Lipooligosaccharide locus class of *Campylobacter jejuni*: sialylation is not needed for invasive infection

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Abstract

Campylobacter jejuni is a highly diverse enteropathogen that is commonly detected worldwide. It can sometimes cause bacteraemia, but the bacterial characteristics facilitating bloodstream infection are not known. A total of 73 *C. jejuni* isolates, consecutively collected from blood-borne infections during a 10-year period all over Finland and for which detailed clinical information of the patients were available, were included. We screened the isolates by PCR for the lipooligosaccharide (LOS) locus class and for the presence of the putative virulence genes *ceuE*, *ciaB*, *fucP*, and *virB11*. The isolates were also tested for γ-glutamyl transpeptidase production. The results were analysed with respect to the clinical characteristics of the patients, and the multilocus sequence types (MLSTs) and serum resistance of the isolates. LOS locus classes A, B, and C, which carry genes for sialylation of LOS, were detected in only 23% of the isolates. These isolates were not more resistant to human serum than those with the genes of non-sialylated LOS locus classes, but were significantly more prevalent among patients with underlying diseases (p 0.02). The fucose permease gene *fucP* was quite uncommon, but was associated with the isolates with the potential to sialylate LOS ($p < 0.0001$). LOS locus classes and some of the putative virulence factors were associated with MLST clonal complexes. Although some of the bacterial characteristics studied here have been suggested to be important for the invasiveness of *C. jejuni*, they did not explain why the clinical isolates in the present study were able to cause bacteraemia.

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Introduction

Campylobacter jejuni is one of the most common bacterial enteropathogens. It causes diarrhoea with fever and abdominal pain, and in many cases the infection resolves spontaneously [1,2]. However, complications such as bacteraemia and post-infection sequelae such as Guillain–Barré syndrome (GBS) and reactive arthritis may occur [3]. There is an increasing body of evidence indicating that the structure of *C. jejuni* lipooligosaccharide (LOS) might have a role in the outcome of infection. Sialylated LOS locus classes have been found more frequently than non-sialylated LOS locus classes in GBS-associated *C. jejuni* strains [4]. Furthermore, it has been reported that *C. jejuni* strains with sialylated LOS are less susceptible to normal human serum (NHS) and have a higher invasion potential in cell cultures, and that inactivation of LOS sialyltransferase results in the loss of such invasiveness [5–8].

The role of different putative virulence factors in bacterial adhesion, colonization, invasion, and ultimately, the outcome of *Campylobacter* infection has yet to be explained. *Campylobacter* invasion antigen (CiaB) and the plasmid pVir are suggested markers for the virulence potential of *C. jejuni* [9–11]. In our study on *C. jejuni* isolates from enteritis patients [12], the putative virulence genes *ceuE*, encoding an enterochelin uptake

protein [13], and *cj0486*, recently demonstrated to be a putative L-fucose permease gene, *fucP* [14,15], were more prevalent in isolates of foreign origin, whereas γ-glutamyl transpeptidase (GGT) was associated with infections acquired in Finland.

In a recent nationwide study conducted over a 10-year period, we collected blood culture isolates of *C. jejuni* and *Campylobacter coli*, clinical features of the corresponding bacteraemic episodes and characteristics of the patients from all over Finland [16]. We found that the patients were moderately young, and mostly without any significant underlying diseases [16]. We recently characterized these particular *C. jejuni* blood culture isolates with respect to their clonal distribution, by using multilocus sequence typing (MLST), and to their serum resistance. We found that nearly half of the isolates belonged to the otherwise uncommon sequence type (ST) 677 clonal complex (CC) (ST-677 CC) [17]. Isolates belonging to this particular CC were more resistant to NHS than the other isolates, but otherwise serum resistance was not a prerequisite for bacteraemic infection [17]. In the present study, we further examined bacterial characteristics that might be important for the ability of *C. jejuni* to enter the bloodstream, and used PCR to screen for LOS locus class and for the presence of the putative virulence genes *ceuE*, *ciaB*, *fucP*, and *virB11*. Isolates were also tested for GGT production. These results were analysed with respect to the clinical characteristics of the patients, and the MLST types and serum resistance of the isolates.

Materials and Methods

The bacterial isolates and the patient data were collected as described previously [16]. Briefly, all patients with known episodes of *C. jejuni* or *C. coli* bacteraemia from the time period 1998–2007 in Finland, and for whom both the bacterial isolates and clinical information were available, were included. The underlying diseases were scored and grouped according to the Charlson weighted index of comorbidity [18]. Of the 76 patients described earlier, three were excluded because of *C. coli* infection.

All except one of the 73 *C. jejuni* bacteraemia isolates had been earlier genotyped by MLST [17]. Among the isolates, the ST-677 CC was the most prevalent (35 isolates, 48%) followed by the ST-45 CC (12 isolates, 16%) and ST-21 CC (10 isolates, 14%). The ST-48 CC and ST-464 CC were both represented by two isolates, and six CCs (ST-52, ST-354, ST-443, ST-460, ST-508, and ST-1332) had one isolate each. Five isolates were in unassigned STs [17].

Serum sensitivity assay had been performed for 73 isolates according to methods described previously [5,17,19]. Susceptibility to NHS varied between the *C. jejuni* isolates belonging to different CCs, such that isolates of the ST-677 CC were significantly less susceptible, whereas the isolates of the ST-45 CC were significantly more susceptible, than all other isolates [17].

LOS biosynthesis locus classification was performed for all 73 *C. jejuni* isolates by PCR screening, according to Parker *et al.* [20,21]. The primers and sequences used are described in Table 2 of reference [21]. LOS locus classes A and B were identified with primers for orf7ab (*cst*II), orf6ab1 (*cgt*B-1), orf6ab2 (*cgt*B-2), and orf5bII (*cgt*AII). LOS locus class C was identified with primers for orf6c (*cgt*B) and orf7c (*cst*III). LOS locus classes E/O and H/P were identified with primers for orf26e and orf27e. Primers for orf18df were used to attribute isolates not covered by the above primers. The orf18df-positive isolates belong to the non-sialylated LOS locus classes D, F, I, J, K, N, S, or Q [20]. All isolates were positive for orf12 (*waaV*).

The presence of the putative virulence genes *ceuE*, *ciaB*, *fucP* and *virB11* was screened for by PCR. The primers used are listed in Table 2 of reference [12]. The reaction mixture contained 1 × AmpliTaq Gold 360 buffer, 1.25 U of AmpliTaq Gold 360 polymerase (Applied Biosystems, Foster City, CA, USA), 200 μM dNTP (Fermentas, St. Leon-Rot, Germany), 0.2 μM each primer (Eurogentec, Ougrée, Belgium) and 5 μL of template DNA, in a total volume of 25 μL. Cycling conditions were as follows: 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, annealing (*ceuE*, 60°C; *virB11*, 53°C; *ciaB* and *cj0486*, 58°C) for 30 s, 72°C for 60 s, and final extension at 72°C for 7 min. For *virB11*, a touch-down protocol was used, with five cycles at 53°C, five cycles at 52°C, and 15 cycles at 51°C. Qualitative detection of GGT activity was performed as described previously [12].

Statistical analyses were performed with Graphpad Prism version 4.03 (Graphpad Software, San Diego, CA, USA) and PASW Statistics 18 (SPSS, Chicago, IL, USA). The χ^2-test and Fisher's exact test were used for comparison of categorical variables. The Mann–Whitney test was used for comparison of continuous variables. All tests were two-sided, and a p-value of <0.05 was considered to be statistically significant.

The study was approved by the Finnish Ministry of Social Affairs and Health.

Results

In this cohort of 73 patients with *C. jejuni* bacteraemia, only 17 (23%) of the isolates belonged to LOS locus classes with the potential to sialylate their LOSs; four to class A1, five to class B2, and eight to class C. Of the remaining isolates 38

belonged to class E/O and 15 to class H/P. Three isolates were positive for orf18. There was no association between the isolates harbouring genes for sialylation of LOS and symptoms of the patients preceding the bacteraemia, but the patients with underlying diseases (Charlson index of ≥1) had significantly more often isolates of LOS locus class A, B, or C, carrying genes for sialylation of LOS (p 0.02; Table 1). Among the *C. jejuni* isolates with the ability to sialylate LOS, those of locus class C showed further specific features; patients infected with isolates of LOS locus class C were hospitalized for a longer period than those infected with other isolates (median duration, 19 days vs. 4 days; p 0.005). This finding seemed to be LOS locus class-specific, as no significant difference could be detected in the hospitalization time between the patients infected with isolates of LOS locus class A, B or C (analysed as a group) and those infected with isolates unable to sialylate LOS (Table 1). In addition, the isolate of the patient who had developed GBS was of LOS locus class C.

Resistance to NHS was compared between isolates according to LOS locus class. There were no significant differences in susceptibility to NHS when the isolates harbouring genes needed for sialylation of LOS (classes A, B, and C) were compared with the isolates unable to sialylate LOS (median bacterial survival in serum, 15% vs. 27%; p 0.28) (Fig. 1). Although isolates of LOS locus class C were associated with longer duration of hospitalization, as mentioned above, these isolates were not more resistant to NHS than other isolates (p 0.315).

We found associations between LOS locus classes and MLST clonal complexes. All isolates of the ST-677 CC and of the ST-45 CC were of LOS locus class E/O or H/P, lacking genes for sialylation of LOS, whereas all isolates of the ST-21 CC belonged to classes enabling sialylation of LOS, including all eight isolates of locus class C (Table 1).

The presence of genes encoding putative virulence factors in the *C. jejuni* isolates were studied by PCR. The *fucP* gene, which is involved in the utilization of L-fucose, was present in 17 (23%) isolates; *ceuE*, encoding a protein involved in iron uptake, was present in 71 (97%) isolates; *ciaB* was present in 73 (100%) isolates; and *virB11* of the pVir plasmid was present in only two (3%) isolates. Production of GGT was detected in 13 (18%) isolates. These isolates predominantly belonged to the ST-45 CC (83% GGT-positive vs. 5% of all other strains; p <0.0001), and were significantly more sensitive to NHS than GGT-negative isolates (median bacterial survival in serum, 1.3% vs. 27%; p 0.0002). The *fucP* gene was significantly more prevalent among isolates with the potential to sialylate LOS than among those lacking genes for sialylation of LOS (p <0.0001; Table 1). This gene was also strongly associated with ST-21 CC isolates (100% *fucP*-positive vs. 14% of all other isolates; p <0.0001). Furthermore, *fucP*-positive isolates were significantly more sensitive to NHS than *fucP*-negative isolates (median bacterial survival in serum, 11% vs. 28%; p 0.039). All 35 ST-677 CC isolates were *fucP*-negative, *virB11*-negative, *ciaB*-positive, GGT-negative, and, except for two isolates, also *ceuE*-positive. Of the other isolates, one ST-45 CC isolate and one ST-21 CC isolate were *virB11*-positive.

Discussion

A unique collection of 73 *C. jejuni* blood culture isolates from patients with bacteraemic infections collected during a 10-year period all over Finland was screened for LOS locus class and putative virulence factors. *C. jejuni* isolates with the potential to sialylate LOS, which have been previously suggested to be connected with invasiveness *in vitro* [7,8] and more severe symptoms in enteritis patients [22], constituted only 23% of the invasive bacteraemia isolates in the present study. These

TABLE 1. Bacterial multilocus sequence type (MLST) clonal complexes (CCs), putative virulence factors and patient characteristics, according to the lipooligosaccharide (LOS) locus classes of corresponding *Campylobacter jejuni* bacteraemia isolates; isolates harbouring genes needed for sialylation of LOS (LOS locus classes A, B, and C; *n* = 17) and those unable to sialylate LOS (all other LOS locus classes; *n* = 56) are shown separately

MLST CC, putative virulence factor, and patient characteristic	*C. jejuni* with LOS locus class A, B, or C, *n* = 17	*C. jejuni* with other LOS locus classes, *n* = 56	p
CC			
ST-677	0	35 (32 E/O, 3 H/P)	<0.0001
ST-45	0	12 (8 H/P, 4 E/O)	0.06
ST-21	10 (8 C, 2 A1)	0	<0.0001
Fucose permease gene, *fucP*	13 (2 A1, 3 B2, 8 C)	4 (2 H/P, 2 18df)	<0.0001
γ-Glutamyl transpeptidase (GGT)	1 (A1)	12 (5 E/O, 7 H/P)	0.28
Age (years), median	49	45	NS
Duration of hospitalization (days), median	5	4	0.18
Significant underlying disease (Charlson index score of ≥1)	9 (2 A1, 2 B2, 5 C)	13 (10 E/O, 3 H/P)	0.02

NS, not significant.

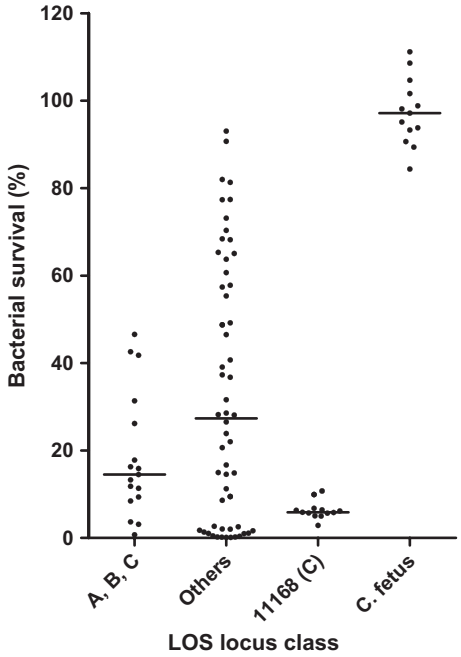

FIG. 1. Scatter plot of bacterial survival (%) in normal human serum of *Campylobacter jejuni* blood isolates. Lipooligosaccharide (LOS) locus classes A, B and C harbour genes needed for sialylation of LOS, and other classes lack such genes. *C. jejuni* 11168 and a blood isolate of *C. fetus* are shown as controls. Values represent means of two or three experiments; medians are indicated by horizontal lines.

isolates were more often detected in patients with significant underlying diseases, and were not more serum-resistant than other isolates, suggesting that sialylation of LOS is not needed for invasive *C. jejuni* infection.

C. jejuni shows a high level of variation in the LOS biosynthesis loci. Locus classes A, B and C possess the genes needed for sialylation of LOS [21,23]. In the current study, the number of *C. jejuni* isolates with genes for sialylation of LOS was very limited (17/73, 23%) as compared with cohorts of enteritis patients, where the prevalence of such isolates has been shown to be *c.* 60% [6,22,24]. This finding is especially interesting with regard to the observation that *C. jejuni* strains expressing sialylated LOS have been shown *in vitro* to be more invasive in intestinal epithelial cell cultures [7,8]. Indeed, blood culture isolates of *C. jejuni* would be expected to be invasive, but, according to the findings of the current study, the *in vivo* invasiveness of these particular isolates did not seem to be based on the potential to sialylate LOS. In further support of this, we found that isolates harbouring genes for sialylation of LOS were significantly more likely to be isolated from patients with significant underlying diseases, presumably predisposing these particular patients to severe infections. Another important finding was that none of the isolates of the most prevalent CC, ST-677 CC, covering almost half of the blood isolates, possessed genes needed for sialylation of LOS [17]. Instead both the ST-677 CC and the other major CC, ST-45 CC, were associated with isolates of LOS locus class E/O or H/P, in line with earlier reports [6,25,26]. Only 15 of the 73 bacteraemia patients had travelled abroad before the onset of the disease, which could explain, to some extent, the present results. However, in our recent study on faecal isolates of *C. jejuni* from consecutive enteritis patients in Finland, the prevalence of isolates capable of sialylating their LOS was 48% among the domestic isolates [24], which is still considerably higher than the prevalence among the blood isolates of domestic origin in the present study (21%).

In general, resistance to complement-mediated killing in serum is important for access of pathogens to the bloodstream. This is also true for *Campylobacter fetus*, the serum resistance of which is attributable to a proteinaceous S-layer that is absent in *C. jejuni* isolates [19,27]. Although data on the role of serum resistance in the invasiveness of *C. jejuni* are scarce [17,19,28], whether some structural components of *C. jejuni* could be associated with serum resistance has been investigated. Guerry *et al.* [5] demonstrated that a *C. jejuni* mutant with a non-sialylated LOS became more susceptible to NHS than the wild type, indicating that sialylated LOS may have a potential role in serum resistance. On the other hand, Keo *et al.* [29] suggested that capsule expression, rather than LOS, would be of importance for complement resistance. In our study on non-selected *C. jejuni* blood isolates, susceptibility to NHS was not attributable to the ability to sialylate LOS, as isolates lacking genes for LOS sialylation showed a wide range in serum susceptibility, and there was no significant difference between such isolates and those carrying genes for sialylation of LOS. It remains to be investigated whether expression of capsule polysaccharides could explain serum resistance in our material.

None of the four putative virulence factors studied seemed to explain the invasive character of the blood isolates. In particular, the fucose permease gene *fucP* was considerably less prevalent among the blood culture isolates in the present study (23%) than in our earlier study on faecal isolates (49%) [12]. This gene was previously suggested to be associated with hyperinvasiveness *in vitro* [10], and with colonization ability in a piglet infection model [15]. In the present study, we found that *fucP*-positive isolates were significantly more sensitive to human serum than *fucP*-negative isolates (p 0.039), suggesting that this gene did not contribute to the invasive phenotype of the blood isolates. Instead, we found that *fucP* was associated with the ST-21 CC, which further corroborates the conclusion by de Haan *et al.* [30] that the gene is connected to particular CCs rather than to specific hosts. In line with this, we also

found that production of GGT was associated with the ST-45 CC, as found in earlier studies [30,31].

In conclusion, the bacterial characteristics previously suggested to be important for the invasiveness of *C. jejuni*, in particular the ability to sialylate LOS, were quite uncommon in our study group consisting of non-selected bacteraemic *C. jejuni* patients, mainly without significant underlying diseases. This suggests that these factors had only a limited role in *C. jejuni* invasion of the intestinal epithelium of those patients. Our study emphasizes the crucial role of well-defined clinical materials in studies on bacterial virulence and understanding human disease.

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Transparency Declaration

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