Campylobacter jejuni capsular genotypes are related to Guillain–Barré syndrome

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Abstract

In about one in a thousand cases, a *Campylobacter jejuni* infection results in the severe polyneuropathy Guillain–Barré syndrome (GBS). It is established that sialylated lipo-oligosaccharides (LOS) of *C. jejuni* are a crucial virulence factor in GBS development. Frequent detection of *C. jejuni* with sialylated LOS in stools derived from patients with uncomplicated enteritis implies that additional bacterial factors should be involved. To assess whether the polysaccharide capsule is a marker for GBS, the capsular genotypes of two geographically distinct GBS-associated *C. jejuni* strain collections and an uncomplicated enteritis control collection were determined. Capsular genotyping of *C. jejuni* strains from the Netherlands revealed that three capsular genotypes, HS1/44c, HS2 and HS4c, were dominant in GBS-associated strains and capsular types HS1/44c and HS4c were significantly associated with GBS (p 0.05 and p 0.01, respectively) when compared with uncomplicated enteritis. In a GBS-associated strain collection from Bangladesh, capsular types HS23/36c, HS19 and HS41 were most prevalent and the capsular types HS19 and HS41 were associated with GBS (p 0.008 and p 0.02, respectively). Next, specific combinations of the LOS class and capsular genotypes were identified that were related to the occurrence of GBS. Multilocus sequence typing revealed restricted genetic diversity for strain populations with the capsular types HS2, HS19 and HS41. We conclude that capsular types HS1/44c, HS2, HS4c, HS19, HS23/36c and HS41 are markers for GBS. Besides a crucial role for sialylated LOS of *C. jejuni* in GBS pathogenesis, the identified capsules may contribute to GBS susceptibility.

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Introduction

Campylobacter jejuni is one of the most frequent causes of bacterial gastroenteritis in humans worldwide [1,2]. In general, *C. jejuni* diarrhoea is self-limiting, but a wide spectrum of serious complications may occur, including bacteraemia, reactive arthritis and peripheral neuropathies, e.g. Guillain–Barré syndrome (GBS) and Miller–Fisher syndrome (MFS) [3].

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GBS is a post-infectious, immune-mediated disease of the peripheral nerves, which is characterized by a rapidly progressive weakness of the limb muscles [4]. About one-quarter of patients develop additional respiratory failure and autonomic dysfunction and 2–10% of the patients die [5,6]. MFS is regarded as a variant of GBS in which the weakness is restricted to the oculomotor muscles [7].

Various studies have investigated which risk factors of *C. jejuni* predispose to the development of GBS [8,9]. Currently, sialylation of lipo-oligosaccharides (LOS) of *C. jejuni* is the best characterized virulence factor associated with GBS [10,11]. The structural similarity (molecular mimicry) between sialylated *C. jejuni* LOS and ganglioside structures present on human peripheral nerves drives a cross-reactive immune response resulting in immune-mediated nerve damage [10]. In *C. jejuni*, genes involved in LOS sialylation are located in the LOS biosynthesis locus. Variability in gene content in *C. jejuni* LOS loci has led to the assignment of several LOS locus classes (A to S) [12]. Genes involved in the synthesis of sialylated LOS and ganglioside mimics are present in the LOS classes A, B, C, M and R [12]. Of these, *C. jejuni* strains with LOS classes A, B and C are frequently isolated from stools of patients with GBS and the LOS classes A and B are associated with GBS and MFS, respectively [11].

The presence of sialic acid in the LOS, however, does not appear to be the only bacterial factor involved in the pathogenesis of GBS. In patients with uncomplicated enteritis, the prevalence of sialylated *C. jejuni* strains has been reported to be up to 63% [11]. We hypothesize that the expression of additional *C. jejuni* virulence factors in combination with the presence of sialylated LOS contributes to the induction of GBS. Factors that increase the virulence of *C. jejuni* include the bipolar flagella, surface and secreted proteins involved in the invasion of the intestinal epithelium [13], and cytolethal distending toxin [14]. Genes that encode these virulence factors, however, are generally conserved and present in strains derived from both clinical and environmental sources [15,16].

The polysaccharide capsule is a virulence factor that is associated with cell invasion [17], complement resistance [18] and immune modulation [19]. The capsule biosynthesis locus is a highly variable region in the genome of *C. jejuni*. Diversity in gene content and gene function, combined with the presence of phase variable genes in the capsule locus, allows the production of a broad repertoire of capsular structures [20]. This feature is the basis of the classical Penner serotyping scheme that divides *C. jejuni* into 47 serotypes [21]. Previous studies based on Penner serotyping showed that the capsular serotypes HS:19, HS:23/36c and HS:41 are associated with GBS in Japan, Bangladesh and South Africa, respectively [22–24]. In contrast, no particular association between the capsular serotype and neuropathogenicity was observed for GBS-associated strains from Europe or North America [8,25]. This discrepancy may be explained by the observation that GBS-associated *C. jejuni* strains in Japan, Bangladesh and South Africa had a genetically related background whereas the strains in Europe and the USA were more heterogeneous.

Recently, capsular genotyping was introduced as a fast, readily available and reliable method to assess the capsular type in *C. jejuni* [26]. Compared with capsule-based serotyping, capsular genotyping is not sensitive to variations in capsule gene expression or influenced by genes or gene products outside the capsule locus. With the availability of two large and geographically distinct GBS-associated strain collections, we determined whether variation in the *C. jejuni* capsular genotype is involved in the risk of developing GBS. The capsular genotype defined by multiplex PCR was determined in collections of *C. jejuni* strains isolated from Dutch patients with GBS or MFS, Dutch patients with enteritis (control patients) and Bangladeshi patients with GBS or MFS. The capsular type was related to the LOS class to determine whether particular LOS class/capsular type combinations were more prevalent among GBS/MFS-associated strains compared with enteritis-associated strains. In addition, multilocus sequence typing (MLST) was performed to assess the genetic relation between strains.

Materials and methods

Strain collection, culture conditions and DNA isolation

In this study, Dutch and Bangladeshi GBS-associated *C. jejuni* strain collections and a Dutch enteritis-associated control strain collection were used. The Dutch GBS collection comprised 32 GBS-associated and five MFS-associated strains, which were isolated from stools of primarily Dutch patients. Among these are three GBS-associated strains from patients from the Netherlands Antilles and one from Belgium. The GBS-associated strain collection from Bangladesh consisted of 30 strains. The Dutch enteritis-associated strains were isolated from patients with diarrhoea in the Netherlands and were from two different provenances. The strains in the first enteritis collection (n = 21) were isolated between 1990 and 1999 and have been previously described [11]. A second enteritis collection originates from a Dutch study performed by the Rijksinstituut voor Volksgezondheid en Milieu in 2002–2003, on behalf of a national *Campylobacter* and *Salmonella* surveillance study [27,28]. From this large collection, we selected 122 enteritis strains that matched our GBS/MFS-associated strains based on age (decade) and sex. Hence, we selected three or four age- and sex-matched enteritis control strains per GBS/MFS case. There were no significant differences in the

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distribution of the various capsular genotypes between the two enteritis collections. Therefore, the two enteritis strain collections were combined in this study and referred to as the uncomplicated enteritis-associated strain collection (n = 143). For the Dutch GBS-, MFS- and enteritis-associated strains, LOS class typing (A, B, C, D and E) was performed previously [11] (A. P. Heikema, D. Horst-Kreft, R. Louwen, R. Huizinga, M. Gilbert, J. Li, C. T. Parker and H. P. Endtz). The LOS class of 10 of the GBS-associated *C. jejuni* strains derived from Bangladesh was determined previously [24]. For the remaining 20 strains, the LOS class was newly assessed. *Campylobacter jejuni* strains were cultured from stocks kept at −80°C and maintained on Colombia blood agar plates (Becton Dickinson BV, Alphen aan den Rijn, the Netherlands) supplemented with 10 mg/L vancomycin in a micro-aerobic atmosphere at 37°C. DNA was isolated from overnight-grown *C. jejuni*, using a DNeasy blood and tissue kit (Qiagen, Venlo, the Netherlands) as described by the manufacturer's protocol.

LOS locus class PCR

Genotyping was performed for LOS classes A, B, C, D and E using standard PCR. Primers used are based on the DNA sequence of unique genes or regions of the LOS locus classes involved and were previously described [11].

Multiplex PCR-based capsule analysis

Capsular genotyping was performed as described previously [26]. In short, based on the sequence of 17 capsule loci, unique regions were identified and specific primer pairs that recognize each Penner serotype were designed. The primer pairs were divided in two mixes in such a way that the expected amplicons differed at least 20 bp from one another. Standard PCR was performed and the PCR products were separated on 2% agarose gels using a 50-bp DNA ladder (Fermentas, Landsmeer, the Netherlands) to assess the size of each amplicon.

Multilocus sequence typing

Multilocus sequence typing, which relies on the partial sequencing of seven housekeeping genes, was performed as described elsewhere [8]. A dendrogram was constructed using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). Pairwise alignment of the sequences of the separate MLST alleles was performed. The dendrogram was generated using the unweighted pair group method with arithmetic mean clustering.

Statistical analysis

Fisher exact and chi-square tests were performed using Prism software (GraphPad, La Jolla, CA, USA). Two-sided $p \leq 0.05$ was considered to be statistically significant.

Results

Validation of the capsular genotyping method

The capsular genotype determined by PCR was compared to the serotype for our *C. jejuni* strains with known Penner serotype (n = 40). The serotype did not match the genotype in six cases and in one case the serotype was not included in the multiplex capsule PCR (data not shown). Full genome sequence data were available for four of six strains with a mismatch between capsular serotype and genotype. Blast analysis of the capsule locus region revealed homology to strains with serotypes that matched the identified genotype and not the serotype in all four cases (data not shown). Overall, we found a specificity of 95% and a sensitivity of 100% for strains (n = 39)

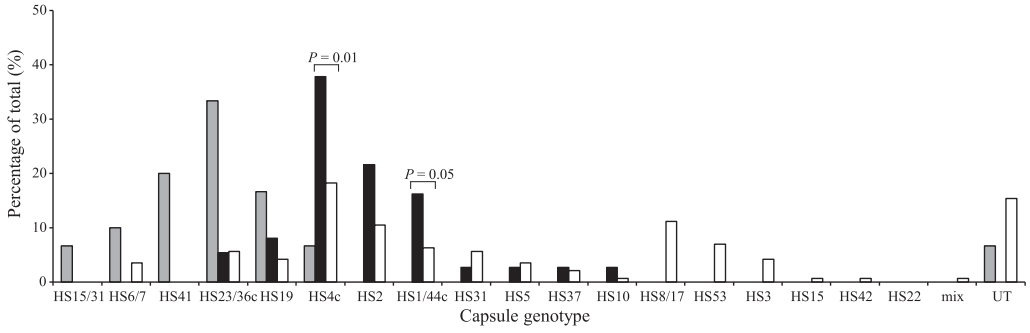

FIG. 1. Capsular genotypes in Guillain–Barré syndrome/Miller–Fisher syndrome (GBS/MFS) -associated and enteritis-associated strains. A multiplex PCR was used to determine the capsular type of 37 GBS/MFS-associated (black bars) and 143 enteritis-associated (white bars) *Campylobacter jejuni* strains derived from Dutch patients and 30 GBS-associated strains derived from patients with GBS from Bangladesh (grey bars). With this PCR, 17 main capsular types can be distinguished. Chi square and Fisher exact tests were used to compare the frequency of specific capsular genotypes between the Dutch GBS/MFS patients and the enteritis group. UT, untypeable; mix, more than one capsular genotype detected. $p \leq 0.05$ was considered as statistically significant.

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with serotypes covered in the genotyping method. Therefore, we concluded that the genotyping method is a reliable method to determine the capsular type in *C. jejuni*.

Six capsular genotypes are dominant in GBS/MFS-associated *C. jejuni* strains

In the Dutch GBS/MFS-associated strains, a capsular genotype could be assigned for all strains whereas in the Dutch enteritis-associated strains a capsular genotype could be assigned for 120 of 143 (83%) of the strains. Twenty-two strains in the enteritis group failed to produce amplicons and in one case a mix of two capsular types (HS1/HS41) was found. In the Dutch GBS/MFS collection, nine different capsular types were identified of which three capsular types; HS4c, HS2 and HS1/44c were dominant (Fig. 1). In the Dutch enteritis strain collection, a broader panel of 15 different capsular types was identified of which capsules HS4c, HS8/17 and HS2 were most prevalent (Fig. 1). When GBS/MFS-associated *C. jejuni* strains and enteritis-associated strains were compared, capsular type HS4c (p 0.01; chi-square test) and HS1/44c (p 0.05; chi-square test) significantly associated with GBS/MFS (Fig. 1). No association with a specific capsular type was observed for the MFS strains. In the five MFS-associated strains, four different capsular types were detected; HS2, HS4c (in two cases), HS10 and HS23/36c. When combined, 28/37 (76%) of the GBS/MFS-associated strains from the Netherlands had capsular type HS1/44c, HS2 or HS4c, compared with 50/143 (35%) in the enteritis control strains ($p < 0.0001$; chi-square test).

To determine whether the dominant capsular types identified in the GBS/MFS-associated strain collection from the Netherlands were also prominent in GBS-associated strains from another geographical area, capsular genotyping was performed on 30 GBS-associated strains isolated from patients from Bangladesh. In these strains, a capsular type could be assigned for 28/30 (93%) of the strains and capsular types HS23/36c, HS19 and HS41 were dominant (Fig. 1). In a randomly selected uncomplicated enteritis control group from Bangladesh, 0/39 (0%) of the *C. jejuni* strains had the capsular type HS19, 11/39 (28%) had HS23/36 and in 1/39 (3%) capsular type HS41 was identified (Z. Islam, personal communications). The capsular types HS19 and HS41 significantly associated with GBS in Bangladesh when compared with enteritis (p 0.008 and p 0.02, respectively; chi-square test). Capsular type HS41 was only found in strains from Bangladesh whereas capsular types HS23/36c and HS19 were also observed in the Dutch strain collections. When combined, 56/67 (84%) of the GBS/MFS-associated *C. jejuni* strains either had capsular type HS1/44c, HS2, HS4c, HS19, HS23/36c or HS41 (from here on referred to as GBS/MFS-related capsular types).

Sialylated LOS classes and specific capsular genotypes are correlated

Upon correlation of LOS class and capsular genotype in the GBS/MFS strain collection from the Netherlands, we observed that all but one strain with the LOS class A, B or C had the GBS/MFS-related capsular types (Fig. 2a). A particular

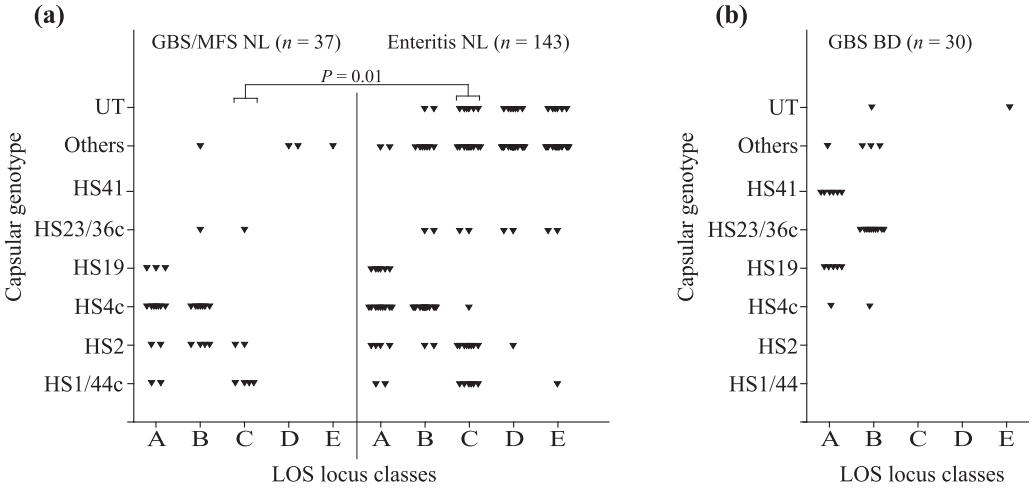

FIG. 2. Correlation of lipo-oligosaccharide (LOS) class and capsular genotype. For each strain, the LOS class was correlated to the respective capsular type. The six main capsular types observed in the Guillain–Barré syndrome/Miller–Fisher syndrome (GBS/MFS) strain collection are depicted. (a) *Campylobacter jejuni* strains from the Netherlands. The chi-square tests were used to assess whether there were significant differences in LOS class/capsular type combinations between the GBS/MFS and enteritis strains. Hereto, the six main capsular types were compered to others and UT. (b) *C. jejuni* strains from BD. Others, capsular types that did not belong to the group of six dominant capsular types observed in the GBS/MFS-associated strain collections but that could be determined with the multiplex capsule PCR; NL, The Netherlands; BD, Bangladesh; UT, untypeable; $p \leq 0.05$ was considered as statistically significant.

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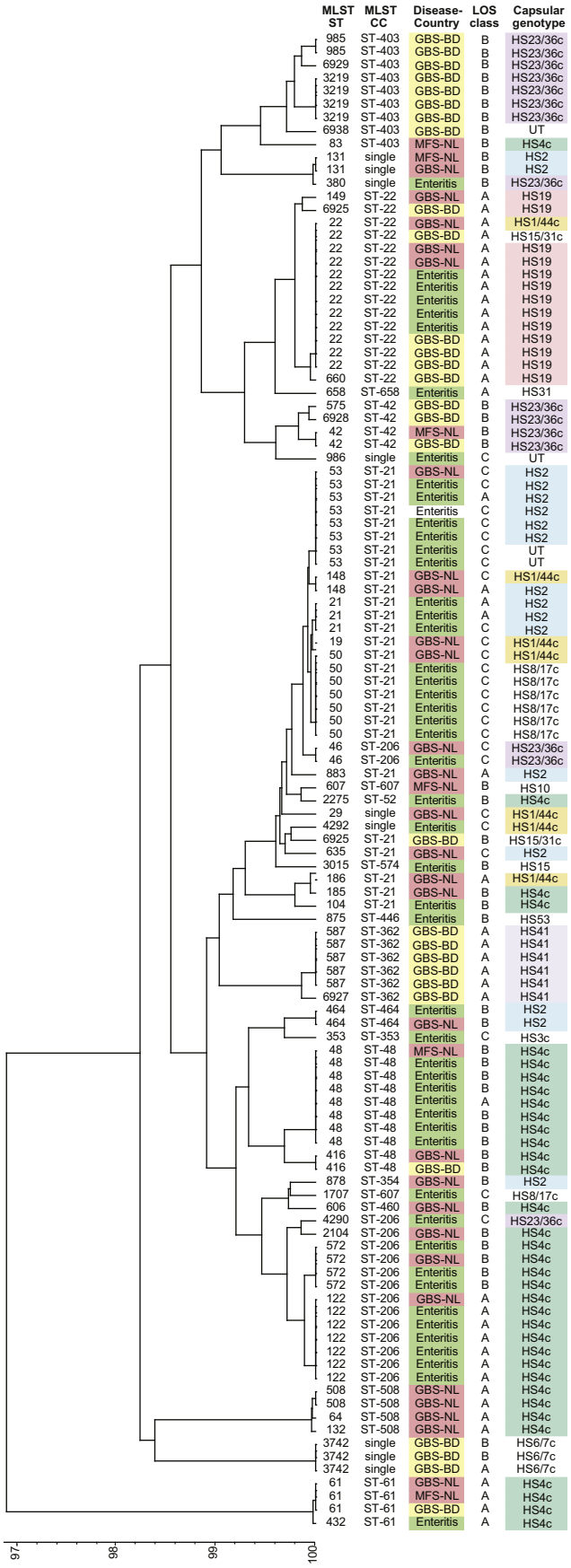

FIG. 3. Dendrogram of MLST genotype variation of *C. jejuni* strains with sialylated LOS loci classes. The dendrogram was generated from MLST sequence type data of 58 GBS-, 5 MFS- and 51 enteritis-associated *C. jejuni* strains with either an LOS class A, B or C. ST, sequence type; CC, clonal complex; GBS, Guillain–Barré syndrome; MFS, Miller Fisher syndrome; NL, Netherlands; BD, Bangladesh.

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distribution pattern was observed when LOS class and capsular type were combined. In the GBS/MFS collection, strains with LOS class A were typed HS1/44c, HS2, HS4c or HS19, in strains with LOS class B, capsular types HS2 and HS4c were the most prominent and LOS class C strains, mostly had capsular type HS1/41 or HS2 (Fig. 2a). In the enteritis control collection, approximately the same capsule distribution was observed for LOS class A strains. In contrast, for the class B and C strains, similar capsular types were only found for a subset of the enteritis strains when compared with the GBS/MFS strains. A distinct capsular type was found in 9/28 (32%) of the LOS class B strains and 18/34; 53% of the class C strains had a capsular type that did not belong to the GBS/MFS-related capsule group (p 0.01; GBS/MFS versus enteritis; chi-square). In enteritis strains with LOS class B, there was diversity in the remaining capsular types but in the LOS class C strains, capsular type HS8/17 was detected in 9/18 (50%) of the strains, which did not belong to the GBS/MFS-related capsule group.

In enteritis strains with LOS class D, capsular type HS53 was dominant (9/31; 29%; HS53 is not present in class D strains of the GBS/MFS collection) whereas capsular types within class E strains were diverse (data not shown). It should be noted that LOS class D and E loci do not possess the genes encoding proteins that are involved in LOS sialylation and are necessary to produce ganglioside mimics.

Lipo-oligosaccharide genotyping of 20 newly isolated GBS-associated *C. jejuni* strains from Bangladeshi patients resulted in the identification of ten strains with LOS class A and ten strains with LOS class B. When LOS class and capsular type of the GBS-associated strains derived from Bangladeshi patients (n = 30) were correlated, all strains with capsular type HS41 or HS19 correlated with LOS class A, whereas all the strains with the HS23/36c capsular type correlated with LOS class B (Fig. 2b).

A genetic linkage between LOS/capsule combinations

Multilocus sequence typing was performed on all (n = 67) GBS-associated and MFS-associated strains and on 84/143 (59%) of the enteritis-associated strains. The 84 enteritis control strains on which MLST was performed comprised 60% sialylated strains and 40% non-sialylated strains. These percentages are representative for the whole enteritis control collection of 143 strains. A total of 76 different sequence types were identified, which were classified into 21 clonal complexes. The *C. jejuni* clonal complex ST-21 was most prominent (29/151; 19%), followed by ST-206 (16/151; 11%) and ST-22 (15/151; 10%). In 13 cases, the MLST represented a singleton. In the GBS collection, the clonal complexes ST-21 and ST-22 were the most common (both 10/67; 15%) followed by ST-403 (9/67; 13%). A minimum spanning tree, in which our MLST data were combined with MLST data of *C. jejuni* isolates from sporadic cases of human enteritis present in the Oxford *C. jejuni* MLST database (n = 4066), showed that our GBS/MFS-associated and enteritis-associated strains were widely spread within the total *C. jejuni* MLST population (see Supplementary material, Fig. 1). GBS/MFS-associated as well as enteritis-associated control strains were present within the same clonal complexes.

To assess whether the LOS class/capsular type co-occurrence within one strain was due to a closely related genetic background, in particular for the strains that carry genes involved in LOS sialylation, a dendrogram was generated using strains from both the Netherlands and Bangladesh, which had LOS loci classes A, B or C (Fig. 3). The same genetic background, ST-21, was frequently observed for strains with capsular types HS1/44c and HS2 and LOS class C (18/29; 62%). Most HS8/17 strains with LOS class C (6/7; 86%) also belonged to ST-21. The HS4c strains with LOS class A or B were predominantly present in four genetic backgrounds: ST-206, ST-48, ST-61 and ST-508 (30/35; 86%). A strong individual genetic relationship was observed for LOS class A strains with capsular type HS41 (n = 6) and HS19 (n = 13). All HS19 strains belonged to ST-22 whereas all HS41 strains belonged to ST-362. The strains with capsular type HS23/36c were more widely spread within the dendrogram. Most (11/14; 78%), however, belonged to two MLST clonal complexes, ST403 and ST-42 (Fig. 3). One MLST clonal complex, ST-508, contained GBS-associated strains only but overall MLST typing did not discriminate strains carrying genes involved in LOS sialylation when GBS/MFS-associated and enteritis-associated strains were compared.

Discussion

We identified a group of six main capsular genotypes HS1/44c, HS2, HS4c, HS19, HS23/36c and HS41 in two large, geographically distinct GBS/MFS-associated *C. jejuni* strain collections. In a predominantly Dutch collection (n = 37), capsular genotypes HS1/44c, HS2 and HS4c were dominant. Of these, capsular type HS4c and HS1 associated with the neuropathogenicity. In a GBS-associated strain collection from Bangladesh (n = 30), three other capsular genotypes, HS19, HS23/36c and HS41 were abundant and capsular type HS19 and HS41 associated with GBS.

In the GBS/MFS group, a capsular type was assigned to all but two strains whereas 22 strains in the enteritis group failed to produce amplicons. The 24 strains in total that did not produce amplicons probably have a capsular type distinct from the 17 major capsular types that were tested. Multiple amplification fragments found in one case might be due to a mix of two strains, gene translocations between particular capsular types or multiple capsule operons in one genome. Earlier, we reported that no

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association was observed between GBS and a particular capsular serotype in a collection of strains from GBS patients in the Netherlands [8]. The discrepancy with the current results may be explained by the fact that serotyping is sensitive to capsule expression whereas genotyping is not. For example, phase variation and intragenic mutations that lead to premature stop codons result in more diversity in capsular serotypes compared with the capsular genotypes [26]. Furthermore, for some serotypes, the LOS plays a role in sero-diversity [29]. Reduction of the number of capsular genotypes compared with serotypes, enhanced sensitivity of the genotyping method compared with serotyping and, in particular, more power due to an expanded GBS/MFS-associated *C. jejuni* strain collection are factors that explain why an association between capsular genotypes and GBS was found in the current study and not in the study performed by Dingle *et al.* [8]. The association with GBS and the capsular types HS1/44c and HS4c reported in our study could be biased by a suboptimal enteritis control group, in which case it would under-represent HS1/44c and HS4c strains. Geographical differences in capsular type distribution has been reported [30] but with 9% of HS1/44c and 26% of HS4c strains, our Dutch enteritis collection certainly does not under-represent the capsule proportion in Europe (9.1% HS1/44c, 17.3% HS4c) and North America (9.3% HS1/44c, 23.4% HS4c) [30].

As great diversity between the capsular loci and multiple rearrangements between capsular genes has been reported for the capsular types HS1/44c, HS2, HS4c, HS19, HS23/26c and HS41 [20,26], it remains unclear whether particular genes or mutations in particular genes within GBS-associated capsular types may promote the development of GBS.

Our current data, derived from a three-fold expanded Bangladeshi strain collection, confirm that HS23/36c is a prevalent capsular type among GBS-associated strains from Bangladesh [24,31]. This capsular type, however, was also frequently observed in enteritis-associated strains from Bangladesh and was not associated with GBS when compared with enteritis.

When we combined the results of the LOS classification with the capsular genotyping, a striking correlation was observed. Capsular types HS19 and HS41 correlated solely with LOS class A, HS23/36c often with LOS class B, HS1/44c was predominantly found in strains with LOS class A and C and capsular type HS4c mostly associated with LOS class A and B strains. In agreement with our data, strains with the HS19 (ST-22), HS23/36c (ST-403) and HS41 (ST-362) capsular type represent genetically related populations [24,32,33]. A correlation between capsular type and LOS class in these strains is therefore to be expected. The frequent sharing of capsular types in GBS-associated strains that differ in MLST, observed in this study, argues more strongly for a role of the capsule in GBS induction. The bi-functionality of some of the enzymes involved in polysaccharide biosynthesis may additionally explain why particular LOS loci and capsule types are linked. In *C. jejuni*, a phosphatase encoded by the LOS locus gene *cj1152* is active in the biosynthesis of both, LOS and capsule [20]. Additionally, *gmhA* in the LOS locus and *gmhA2* in the capsule locus have duplicate functions [20]. More in-depth studies on gene functionality of LOS and capsule biosynthesis genes are needed to explain why certain LOS classes and capsules are genetically linked together. MLST analysis showed that the Dutch and Bangladeshi HS19 (ST-22) strains and the Bangladeshi HS41 (ST-362) strains originate from a similar genetic lineage to that of the HS19 strains frequently isolated in Japan [34] and HS41 strains that were associated with GBS in South Africa, respectively [35]. Moreover, the Japanese and South African GBS strains were all LOS class A. The preservation of stable lineages in HS19 and HS41 strains is probably linked with DNase activity or defects in the DNA transformation mechanism resulting in non-naturally transformable strains [36]. The described feature of *C. jejuni* to exchange genes and even entire loci due to horizontal gene transfer [20,37] explains the observed variations in LOS class and capsular type within identical MLST clonal complexes. Overall, MLST failed to segregate strains associated with GBS from strains associated with gastroenteritis.

Does capsule type discriminate between neuropathogenic and uncomplicated diarrhoeal strains of *C. jejuni*? In particular for the LOS classes B and C the answer is affirmative. LOS class B and C strains with capsular types other than HS1/44c, HS2, HS4c or HS23/36c were predominantly detected in the enteritis-associated control strain collection. Capsular type HS8/17 and HS31 for example were observed in enteritis-associated strains with LOS class A, B or C but not in GBS-associated strains with similar LOS classes. Such strains may therefore be considered to be less potent with regard to GBS induction. Certainly, more comparative capsule studies, including functional studies, are needed to determine if and how a particular capsular type might contribute to GBS development. In conclusion, we demonstrate that the combination of LOS loci that contain genes involved in LOS sialylation with the capsular types HS1/44c, HS2, HS4c, HS19, HS23/36c and HS41 are associated with the development of GBS and MFS and are therefore markers for these neurological diseases. The observation that similar capsular types are prevalent in GBS-associated strains with genetically unrelated lineages strongly argues for a role of the capsule in GBS inductions. A distinct capsular type other than the six capsular types identified in this study may in part explain why not all infections with *C. jejuni* that carry ganglioside mimics lead to GBS. The geographical differences in *C. jejuni* capsular type distribution in GBS patients reported here needs to be confirmed in other comparative studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.05.031.

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