), *Sal*I (B), *Kpn*I (C) and *Bam*HI (D). The numbers below each photograph refer to study numbers listed in Table 1. Tracks labelled R denote a standard reference strain marker (*Sma*I digest, *C. hyointestinalis* subsp. *hyointestinalis* CCUG 14169^{T}). λ indicates molecular weight marker (λ ladder).

strains were shown to be from the same source in this study, we identified identical *C. jejuni* isolates from humans, poultry and cattle, isolated by purely random sampling. These data provide substantial genotypic evidence for a link between sporadic human campylobacteriosis and foodstuffs. Although poultry are traditionally regarded as the principal source of such infection [1, 2], cases linked to bovine sources have been described previously [21, 22]. These data confirm the widely accepted view that some cases of sporadic

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human campylobacteriosis are mediated by the consumption of contaminated poultry products, but also indicate the need for further investigations concerning the infectious potential of other foodstuffs such as beef as a source of human infection or as a reservoir for *C. jejuni* strains pathogenic to humans. Conversely, it is also noteworthy that our data suggest several clones occur only in humans and that certain strains have been isolated only from animal sources. Similar observations have been noted in several other studies where a variety of phenotypic and genotypic typing methods have been used to investigate inter-strain relationships of *C. jejuni* from various sources [6, 7, 9]. These data, when considered with studies demonstrating that *C. jejuni* strains may significantly differ in their ability to produce various toxins [23, 24], may indicate that not all strains occurring in animals may be pathogenic for humans. The ability to identify rapidly certain clones of known pathogenicity may thus be more relevant to public health protection than simply detecting the presence of *C. jejuni*. The possibility of competitive exclusion of human pathogenic strains by non-pathogenic isolates may also be of interest.

The principal use of typing methods is to investigate the relationships of strains believed to be involved in an outbreak [5, 10]. Several investigators have published excellent guidelines concerning the interpretation of typing data where used to investigate putative outbreaks [5, 10]. However, these guidelines require revision where relationships between large populations of strains over extended periods of time are examined, and the use of several endonucleases and/or analyses have been suggested for such purposes [10]. The latter view is supported by our data, and that of Gibson and colleagues [8], and we consequently recommend that *C. jejuni* strains showing identical or similar *Sma*I MRPs be subjected to further analysis with additional enzymes such as *Bam*HI or *Kpn*I, since these demonstrate a high discriminatory potential. Furthermore, our data indicate that serotyping can be a sensible and cost-effective means of initially identifying randomly-isolated strains with a common origin. Some studies have suggested that antigenic variation may occur between genotypically related campylobacters [6, 7, 11, 12]. Our data indicate that Penner serotypes are stable between randomly isolated strains subsequently shown to be genetically identical by our criteria, although MRP–PFGE is also capable of additional discrimination within the serotype. Therefore, the combination of both serotyping and genotyping offers the most appropriate means of determining strain relationships between sporadic isolates of *C. jejuni*. An epidemiological investigation of *C. jejuni* Penner serotype 2 strains (the most prevalent serotype in Denmark) is presently underway in our laboratory.

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