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Comparative Typing of *Campylobacter jejuni* by Heat-Stable Serotyping and PCR-Based Restriction Fragment Length Polymorphism Analysis

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***Campylobacter jejuni* has become the most common bacterial cause of human gastroenteritis worldwide. Rapid, discriminatory typing methods are required to identify potential clusters of infections. The major disadvantage of the well-evaluated and widely used Penner heat-stable serotyping method is the high level of nontypeability. The correlation of the types determined by the Penner heat-stable serotyping method and PCR-based restriction fragment length polymorphism (RFLP) analysis of the lipooligosaccharide (LOS) biosynthesis genes of *C. jejuni* was studied with 149 *C. jejuni* strains. Of these strains, 79 were patient strains belonging to 25 Penner serotypes, 60 were nontypeable patient strains, and 10 were reference strains. A 9.6-kb DNA fragment of the LOS gene cluster was amplified and digested with the restriction enzymes HhaI and DdeI. Altogether, 39 different RFLP types (including 30 HhaI profiles and 32 DdeI profiles) were identified. Type Hh1Dd1 was the most common type, with 36% of the strains and strains of 12 serotypes being of this type. A high level of discrimination was obtained, and a correlation between the Penner serotypes and the PCR-RFLP types could be seen. Also, variation in the LOS biosynthesis genes within a single Penner serotype was found. Although the PCR-RFLP method may not be sufficient to compensate for Penner serotyping, it can give valuable information about nontypeable strains and further characterize strains of common serotypes.**

Campylobacter jejuni has become the most common bacterial cause of human gastroenteritis in many developed countries, including Finland (3). Human campylobacter infections in Northern Europe have shown a peak in the summer months of July, August, and September (12; http//www.ktl.fi/ttr). Also in Finland, most domestic campylobacter infections have occurred from July to August [4, 19; U.-M. Nakari, M. Korkeila, K. Laaksonen, P. Ruutu, and A. Siitonen, Int. J. Med. Microbiol. **293**(Suppl. 35)**:**136, 2003, abstr.]. The majority of infections have appeared to be sporadic, and in most cases the source of infection has remained unknown. Rapid, discriminatory typing methods are required to identify potential clusters of infections.

The most widely accepted and well-evaluated phenotypic method for the typing of *C. jejuni* strains is the Penner serotyping technique, based on passive hemagglutination. It differentiates the strains on the basis of soluble heat-stable (HS) antigens (15). The antigenic basis of the Penner serotyping system was thought to be the expression of somatic lipopolysaccharide (LPS) (7–10, 16–18). However, more recent studies have found that *C. jejuni* strains carry lipooligosaccharide (LOS) molecules instead of LPS molecules. Moreover, the high-molecular-weight O polysaccharide accounting for the Penner serotype specificity in some strains has been shown to be capsular in nature. This polysaccharide is not linked to LPS or LOS of *C. jejuni* (6, 22).

High levels of nontypeability (up to 63%) of human isolates by the Penner serotyping technique have been reported from some countries, but in general, the rate of nontypeability has been less than 20% (11). *C. jejuni* is antigenically complex, as demonstrated by the presence of at least 48 HS serotypes (11). The antisera included in commercially available serotyping kits do not recognize all of these serotypes. In addition, the antisera are expensive. We used a PCR-restriction fragment length polymorphism (RFLP) method based on the LOS biosynthesis genes (20) to study the correlation between Penner serotypes and PCR-RFLP types and to see if this method was applicable to typing of the strains which are not typeable by serotyping.

MATERIALS AND METHODS

Bacterial strains. A large collection of *C. jejuni* strains, consisting of recent isolates from Finnish patients submitted by clinical microbiological laboratories, was serotyped, and 139 of them were selected for PCR-RFLP analysis (Table 1). Of these 139 strains, 60 were not typeable with the antisera used (see below). In addition, 10 National Collection of Type Cultures (NCTC; Health Protection Agency, London, United Kingdom) reference strains belonging to known Penner serotypes were studied.

Penner serotyping. For Penner serotyping, the strains were subcultured twice on blood agar and grown for 48 h at 42°C in a microaerobic atmosphere. Serotyping was performed according to the Penner serotyping scheme (15) with a commercially available set of antisera (Denka-Seiken Co., Ltd., Tokyo, Japan). Briefly, HS antigens of *Campylobacter* were extracted with nitrite and absorbed onto fixed chick red blood cells. The sensitized red blood cells were tested for agglutination with homologous antisera. The Denka-Seiken serotyping kit contains 25 absorbed antisera against the following HS serotypes (grouped antisera are given in parentheses): (1,44), 2, 3, (4, 13, 16, 43, 50), 5, (6,7), 8, 10, 11, 12, 15, 18, 19, 21, (23,36,52), 27, 31, 32, 37, 38, 41, 45, 52, 55, and 57. Some *Campylobacter* strains may react with more than one antiserum. Nontypeable strains were called NT.

Amplification of LOS gene cluster. The genomic DNA was isolated with a Genomic DNA Purification kit (MBI Fermentas, Vilnius, Lithuania), and 200 ng of the DNA was used as the template in the PCR. A 9.6-kb DNA fragment of the LOS gene cluster was amplified with primers galE1 and wlaH3 (20) (Table 2). The large size of the amplified fragment placed special requirements on the PCR protocol used. Specific amplification products were obtained only after careful optimization (see Discussion). PCR was performed in a 50-μl reaction volume (with a PCR Sprint thermal cycler [Hybaid, Ashford, United Kingdom]) in thin-walled PCR tubes. The reaction mixtures consisted of 1× reaction buffer

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TABLE 1. HS serotypes and RFLP types of 139 patient strains and 10 reference strains

Identification no.	Serotype(s)	RFLP type
NCTC 12500	HS 1	Hh7Dd7
CH[a] 470, 476	HS 1,44	Hh7Dd7
CH 490	HS 1,44 + HS 4 complex	Hh3Dd3
CH 14, 92	HS 2	Hh2Dd2
CH 7, 34	HS 2	Hh7Dd7
CH 106	HS 3	Hh8Dd12
CH 600	HS 3	Hh17Dd17
CH 394	HS 4 complex	Hh3Dd3
CH 25, 46, 74, 81, 404, 617	HS 4 complex	Hh8Dd12
CH 615	HS 5 + HS 12	Hh16Dd12
NCTC 12505	HS 6	Hh1Dd1
CH 1, 2, 3, 10, 30, 55, 73	HS 6,7	Hh1Dd1
NCTC 12506	HS 7	Hh25Dd27
CH 22, 270, 291	HS 8	Hh7Dd7
CH 475	HS 8	Hh17Dd20
CH 138, 140	HS 10	Hh1Dd1
CH 19	HS 10	Hh3Dd3
CH 105	HS 11	Hh12Dd2
CH 449, 455	HS 11	Hh14Dd14
NCTC 12511; CH 6, 23, 32, 54	HS 12	Hh1Dd1
CH 50	HS 12	Hh21Dd23
CH 625	HS 15	Hh1Dd1
CH 8	HS 15	Hh10Dd13
CH 115	HS 15	Hh13Dd11
CH 558	HS 18	Hh10Dd16
NCTC 12517; CH 306, 674	HS 19	Hh5Dd5
CH 62	HS 19	Hh18Dd9
CH 36, 113, 432	HS 21	Hh1Dd1
CH 628	HS 23,36,53	Hh1Dd1
CH 20, 28	HS 23,36,53	Hh15Dd15
NCTC 12521; CH 44, 69, 327, 478	HS 27	Hh1Dd1
CH 279	HS 27 + HS 31	Hh1Dd1
CH 317	HS 31	Hh3Dd3
CH 322	HS 31	Hh17Dd19
CH 568	HS 32	Hh15Dd13
CH 517	HS 32	Hh19Dd21
CH 200, 259, 504	HS 37	Hh15Dd18
CH 35, 37, 49, 56, 66	HS 38	Hh8Dd8
NCTC 12542	HS 41	Hh6Dd6
CH 38, 61, 477	HS 41	Hh9Dd9
NCTC 12549	HS 44	Hh3Dd3
CH 227, 247, 292, 436	HS 55	Hh1Dd1
NCTC 12552; CH 633	HS 57	Hh1Dd1
CH 63, 109	HS 57	Hh16Dd12
NCTC 12553	HS 58	Hh24Dd26
CH 99, 137, 168, 177, 260, 387, 623, 634, 755, 947, 987, 1018, 1044, 1062, 1083, 1085, 1112, 1117, 1161, 1220, 1232	NT	Hh1Dd1
CH 241	NT	Hh2Dd2
CH 358	NT	Hh3Dd3
CH 1198, 1205, 1210	NT	Hh3Dd16
CH 16	NT	Hh4Dd4
CH 1149	NT	Hh7Dd7
CH 361	NT	Hh7Dd25
CH 51, 53, 75	NT	Hh8Dd8
CH 71, 187, 314, 354, 401, 1056, 1072, 1107, 1119, 1152	NT	Hh8Dd012
CH 77	NT	Hh9Dd9
CH 472	NT	Hh10Dd16
CH 965	NT	Hh10Dd29
CH 793	NT	Hh11Dd2
CH 151, 1012	NT	Hh16Dd12
CH 45	NT	Hh20Dd22
CH 64, 158	NT	Hh22Dd10
CH 360, 1047, 1093, 1113	NT	Hh23Dd24
CH 769	NT	Hh26Dd28
CH 1237	NT	Hh27Dd31
CH 1226	NT	Hh28Dd30
CH 149	NT	Hh29Dd1
CH 938	NT	Hh30Dd32

[a] CH, patient strain.

TABLE 2. Primers used for amplification of the LOS gene cluster of *C. jejuni*[a]

Primer	Orientation	Position[b]	Sequence
galE1	Forward	1185–1206	GCGGTGGTGCAGGTTATATAGG
wlaH3	Reverse	10783–10806	TCAGTTCTTGCCATTAAATTTCTC

[a] The primers were originally described by Shi et al. (20).
[b] Nucleotide positions are according to the sequence from the National Center for Biotechnology Information with GenBank accession number Y11648.

with 2.0 mM $MgCl_2$ (Finnzymes, Espoo, Finland), 1.0 U of DynaZyme Ext DNA polymerase (Finnzymes), 200 ng of forward and reverse primers, 0.2 mM concentrations of deoxynucleoside triphosphates, 0.8 M betaine (*N*,*N*,*N*-trimethylglycine; Sigma, St. Louis, Mo.), and the template DNA (200 ng). The reaction included an initial denaturation of the DNA at 94°C for 2 min, after which the enzyme was added and then 10 cycles of denaturation (25 s, 94°C), primer annealing (30 s, 57°C), and chain extension (7 min, 69°C) were carried out. In cycles 11 to 22 the chain extension time was increased by 20 s/cycle. A final elongation step was performed for 10 min at 69°C.

RFLP analysis. After amplification, 10 μl of the PCR product was digested with 10 U of each of the restriction enzymes HhaI and DdeI (Promega, Madison, Wis.) in separate reactions in a total volume of 20 μl with 2 μg of bovine serum albumin for 3 to 4 h at 37°C. The digest was analyzed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. A GeneRuler DNA ladder mix (MBI Fermentas) was used as a reference size marker. Electrophoretic patterns were entered into BioNumerics gel analysis software (Applied Maths, Kortrijk, Belgium). The types were called Hh and Dd and were numbered separately according to their order of appearance (types Hh1 to Hh30 and Dd1 to Dd32), and the two types were combined to give RFLP types (HhDd types).

RESULTS

Of the 139 patient strains selected for the study, 79 were typeable and belonged to 25 different HS serotypes or combinations of serotypes (Table 1). Each serotype contained one to seven strains. Sixty strains were NT by serotyping. The strains within the serotypes containing more than one strain divided into one to three RFLP types (Table 1). Eight serotypes, HS 1,44, HS 21, HS 27, HS 37, HS 38, HS 41, HS 55, and HS 6,7, with two to five strains each, included only one RFLP type. Serotypes HS 3, HS 31, and HS 32 each included two strains and two RFLP types. Serotypes HS 10, HS 11, HS 19, HS 23, 36, 53, and HS 57 included three strains and two RFLP types. Among the serotypes with more than three strains, strains of HS 2 were equally divided into RFLP types Hh2Dd2 and Hh7Dd7, whereas the predominant RFLP types (Hh8Dd12, Hh7Dd7, and Hh1Dd1) were found in serotypes HS 4 complex, HS 8, and HS 12. HS 15 was the most polymorphic serotype: each of the three HS 15 strains analyzed was of a different RFLP type.

All strains tested were typeable by PCR-RFLP analysis. Among the 89 serotypeable strains (10 NCTC reference strains and 79 patient strains) and the 60 NT strains, 26 and 22 RFLP types, respectively, were found (Table 3). Nine of these were found among both serotypeable and NT strains. Thereby, altogether 39 different RFLP types (including 30 Hh types and 32 Dd types) were identified. Type Hh1Dd1was the most common RFLP type. It accounted for 36% of the strains and contained strains from 12 HS serotypes (including the HS 6 reference strain and NT strains). Four RFLP types (Hh3Dd3, Hh7Dd7, Hh8Dd12, and Hh16Dd12) contained strains belonging to two to five HS serotypes, and 21 RFLP types contained only one serotype, without counting NT strains. Only NT strains were found in 13 RFLP types.

DISCUSSION

We studied the LOS biosynthesis genes of 89 serotypeable and 60 NT *C. jejuni* strains by PCR-RFLP analysis. The strains were recent isolates from Finnish patients. Ten NCTC reference strains were also included. The aim of the study was to see if there was a correlation between Penner HS serotypes and RFLP types, as previously described by Shi et al. (20). We were especially interested in finding out if this method would be applicable to the serotyping of NT strains by genotypic means. We typed a large number of NT strains by PCR-PFLP analysis and achieved 100% typeability.

The large size of the amplified fragment placed special requirements on the PCR conditions and reagents used. We used DyNazyme EXT DNA polymerase, which is a mixture of *Taq* polymerase and a proofreading polymerase specifically designed for long PCR. At first, the amplification conditions described by Shi et al. (20) were used. However, no specific amplification products were obtained. Therefore, the amplification conditions were optimized. As high temperatures damage the PCR product during the long amplification process, it was important to keep the denaturation time as short as possible (25 s). Also, the elongation temperature was lowered from 72 to 69°C. Irrespective of these optimization steps, much smearing occurred at first. The addition of 0.8 M betaine to the PCR mixture significantly decreased the smearing, and specific amplification products were obtained. Betaine has been reported to decrease the smear produced by DyNazyme EXT DNA polymerase when long extension times are used (1). Moreover, a hot start was found to be necessary.

The discrimination indices (5) of the Penner serotyping method were 0.96 for the typeable patient strains and 0 for the NT strains. The discrimination index for the PCR-RFLP typing system was 0.85 for both serotypeable and NT strains. Hence, the PCR-RFLP typing system seems to be most applicable for the typing of NT strains, whereas serotypeable strains are slightly more effectively discriminated by Penner serotyping. However, the isolates from patients represented a selected subpopulation, and therefore, the discriminatory abilities of these methods might be different during routine use.

A correlation between Penner serotypes and RFLP types could be seen. Some typing results were very clear; for example, all HS 37 strains were Hh15Dd18, and this RFLP type was not found among the other serotypes. All HS 38 strains and some NT strains were Hh8Dd8. However, not all serotypes could be distinguished by PCR-RFLP analysis. For example, the most common RFLP type, Hh1Dd1, contained strains belonging to several serotypes.

Antisera against HS serogroups 1,44 and 6,7 are included in the Denka-Seiken serotyping kit, but individual serotypes within these serogroups cannot be determined. The PCR-

TABLE 3. RFLP types found among 149 *C. jejuni* strains and their HS serotypes

RFLP type (% of strains)	HS serotype	No. of strains tested (n = 149)
Hh1Dd1 (36; n = 53)	HS 6,7	7
	HS 12	5
	HS 27	5
	HS 55	4
	HS 21	3
	HS 10	2
	HS 57	2
	HS 6	1
	HS 15	1
	HS 23,36,53	1
	HS 27 + HS 31	1
	HS NT	21
Hh2Dd2 (2.0; n = 3)	HS 2	2
	HS NT	1
Hh3Dd3 (4.0; n = 6)	HS 4 complex	1
	HS 10	1
	HS 31	1
	HS 44	1
	HS 1,44 + HS 4 complex	1
	HS NT	1
Hh3Dd16 (2.0; n = 3)	HS NT	3
Hh4Dd4 (0.7; n = 1)	HS NT	1
Hh5Dd5 (2.0; n = 3)	HS 19	3
Hh6Dd6 (0.7; n = 1)	HS 41	1
Hh7Dd7 (6.0; n = 9)	HS 8	3
	HS 1,44	2
	HS 2	2
	HS 1	1
	HS NT	1
Hh7Dd25 (0.7; n = 1)	HS NT	1
Hh8Dd8 (5.4; n = 8)	HS 38	5
	HS NT	3
Hh8Dd12 (11; n = 17)	HS 4 complex	6
	HS 3	1
	HS NT	10
Hh9Dd9 (2.7; n = 4)	HS 41	3
	HS NT	1
Hh10Dd13 (0.7; n = 1)	HS 15	1
Hh10Dd16 (1.3; n = 2)	HS 18	1
	HS NT	1
Hh10Dd29 (0.7; n = 1)	HS NT	1
Hh11Dd2 (0.7; n = 1)	HS NT	1
Hh12Dd2 (0.7; n = 1)	HS 11	1
Hh13Dd11 (0.7; n = 1)	HS 15	1
Hh14Dd14 (1.3; n = 2)	HS 11	2
Hh15Dd13 (0.7; n = 1)	HS 32	1
Hh15Dd15 (1.3; n = 2)	HS 23,36,53	2
Hh15Dd18 (2.0; n = 3)	HS 37	3
Hh16Dd12 (3.4; n = 5)	HS 57	2
	HS 5 + HS 12	1
	HS NT	2
Hh17Dd17 (0.7; n = 1)	HS 3	1
Hh17Dd19 (0.7; n = 1)	HS 31	1
Hh17Dd20 (0.7; n = 1)	HS 8	1
Hh18Dd9 (0.7; n = 1)	HS 19	1
Hh19Dd21 (0.7; n = 1)	HS 32	1
Hh20Dd22 (0.7; n = 1)	HS NT	1
Hh21Dd23 (0.7; n = 1)	HS 12	1
Hh22Dd10 (1.3; n = 2)	HS NT	2
Hh23Dd24 (2.7; n = 4)	HS NT	4
Hh24Dd26 (0.7; n = 1)	HS 58	1
Hh25Dd27 (0.7; n = 1)	HS 7	1
Hh26Dd28 (0.7; n = 1)	HS NT	1
Hh27Dd31 (0.7; n = 1)	HS NT	1
Hh28Dd30 (0.7; n = 1)	HS NT	1
Hh29Dd1 (0.7; n = 1)	HS NT	1
Hh30Dd32 (0.7; n = 1)	HS NT	1

RFLP profiles of the HS 1 and HS 44 reference strains were distinguishable from each other, and also, serotypes 6 and 7 were differentiated by PCR-RFLP analysis. The PCR-RFLP profiles of clinical isolates belonging to serogroup 1,44 were of type Hh7Dd7, as was the HS 1 reference strain, and HS 6,7 clinical isolates were of RFLP type Hh1Dd1, as was the HS 6 reference strain.

Recent genetic analysis of the genes involved in LOS biosynthesis has shown that there can be variation within a single Penner serotype (2). Our study also demonstrated that there is variation in the LOS biosynthesis genes within a single Penner serotype. For example, HS 12, one of the most common serotypes among Finnish *Campylobacter* isolates [21; Nakari et al., Int. J. Med. Microbiol. **293**(Suppl. 35)**:**136, 2003, abstr.], was divided into RFLP types Hh1Dd1 and Hh21Dd23.

It has been demonstrated that loci for both LOS and the capsule are present in the *Campylobacter* genome (14), and the *C. jejuni* HS antigens that form the basis of the Penner sero-

typing system are considered to include both LOS and the capsule (6, 10, 13, 18). It has been suggested that some clonally related isolates share the same capsule and LOS antigens, while other strains appear to have a common capsule antigen but differ in their LOS antigens, or vice versa (13). The results of our study were concordant with this suggestion. Namely, some strains were distinguished by Penner serotyping but not by PCR-RFLP analysis of the LOS gene cluster, whereas some serotypes included more than one RFLP type.

In a previous study (20), a correlation between Penner serotypes and PCR-RFLP types and variations in the RFLP types of strains within a single Penner serotype were also observed. In addition to confirming these findings, our study demonstrated that the PCR-RFLP method is applicable to the typing and classification of NT strains. Comparison of the LG genotypes reported by Shi et al. (20) to our HhDd profiles was found to be difficult, despite the use of the same primers and restriction enzymes. Three RFLP types with identical HhaI and DdeI banding patterns were found. Our most common RFLP type, Hh1Dd1, was identical to genotype LG 5. This RFLP type was associated with serotypes HS 6, HS 12, HS 21, HS 27, HS 55, and HS 57 in both studies. Type Hh2Dd2 was identical to genotype LG 2 and was associated with serotype HS 2, and Hh5Dd5 was identical to genotype LG 13 and was associated with HS 19. Many more identical patterns likely exist. Extensive and reliable comparison of the RFLP banding patterns between laboratories would require interlaboratory standardization of technical procedures and products, particularly electrophoretic conditions and gel analysis software.

Although it has been demonstrated that the HS serotype would be determined by the capsular biosynthesis-related genes (6) instead of the LOS biosynthesis genes, the PCR-RFLP patterns reflect the HS serotypes at least to a partial degree. The PCR-RFLP method may not be sufficient to compensate for the Penner serotyping method, but it can give valuable information about NT strains and further characterize strains of common serotypes.

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