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Restriction fragment length polymorphisms among the flagellar genes of the Lior heat-labile serogroup reference strains and field strains of *Campylobacter jejuni* and *C. coli*

A. P. BURNENS[1], J. WAGNER[1], H. LIOR[2], J. NICOLET[1] AND J. FREY[1]

[1] *National Reference Laboratory for Foodborne Diseases, University of Berne, Länggass-Str. 122, CH-3012 Berne, Switzerland*

[2] *Laboratory Centre for Disease Control, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada*

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SUMMARY

Several typing systems have been described for *Campylobacter jejuni* and *C. coli*, to assess the complex epidemiology of these important enteric pathogens. In the present study two typing methods, slide agglutination according to the Lior scheme, and the demonstration of restriction-fragment length polymorphisms (RFLP) of flagellar genes, have been used in parallel on a set of 194 strains. This set comprised 118 sero-reference strains of *C. jejuni* and *C. coli* of the Lior scheme, as well as 76 clinical isolates. All isolates were serotyped and subjected to PCR for amplification of flagellar genes, and the PCR product was restricted with *Alu* I. Flagellar genes could be amplified in 152 strains. Among 85 seroreference strains, 74 different RFLP patterns were observed, and among 67 clinical isolates, there were 36 patterns. There was only limited correlation between flagellar RFLP and the Lior serogroup, and the variability of patterns in serogroups HL2 and HL4 were as marked as the variability between serogroups. Flagellar gene RFLP patterns are shown to be stable, highly discriminatory epidemiologic markers.

INTRODUCTION

Campylobacter jejuni and *C. coli* are considered to be among the most important enteric pathogens worldwide. In order to understand the epidemiology of these pathogens, many typing systems have been devised and have recently been compared for discriminatory power [1]. One of the schemes proposed for typing of *C. jejuni* and *C. coli* has been the heat-labile serotyping scheme of Lior [2]. It is based on slide agglutination of not-yet defined heat-labile antigenic surface determinants of campylobacters. The polar flagella of campylobacters are strongly immunogenic and heat-labile [3], but their contribution to the agglutination reaction in the Lior serotyping scheme has been controversial [4]. Another recently developed approach to typing of *C. jejuni* and *C. coli* has been the analysis of polymorphisms among their flagellar genes. The method was based on demonstration of restriction-fragment length polymorphisms (RFLP) of the flagellar genes amplified by the polymerase chain reaction [5–7]. In salmonella, where determinants of seroreactivity are well known, a clear correlation was

shown to exist between serological reaction of the phase 1 flagella (*fliC*) and restriction-fragment length polymorphisms of the respective *fliC* gene, amplified by PCR [8]. We therefore tried to look at the relationship between Lior heat-labile (HL) serogroups of *C. jejuni* and *C. coli* and their flagellar genes, trying to establish a possible correlation between restriction-fragment length polymorphisms of the flagellar genes and the Lior serogroup. Using this approach, we tried to estimate the contribution of flagella among the otherwise unknown heat-labile antigens involved in the agglutination reaction. The seroreference strains having been selected on the basis of antigenic diversity we further hoped that they would constitute a convenient basis for the establishment of an exhaustive catalogue of restriction-fragment length polymorphisms among flagellar genes of *C. jejuni* and *C. coli*.

METHODS

Bacterial strains

The complete set of serogroup reference strains for the Lior HL serotyping system strains up to serogroup HL122 was purchased from the Culture Collection, University of Göteborg (CCUG), Sweden. Four serogroups (HL3, HL37, HL43, HL58) have been deleted from the scheme. The ten *C. lari* strains (HL31, HL34, HL35, HL56, HL64, HL73, HL81, HL93, HL116, HL121) included in the scheme did not give flagellar gene amplification and were not further used in the study, and strain *C. coli* HL75 was not available from the CCUG, leaving 107 strains for analysis. In addition, to assess reproducibility of the results, the reference strains for the ten most frequent HL serogroups worldwide were also directly obtained from the collection of the Laboratory Centre for Disease Control (LCDC), Ottawa, Canada. The reference strain for HL75 was also obtained from this collection (LCDC 8682), as were 19 clinical isolates of serogroups HL2, HL4, and HL6. Finally, 57 fresh clinical isolates of *C. jejuni* and *C. coli* from Switzerland: 11 from humans, 17 from household pets, 3 from cattle, 3 from sheep, 5 from pigs, and 18 from chickens were also analysed. *Campylobacter jejuni* strain 81116 (NCTC 11828), strain IN1 (directly obtained from I. Nachamkin [9]), and strain TGH9011 (CCUG 10937), for which flagellar gene sequence data are known, served as controls. The flagellar genes of one strain of *C. coli* have also been sequenced [10]; the strain is part of the seroreference strains of the Lior scheme (strain VC167, LCDC 52, reference for the HL8 serogroup).

Serotyping

All 194 strains listed above were either reference strains for the Lior serotyping system or have been serotyped at the LCDC by standard protocols which include slide agglutination of living cells in the appropriate pooled and monovalent rabbit sera [2].

PCR primer selection

The nucleotide sequences of the flagellar genes of three strains of *C. jejuni* [9, 11, 12] and one strain of *C. coli* [10] have been recently published, thus permitting the design of suitable PCR primers. The primers were chosen to amplify the entire coding sequence of the *flaA* gene of *C. jejuni* strain 81116 and were thus

complementary to nucleotides 83–100 (upper strand) and 1791–1813 (lower strand) respectively of the sequence (EMBL/GenBank J05635). The sequences of the oligonucleotides were as follows: 5′-ccg gat ccc ATG GGA TTT CGT ATT AAC-3′ (upper primer) and 5′-t tcg aat tCT ATT GTA ATA ATC TTA AAA CAT-3′ (lower primer) with the sequence shown in uppercase letters providing the specific priming for the PCR. The nucleotide extension shown in lowercase was provided to allow cutting of the PCR-product with the restriction enzymes *Bam* HI and *Eco* RI, in order to permit cloning of the product, if necessary. The primer sequences were expected to anneal to the 3′ and 5′ end of the *flaA* genes of *C. jejuni* strain 81116 [11], and *C. coli* VC167 [10] with a perfect match, and *C. jejuni* strain IN1 with 1 mismatched base for the lower primer [9]. In contrast, the upper and lower primers would give 4 and 5 mismatches with the *flaB* gene of *C. jejuni* 81116, as well as 4 and 7 mismatches with the *flaB* gene of *C. coli* respectively. For *C. jejuni* TGH9011, the upper primer perfectly matches the published sequence of the flagellar gene, whereas no match can be found for the lower primer. It has been hypothesized that the flagellar gene sequenced in *C. jejuni* TGH9011 may represent the *flaB* homologue of that strain [12], and this would coincide with the sequence divergence observed, and the lack of nucleotide match with the lower primer.

PCR reaction and restriction fragment analysis

Total genomic DNA of all strains used for the study were extracted by a modified guanidium thiocyanate micromethod [13]. Bacterial growth from blood agar plates was suspended in 0·9% NaCl and the bacterial pellet was processed as described. In order to completely abolish residual nuclease activity sometimes present in the extracted DNA, the chloroform extraction step of the original procedure was replaced by an extraction with phenol-chloroform [14]. About 1% of the yield of such a DNA preparation was used as a template for PCR. The PCR reaction mixture contained in a volume of 100 μl: 230 μM of dATP/dTTP, 120 μM of dCTP/dGTP, 1 μM of primers, and 2·5 U of Taq polymerase (Boehringer, Rotkreuz, Switzerland) in 10 mM-Tris-HCl pH 8·3, 1·5 mM-$MgCl_2$, 50 mM-KCl, and 0·005% each of Tween 20 (Calbiochem 655206, Lucerne, Switzerland), and NP-40 (Calbiochem 492017). The reaction was overlaid with mineral oil and subjected to 35 cycles of amplification in a Perkin-Elmer Cetus thermocycler with denaturation for 1 min at a temperature of 94 °C, annealing for 1 min at 46 °C, and an elongation for 1 min at 74 °C. The PCR product was precipitated by adding 0·1 volume of 3 M sodium acetate and 1 volume of isopropanol at −20 °C and redissolved in 10 mM-Tris-HCl pH 7·5 prior to digestion with the restriction enzyme *Alu* I under the conditions specified by the manufacturer (Boehringer, Rotkreuz, Switzerland). The restriction digest was loaded onto a 8% acrylamide gel and the fragments were separated by electrophoresis in Tris-borate buffer and visualized under UV-transillumination after staining by ethidium bromide [14].

RESULTS

With the conditions described above, 53 of the 69 seroreference strains of *C. jejuni* (77%), and 32 of the 39 strains of *C. coli* (82%) gave a PCR product. The following strains did not give an amplification reaction: *C. jejuni* HL7, HL13,

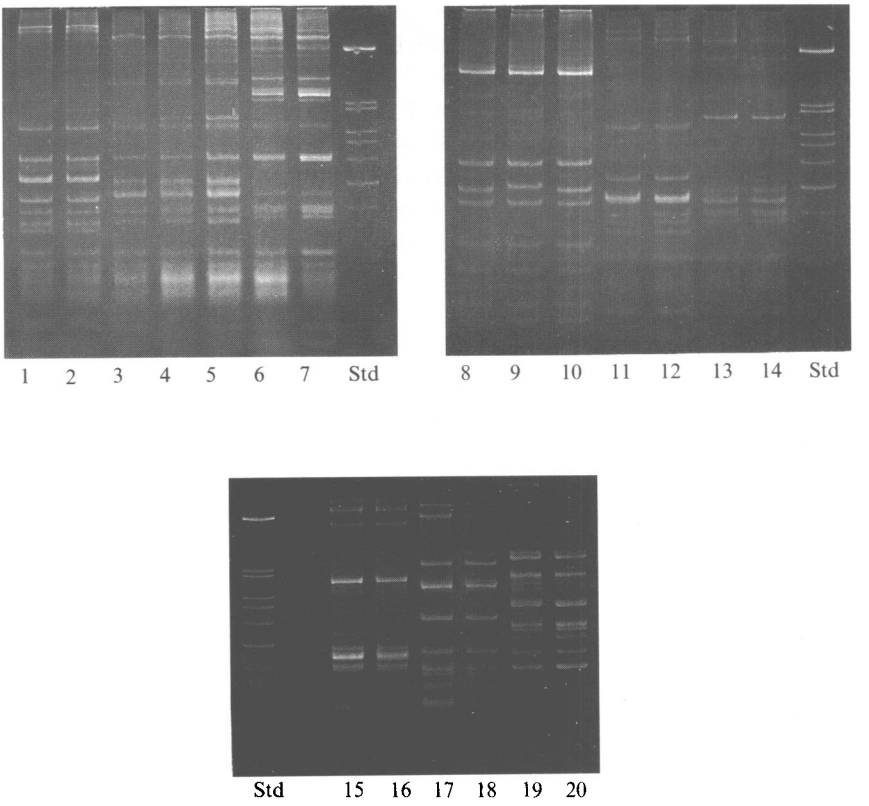

Fig. 1. Flagellar gene *Alu* I restriction fragment patterns of HL seroreference strains showing common profiles. Lane 1 contains the reference strain for serogroup HL32, CCUG 15032, human *C. jejuni*, lane 2: HL11, CCUG 12082, human *C. jejuni*, lane 3: HL10, CCUG 12081, human *C. jejuni*, lane 4: HL79, CCUG 20579, human *C. jejuni*, lane 5: HL1, CCUG 12074, human *C. jejuni*, lane 6: HL28, CCUG 15028, human *C. jejuni*, lane 7: HL53, CCUG 15053, human *C. jejuni*, lane 8: HL6, CCUG 12066, human *C. jejuni*, lane 9: HL50, CCUG 15050, *C. jejuni* source unknown, lane 10: HL88, CCUG 20588, *C. coli* source unknown, lane 11: HL91, CCUG 20591, canine *C. coli*, lane 12: HL99, CCUG 25899, *C. jejuni* source unknown, lane 13: HL49, CCUG 15049, human *C. jejuni*, lane 14: HL112, CCUG 25912, *C. jejuni* source unknown, lane 15: HL47, CCUG 15047, *C. coli* source unknown, lane 16: HL44, CCUG 15044, *C. coli* source unknown, lane 17: HL71, CCUG 19526, human *C. jejuni*, lane 18: HL66, CCUG 19521, human *C. jejuni*, lane 19: HL19, CCUG 15116, chicken *C. jejuni*, lane 20: HL18, CCUG 12078, chicken *C. jejuni*, Std.: molecular weight standard, pBR322 plasmid DNA cut with *Hin* fI.

HL15, HL23, HL27, HL33, HL38, HL39, HL40, HL60, HL67, HL74, HL77, HL84, HL92, and HL96, and *C. coli* HL12, HL25, HL45, HL70, HL80, HL95, HL122. The PCR products were of the expected size of 1731 bp for strains giving an amplification reaction, with the exception of *C. coli* HL109, which yielded a product of *ca.* 1900 bp.

The PCR reaction with *C. jejuni* 81116 as well as *C. coli* VC167 amplified the *flaA* gene only, as shown by restriction analysis of the products with the enzymes *Alu* I, *Dde* I, *Hae* III, and *Hin* fI which always yielded fragments of size expected

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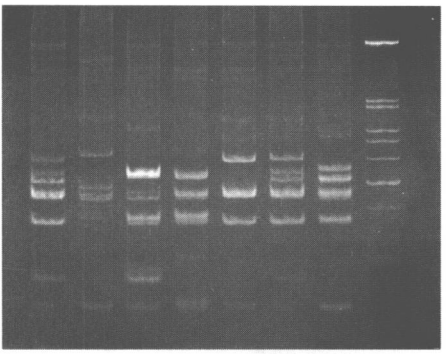

Fig. 2. Flagellar gene *Alu* I restriction fragment patterns of different *C. jejuni* isolates sharing the same HL2 serogroup. Lane 1 contains *C. jejuni* strain LCDC 13050 (human), lane 2: 4281-91 (human), lane 3: 4565-91 (human), lane 4: LCDC 12599 (human), lane 5: LCDC 12372 (human), lane 6: LCDC 12314 (human), lane 7: reference isolate for the HL2 serogroup. Std.: molecular weight standard, pBR322 plasmid DNA cut with *Hin* fI.

on the basis of the *flaA* gene sequence, and no additional bands which could have resulted from co-amplification of *flaB*. Similarly with strain *C. jejuni* IN1 and the same enzymes, only the product corresponding to the flagellar gene sequence was obtained, based on the expected size of restriction fragments according to the published nucleotide sequence. No amplification product could be detected with *C. jejuni* TGH9011 as expected, due to the mismatch of the lower primer.

Among the 85 seroreference strains giving a flagellin gene PCR product, 74 different restriction fragment patterns (RFLP patterns) were observed. The ten strains of serogroups HL, obtained directly from the LCDC collection gave the same patterns as the strains obtained through the CCUG collection, indicating stability of the RFLP pattern over time. There were identical patterns observed in widely divergent serogroups of campylobacter, as shown in Figure 1. There were 20 seroreference strains which could be placed in 9 groups based on identical RFLP patterns. These were strains of the serogroups HL1, HL10 and HL79, serogroups HL6, HL50 and HL88, serogroups HL11 and HL32, serogroups HL18 and HL19, serogroups HL28 and HL53, serogroups HL44 and HL47, serogroups HL49 and HL112, serogroups HL66 and HL71, and serogroups HL91 and HL99. The remaining 65 strains gave unique patterns. Whereas some strains belonging to divergent serogroups gave identical RFLP patterns, as illustrated above, clinical isolates belonging to the same serogroup gave divergent patterns. This was observed for serogroups HL2 and HL4 as illustrated in Figure 2 for the HL2 serogroup. In contrast, the reference strains of HL6 as well as 10 clinical isolates of this serogroup showed a conserved pattern, including *C. jejuni* strain 81116, from which the flagellar genes were cloned and sequenced and which also belongs to the same serogroup (Fig. 1).

Among the 57 fresh clinical isolates from Switzerland, there were 49 strains of *C. jejuni*, of which 40 (82%) gave a flagellin-PCR product, whereas 6 of the 8 (75%) *C. coli* gave an amplification reaction. There were 29 different RFLP patterns among the 46 strains giving a PCR product. Of these 29 patterns, 12

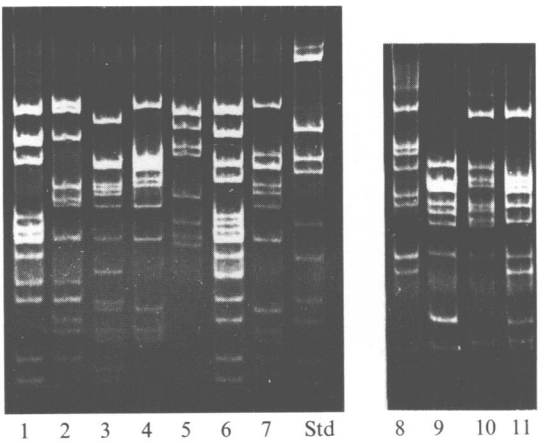

Fig. 3. Flagellar gene *Alu* I restriction fragment patterns of clinical isolates from Switzerland, showing unique patterns not represented among seroreference strains. Lane 1 contains *C. jejuni* isolate D 272-90 (feline), serogroup HL76, lane 2: E 1807-91 (canine), HL16, lane 3: E 1657-90 (feline), HL67, lane 4: K 5-1 (calf), HL4, lane 5: H 111 (chicken), HL1, lane 6: 4281-91 (human), HL2, lane 7: 4420-91 (human), HL1 lane 8: P 1-1 (porcine, *C. coli*), HL20, lane 9: P 11-1 (porcine, *C. coli*), HL20, lane 10: P 32-1 (porcine), HL55, lane 11: H 12-1 (chicken), HL23. Std.: as a standard, the flagellin gene of *C. jejuni* 81116 cut with *Alu* I was included. The molecular weight of the fragments in bp (as given by the sequence) is: 760, 280, 183, 158, 83, 68, 51, 45, 36, 32, 23.

could be assigned to a pattern observed among the seroreference strains and 17 were unique. There were 18 chicken strains from 5 flocks. The 3 chicken strains from 1 flock uniformly failed to give a PCR amplification product. The 15 chicken strains from the remaining 4 flocks gave a distinct profile for each flock, with all strains within an individual flock (1 flock with 6 isolates, and 3 flocks with 3 isolates each) giving the same RFLP profile. Of the 57 strains, 17 were untypeable in the Lior system, and 2 belonged to new serogroups, HL131 and HL133, not represented in the strain collection above. In all, 34 strains were typeable both by the Lior system as well as by PCR–RFLP. Of these, 17 gave RFLP patterns matching those of their respective seroreference strains. In the 17 remaining isolates, 11 new and unique RFLP patterns were observed (Fig. 3). Two of the new patterns were observed among broiler isolates; in each flock there was only 1 type of RFLP pattern, i.e. 1 of the patterns was observed in 3 out of 3 broiler isolates in 1 flock, and the other was found in 5 out of 5 chicken isolates from the remaining flock.

DISCUSSION

Typing of campylobacter isolates is important to understand more clearly the complex epidemiology of these prevalent enteric pathogens [15]. Two serological typing schemes have been developed and widely used, one based on heat-stable [16] and the other based on heat-labile antigens [2]. The determination of antigens involved in the heat-labile serotyping reactions would be of major interest, permitting the precise description of the molecular variability underlying the observed agglutination reactions. To take salmonellae as an example, the

construction of the Kauffmann–White scheme and the understanding of cross-reactions among antigens would not have been possible without knowledge of the antigens involved in the agglutination reactions. One of the aims of the study had therefore been to find a possible relation between serological reactivity, i.e. the heat-labile Lior serogroup of campylobacters and their flagellar genes. The antigens involved in the slide agglutination reaction used for typing are still unknown, and the contribution of flagella is controversial [4]. The data presented here are addressing the question for the first time for a large number of strains representing almost the complete Lior HL-system as well as fresh clinical isolates. In salmonellae, where the contribution of flagella to the slide agglutination reaction used for serotyping have been studied extensively for many decades [17], the restriction-fragment length polymorphisms of the *fliC* flagellar gene amplified by PCR was shown to match the serological specificity very closely [8]. We therefore believe that restriction-fragment length polymorphisms of PCR-amplified flagellin genes are a good indicator of sequence heterogeneity [18]. On the other hand, sequence variation among flagellar genes as shown by RFLP, will also most likely result in serological differences among flagellins. The issue however is complicated by the fact that many strains of *C. jejuni* and *C. coli* have been shown to possess two different flagellar genes [7]. A possible functional importance for the presence of two flagellar genes in *C. jejuni* and *C. coli* has been proposed. Whether both flagellar genes are usually transcribed has not been established except for two strains, and the contribution of the *flaA* and *flaB* products to the construction of the flagellar filament seems to differ between strains. Whereas in *C. coli* VC167, the flagellum seems to be a heteropolymer of the *flaA* and *flaB* products, the flagellar filament in *C. jejuni* 81116 is exclusively built from *flaA* flagellin monomer units. However, the *flaA* product seems always to be the predominating flagellin species in the flagellum and should, therefore largely determine the serological reactivity of the filament. Whether the PCR product obtained with a particular strain in this study corresponds to the *flaA* or *flaB* gene or both cannot be decided, except for strains where both sequences are available. The PCR described above clearly amplifies only the *flaA* gene in *C. jejuni* 81116 and *C. coli* VC167, and also only one of the flagellar genes in *C. jejuni* IN1. Because the sum of the molecular weight of the fragments obtained by *Alu* I digestion of the PCR amplification products never significantly exceeded 1·7 kbp, we believe that the PCR described above always amplifies only one flagellin gene. Without further evidence it can clearly not be decided whether the flagellin gene amplified is the one expressed. However, different *fla*-gene RFLP were found in isolates of the same HL serogroup. Conversely, identical *fla*-gene restriction patterns were present in widely different HL groups. Therefore, the involvement of flagellar epitopes in the agglutinating reaction for more than a few HL serogroups of the Lior scheme seems unlikely. For this reason, the antigens involved in the agglutination reaction used for typing of *C. jejuni* and *C. coli* in the Lior scheme still remain to be determined.

Taken on its own, a typing of *C. jejuni* and *C. coli* based on RFLP of flagella has been shown to give epidemiologically useful distinctions by several investigators [5–7]. The most significant recent addition has been the paper by Owen and colleagues in the pages of this Journal. They showed for the first time that flagellin

gene polymorphisms of human and animal isolates of *C. jejuni* partially overlap, thereby providing molecular evidence linking human disease with campylobacter carriage in animals, especially poultry [19]. These findings have been corroborated and extended to *C. coli* by the present study. In particular, the RFLP patterns were shown to be stable when serostrains obtained through the CCUG collection and the LCDC collection gave identical results. In the CCUG collection, the strains have been maintained lyophilized since 1982, whereas the strains obtained from the LCDC for comparison have been serially passaged during this time, i.e. over 10 years. The epidemiological usefulness of the RFLP data is also shown by the fact that chicken reared in the same flocks were carrying campylobacters with identical RFLP types, as expected by the horizontal spread of campylobacter infection in flocks [20]. Furthermore, the RFLP types of flagellar genes were also very discriminatory, with 91 different patterns observed among the 194 strains analysed. Thus, flagellar gene RFLP provide a marker which is both stable and highly discriminatory. Finally, the typeability was 79%, i.e. this proportion of isolates of *C. jejuni* and *C. coli* gave a PCR product with the primers and PCR conditions described above.

Other primers could eventually have given an increased number of strains yielding a PCR product, increasing typeability of strains. In the present study, for the reasons outlined above, preference was however given to a primer pair which would amplify only one of the flagellar genes and thereby simplify interpretation of the results. For use as a general typing system, the level of discrimination provided by *Alu* I restriction was too high to be practical, with 63% of strains giving unique patterns. Our method has, however been very useful for strain discrimination even within some HL serogroups. We have found *Dde* I to give simple, easily recognizable RFLP patterns with flagellar genes of *C. jejuni* and *C. coli*. The patterns could be visualized on agarose gels, as had been shown by others [6]. For wider use of the flagellar gene RFLP typing, simplified protocols for DNA preparation could be used. The flagellar gene RFLP-typing itself is inherently simple and useful and other primers have meanwhile been designed based on a sequence comparison between flagellar genes of *C jejuni* and *Helicobacter pylori* which give amplification also with strains non-typeable based on the above method. Thus, typeability by flagellar gene RFLP will approach 100%. We have found the distribution of HL serogroups to be obviously very local, with only 36% of the Swiss strains belonging to the 10 most prevalent HL-types worldwide. A similar observation has already been reported for the USA [21]. Therefore the HL seroreference strains used for the study have given an overall impression of the variability of flagellar genes in *C. jejuni* and *C. coli*, as well as of the usefulness of the method. Based on the observations above, typing of campylobacter with *fla*-gene RFLP necessitates setting up a catalogue of restriction patterns taking account of locally prevalent strains.

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