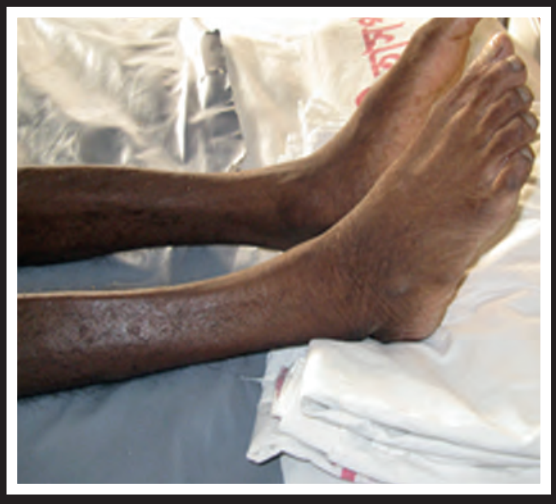
Campylobacter Infection and Guillain-Barré Syndrome in Bangladesh:

Clinical epidemiology and comparative microbial genomics



Zhahirul Islam

Campylobacter Infection and Guillain-Barré Syndrome in Bangladesh: Clinical epidemiology and comparative microbial genomics

Campylobacter infecties en het syndroom van Guillain-Barré in Bangladesh: Klinische epidemiologie en vergelijkend microbieel genoomonderzoek

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To my wife who always gives me endless support to go ahead and to my little boy, "Zarif", who provided the wonderful dimension to my life mission……

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Campylobacter

Campylobacter spp. are small motile, microaerophilic, S-shaped or spiral rods (0.2-0.5 µm wide by 0.5-5 µm long), gram-negative bacteria (Fig. 1). *Campylobacter* was first described in 1886 by Theodore Escherich (1) in the colon of children who had died of 'cholera infantum'. The name *Campylobacter* is derived from the Greek word 'καμπυλοσ' which means curved. In 1962, *Campylobacter*, then still known as 'related Vibrio' was described as a rare and opportunistic human pathogen that was isolated from blood culture of humans (2). In 1972, *Campylobacter jejuni* was first isolated from human diarrheal stools by applying a filtration technique (3). The subsequent development of selective *Campylobacter* stool culture techniques (4) led to the recognition that *C. jejuni* was a more common cause of human diarrheal illness.

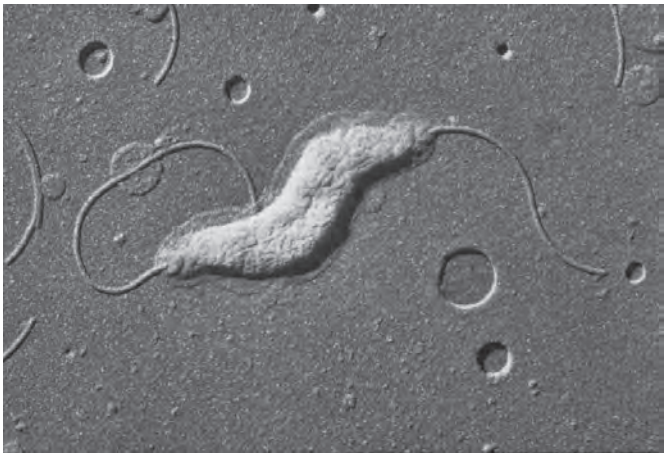

Figure 1: Scanning electron microscope micrograph of *C. jejuni*. Adopted from Jean-Paul Butzler.

Though *C. jejuni* is identified as the most frequent cause of bacterial gastroenteritis in developed countries (5-8), it is a common cause of diarrheal illness in the developing world, particularly in the first few years of life (9). In Bangladesh, *C. jejuni* has been reported as the second (17%) commonest enteropathogen in diarrhoeal patients of young children after rotavirus (20%) (10). Substantial variation in incidence rates has been observed among *Campylobacter* related-gastroenteritis in many developed countries (11). The incidence of symptomatic infections is highest among young children followed by young adults. In addition, there is a strong seasonality with a summer peak from June to September (12). The vast majorities of *Campylobacter* infections are not linked to outbreaks and occur as sporadic infections. Contact with farm animals, especially poultry, cattle and pigs are considered the main source of *Campylobacter* infection (13, 14). Consumption of untreated water, raw milk, or milk products are important additional sources of infection (12).

Campylobacter spp. are difficult to identify phenotypically because of relative biochemical inactivity, special growth requirements and complex taxonomy. *Campylobacter* can be isolated from fecal specimens using selective media that inhibit growth of normal gut flora. Most *Campylobacter* species require a microaerobic atmosphere and an incubation temperature of 37^0 to 42^0 for optimal growth. In most routine laboratories, the identification of *Campylobacter spp*. is based on growth on selective media, colony morphology, gram stain and a combination of biochemical test. Many studies report that the use of a combination of media, including either charcoal cefoperazone deoxycholate agar (CCDA) or charcoal-based selective medium, achieves a higher yield of *Campylobacter* from stool samples than the use of only a single medium (15, 16). As a single medium, CCDA medium was found to be the most sensitive for detecting *C. jejuni* and *C. coli* compared with Skirrow's medium, CAT agar, and filtration technique (17). After the acute stage of disease when the concentration of organisms decreases, enrichment cultures may be necessary to recover *Campylobacter* (18). Stool antigen tests for *Campylobacter* have been developed, and are commercially available. The ProSpecT *Campylobacter* immunoassay varies in sensitivity from 80% to 96% and has a specificity of >97% (19, 21). A large number of samples has been evaluated by Ridascreen *Campylobacter* (R-Biopharm. Darmstadt, Germany) immunoassay comparison with culture technique, and demonstrate that this technique has a sensitivity of 69% and a specificity of 87% (22). In addition, PCR-based detection, identification to the species level, and typing of *Campylobacter* directly from stool samples have been evaluated but are currently not frequently used in routine diagnostic procedures (23-25). During the first few weeks of infection, fecal and serum immunoglobulin (IgA) antibodies are apparent and then decrease precipitously, and expected serum IgG, IgM, and IgA level increase in response to infection (26, 27). The presence of serum IgM, IgA, and/or high titres of IgG antibodies against *C. jejuni* is strongly supportive for an acute infection (28-30). However, serologic testing is not recommended for routine diagnosis of *Campylobacter* infection but appears to be useful for epidemiologic investigations (31).

The clinical aspects of infection with *C. jejuni* are acute gastroenteritis, and extra-intestinal infections and post-infectious complications (Fig. 2). No specific treatment is required for most patients with *Campylobacter* enteritis, other than the oral replacement of fluid and electrolytes lost through diarrhea and vomiting (32). Due to increasing resistant against fluoroquinolones, a macrolide antibiotic such as clarithromycin is first choice when antibiotic treatment is indicated (32). Reactive arthritis, Reiter's syndrome, and Guillain-Barré syndrome (GBS) are post-infectious complications of *Campylobacter* infection. The association of *Campylobacter* infection with GBS, which emerged during the mid-1980s, greatly improved the understanding of the morbidity of the disease. In view of the importance of GBS, and the high incidence of acute flaccid paralysis (33), in this thesis we described *C. jejuni* infection and GBS in Bangladesh.

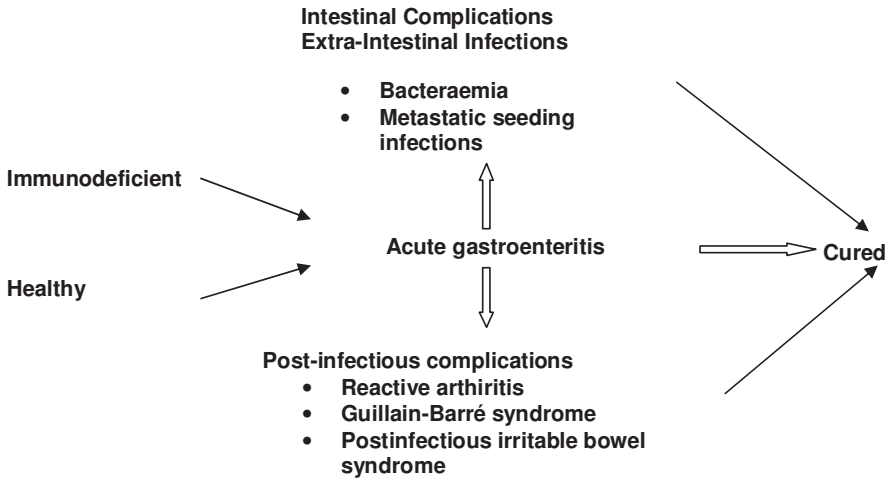

Figure 2. Diagram illustrating an overview of illnesses due to *C. jejuni*

Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) is an immune-meditated polyradiculoneuropathy, a disease of the peripheral nervous system with a broad clinical spectrum and specific pathophysiological and electrophysiological features (34, 35). At present, it is the world most frequent cause of acute paralysis after the extinction of poliomyelitis (36). Since 2000, no virally and clinically confirmed polio cases were found in Bangladesh; however, non-polio acute flaccid paralysis (AFP) is frequently diagnosed (33). Most of the non-polio AFP is thought to be GBS in Bangladesh (37). In the past two decades, much progress has been made in understanding the pathophysiology and epidemiology of GBS in the developed world. There is a scarcity of report on GBS from developing world including Bangladesh.

In the following part of this chapter, we will provide a comprehensive framework for reading the remaining chapters of this thesis. First, it will discuss the history and clinical aspects of GBS. Next, *C. jejuni*-related GBS and pathogenesis of this disease will be addressed in details. The role of GBS-related *C. jejuni* strains and molecular epidemiology of *C. jejuni* will be discussed separately. Prime research objective in this thesis is that *C. jejuni* might play a key role to the development of GBS in Bangladesh. At the end of this chapter the scope and major objectives of the studies described in this thesis will be presented in brief.

History

The first accurate description of GBS was made in 1859 by Jean Baptiste Octave Landry de Thezillat (38). By 1876, the phrase 'Landry's ascending paralysis' became the standard name for the disease. The syndrome was named after G. Guillain and J. A. Barré, two French army neurologists, who in 1916 described, together with A. Strohl, the typical findings in the cerebrospinal fluid (39). They reported the combination of increased protein concentration with a normal cell count in the cerebrospinal fluid (CSF), or albuminocytological dissociation, which differentiated the condition from poliomyelitis (39). In 1927, H. Draganesco and J. Claudian coined the term 'Guillain-Barré Syndrome' (GBS), and regretfully failed to acknowledge Strohl's contribution to the research. Until now, GBS is a descriptive disease entity defined by a set of clinical, laboratory, and electrodiagnostic criteria for which there are no specific diagnostic tests (36). To date, GBS is no longer considered as one single disease entity, but as a continuous spectrum of distinct or overlapping variantss, ranging from a pure motor form to a combination of motor and sensory nervous system disturbance with or without cranial nerve involvement.

Epidemiology

The annual incidence of GBS is reported to be 0.6 to 4.0 cases per 100,000 per year throughout the world (40, 41), but well-controlled population-based studies in Europe report an incidence of 1.2 to 2.3 per 100,000 per year (34, 41-46). Atypical presentations, such as the Miller Fisher syndrome (MFS), are much less frequent, with an incidence of 0.1 per 100,000 (44). In some areas of the world, the incidence of GBS may be higher, as has been suggested for endemic forms in rural areas in northern China (47). A study carried out by the Dutch GBS group, observed a temporary rise in incidence of GBS from 1.6 to 3.1 per 100,000 per year over the period from 1987 to 1999 on the Caribbean island of Curaçao (48). However, a recent review reports that the temporarily increased incidence in Curaçao had nearly returned to normal by 2006 (36). Considering the above-mentioned incidence rates, 40,000 to 120,000 new cases are being diagnosed per year worldwide (49). The incidence increases with age from 1 per 100,000 per year in persons aged below 30 years, to about 4 cases per 100,000 per year in persons older than 75 years (44). Men are 1.5 times more frequently affected than women (41, 42, 44). Multiple cases of GBS within one family have been described, although this appears to be an infrequent event and may represent a chance finding (50).

Clinical features of GBS

Diagnosis

The reason for developing diagnostic criteria for GBS was related to its increasing incidence during the swine flu vaccination period in 1976 and 1977. The criteria were first published in 1978 at the request of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) (51). The criteria were reaffirmed in 1990, and the electrodiagnostic criteria were expanded. These diagnostic criteria are widely accepted and seem appropriate to clinically define GBS (52).

Diagnostic criteria for typical GBS are shown in Table 1. The patient may notice numbness or tingling in the arms or legs with symmetrical loss of strength in hands or feet. The weakness spreads during the next few hours to weeks and may finally lead to a paralysis of limb, trunk, extraocular, facial, pharyngeal, and tongue musculature. Up to one-third of patients need to be ventilated due to the involvement of respiratory muscle (53, 54). Patients may suffer from severe pain and show reduced sensibility or autonomic dysfunction (53-55). Clinical examination of the patients shows poor or absent tendon reflexes. In general, CSF examination shows an increased protein level with normal white cell count. A common misunderstanding is that CSF protein concentration is always increased in GBS; many patients with GBS have a normal protein level in the first week of disease, but this proportion increase in more than 90% at the end of the second week (56). Electrophysiological investigation further supports the diagnosis of GBS with the features of demyelination and axonal degeneration.

Disease course and prognosis

The clinical severity, course and outcome of GBS is highly variable (36). The maximum level of weakness is reached within 4 weeks after onset of symptoms followed by spontaneous recovery (Fig.3). It seems to be a general believe that most patients suffering from a mild clinical course of disease may show a neurological good recovery. However, patients with a mild course of disease may suffer from residual morbidity (57). Many case series report that advanced age is indicative of a worse prognosis (34, 58, 59).

In children, recovery is more rapid and more likely to be complete and death is exceptional (41). Respiratory failure occurs in 25% of patients and may need mechanical ventilation for a prolonged period (60-63). Prognosis ranges from complete neurological recovery with or without fatigue (64), to wheelchair dependency or death. Approximately 20% of patients have a severe functional deficit, and remain unable to walk or bedridden, and they even may need continuous artificial ventilation (65, 66). The mortality usually ranges from 2 to 13% (53, 67-69) and is mostly the result of respiratory failure, cardiovascular and autonomic disturbances (34, 58, 59).

A prognostic model was developed that accurately predicts which patients will have a poor outcome (66). The worse prognostic factors include older age, severe and rapid onset of weakness, a preceding gastrointestinal illness or recent CMV infection, severely reduced compound muscle action potential amplitude, and a low Medical Research Council (MRC) sum core at randomization (53, 64, 66, 70-72).

Table 1. Diagnostic criteria for Guillain-Barré syndrome[a]

Features required for diagnosis

- Progressive motor weakness of more than one limb
- Low or absent reflexes
- No other identifiable cause

Features that strongly support the diagnosis

Clinical

- Progression of symptoms over days to 4 weeks
- Relative symmetry of symptoms
- Mild sensory symptoms or signs
- Cranial nerve involvement, especially bilateral weakness of facial muscles
- Onset of recovery 2-4 weeks after progression stops
- Autonomic dysfunction
- Pain (often present)

Cerebrospinal fluid (CSF)

- High concentration of CSF protein after the first week
- Less than 50 mononuclear leukocytes per µl CSF

Electrodiagnosis

- Conduction slowing or block

Features casting doubt on the diagnosis

- Bladder or bowel dysfunction at onset
- Sharp sensory level
- Marked persistent asymmetry of weakness
- Persistent bladder or bowel dysfunction
- More than 50 mononuclear leukocytes per µl CSF
- Presence of polymorphonuclear leukocytes in CSF

[a]Adapted from the criteria supported by the National Institute of Neurological and Communication Disorders and Stroke (52).

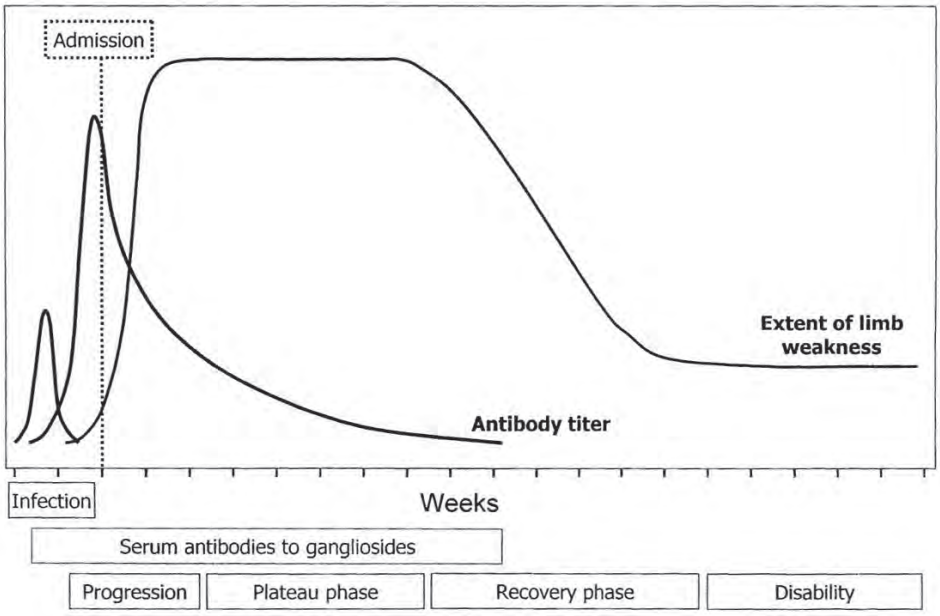

Figure 3: Sequence of events in a typical case of *C. jejuni*-related GBS. Adopted from Jacobs et al. (49).

General treatment

The most important reduction of mortality in GBS has been due to advances in supportive care of critically ill patients (41). Patients with GBS need to be admitted to a hospital for close observation (58, 59). General medical support is extremely important, especially to prevent secondary complications such as infections and decubitus (49, 73). Respiratory failure occurs in 25% of patients and is more likely in cases with rapid progression, bulbar palsy, upper limb involvement, and autonomic dysfunction. Regular monitoring is essential for vital capacity, and early transfer to an intensive therapy unit for prophylactic intubation. All severe patients should be monitored for possible cardiac arrhythmia. In non-ambulant adult patients, subcutaneous heparin and graduated compression stockings should be used to prevent deep vein thrombosis. Wide fluctuations of pulse rate and blood pressure occur in severe cases and sustained autonomic failure. Patients need to be checked regularly for urinary tract infections, gastrointestinal dysfunction, and hyponatremia. Many patients suffer from serious pain, varying from severe back pain, radicular pain or dys- and parethesias (74). Throughout the illness, psychological aberrations and communication problems must be addressed (60, 75).

Increased understanding of the immunologic basis of this disease over the past 15 years has allowed changing the natural course of this disease. Plasma exchange became accepted as the gold standard treatment for GBS almost 20 years ago (41). Since a randomized controlled trial showed that

intravenous immunoglobulins (IVIg) has a similar efficacy as plasma exchange (62), IVIg (0.4 g/kg body weight for 5 days) has replaced plasma exchange as the preferred treatment for severe GBS in most hospital because of its greater convenience (76). The mechanism of action of IVIg is probably multifactorial, possibly involving blockade of Fc receptors, provision of anti-idiotypic antibodies, interference with complement activation, and T-cell regulation (77). Corticosteroids do not have a beneficial effect but in combination with IVIg there may be a synergistic effect (78-80).

Although the past 13 years have seen the emergence of treatments that at least shorten the duration of GBS, 20% of patients are left disabled, and a considerable proportion of these patients has a persistent severe disability (41). Some patients with severe disease may benefit from repeating IVIg treatment, although such an additional therapeutic effects still needs to be demonstrated. Further research is needed to identify the key mechanisms at work in the different subtypes of the disease. Complement-mediated mechanisms have been implicated in all subtypes and drugs that inhibit the complement cascade should be considered. In those patients with detectable antibodies against gangliosides, a novel approach is to absorb the antibodies on a column that has a specific affinity for the individual ganglioside (81).

Variants of GBS

GBS comprises cases in which the type of nerve fiber injury is predominantly caused by demyelination as well as cases in which the primary pathological process is primary axonal degeneration (82). The most common subtype of the syndrome is acute inflammatory demyelinating polyradiculoneuropathy (AIDP) (82), and another subtype described in China, in which the neurological deficit is purely motor, has come to be known as acute motor axonal neuropathy (AMAN) (83-85). Patients in which the axonal degeneration is present in both motor and sensory nerve fibres, have a so called acute motor and sensory axonal neuropathy (AMSAN) (86). In Europe and North America the most frequent pattern is AIDP, present in 90% of GBS cases. In Asia, South and Central America, however, AIDP is relatively rare, and the predominant type is the axonal form of GBS which constitutes 30% to 47% of cases (41). The axonal subtype is rare in North America and Europe (5%-10%). Individual patients with demyelinating or axonal forms of GBS cannot be distinguished based on clinical features, but only by electrophysiology. Both axonal degeneration and very distal demyelination may lead to inexcitable nerves (87, 88). In addition to the variation in clinical symptoms between GBS patients, several cranial nerve variant of GBS can be distinguished of which the MFS occurs most frequently (89). In 1956, C Miller Fisher described the combination of acute ophthalmoplegia, limb ataxia, and areflexia in the absence of limb weakness known as MFS and postulated that this set of features was a form of GBS (90). Patients with MFS may have facial and lower cranial-nerve involvement. Some patients have severe swallowing problems for which they require temporary intubation. In general, however, patients with MFS show a good recovery. Overlap cases between MFS and GBS with a combination of oculomotor and limb weakness also occur.

Pathogenesis

Antecedent illness and preceding infections associated with GBS

Two-thirds of GBS patients have symptoms suggestive of an infection in the 3 weeks before the onset of weakness (36, 41, 91). In a prospective study conducted in England a respiratory infection occurred in 38% of the GBS patients and 12% of the controls, and gastrointestinal infections in 17% of the patients and 3% of the controls (71). Respiratory infections generally started within 1 to 2 weeks before neuropathy onset, gastrointestinal infections usually only 1 week or less before the start of neurological symptoms (54). The most frequent antecedent symptoms in GBS and related disorders were fever (52%), cough (48%), sore throat (39%), nasal discharge (30%), and diarrhoea (27%) (92). In most GBS studies, symptoms of a preceding infection in the upper respiratory tract or gastrointestinal tract predominate, but various infections and other preceding events such as vaccination, operation or stressful events have been reported (41, 71, 93).

Several reports have documented the occurrence of GBS shortly after vaccinations, operations, or stressful events, but the specific relation with GBS is still debated (34, 41, 94-96). There was an increase in incidence of GBS after the swine influenza vaccination program in the USA in 1976 (97). Other influenza vaccines have not been associated with the same risk (94). A retrospective study of the 1992 to1994 vaccine campaigns in the USA identified that vaccines were associated with a very small but significant increased risk of developing GBS of about one GBS case per million vaccines above the background incidence (96). A case–control from the UK did not show any significant association between GBS and previous immunization (95). However, in a recent US report on vaccinations and their side-effects, not only influenza vaccinations but also hepatitis vaccinations were suggested to be associated with the occurrence of GBS (98).

GBS is a heterogeneous syndrome with a wide spectrum of clinical, immunological, electrophysiological and pathological findings (49, 117). A wide variety of infectious agents have been proposed as a possible triggering factor in patients with GBS. In the United Kingdom and The Netherlands, *C. jejuni* infection was identified as the predominant antecedent infection and cytomegalovirus (CMV) as the second most prevalent (53, 93) (Table 2). Other infections associated with GBS were Epstein-Barr virus (EBV) and *Mycoplasma pneumonia* (53, 93, 109). A very recent prospective multicentre study in children with GBS demonstrated that coxsackie viruses were associated as antecedent infection (116). The relative frequency of antecedent infection however may differ between countries. In Northern China, *C. jejuni* infections occur in up to 66% of the GBS.

C. jejuni and GBS

C. jejuni can be cultured from stool specimens of GBS patients, although this appears to necessitate laborious isolation protocol including enrichment media. *C. jejuni* was first isolated from stool specimen of GBS patient in 1982 (118). Subsequently, *C. jejuni* infections were frequently identified in GBS patients by culture or serology in case-reports and small series (Table 3). Still, stool cultures are often negative at the onset of the neurological symptoms, considering the mean period of bacterial excretion after onset of diarrhea is only 16 days (119). GBS typically starts 1 to 3 weeks after

a *Campylobacter* infection. Therefore, studies based on stool culture alone will underestimate the frequency of previous *C. jejuni* infections in GBS. However, recent studies report that *Campylobacter* serology is highly sensitive and specific in GBS patients (30). In Northern China, *C. jejuni* infections occur in up to 66% of the GBS patients, depending on the serological assays and criteria for

Table 2. Antecedent infections in patients with the Guillain-Barré syndrome

Infectious agents	Frequency	References
C. jejuni	14-66%	47, 53, 71, 93, 99-107
Cytomegalovirus	5-15%	53, 108-112
Epstein-Barr virus	0-10%	53, 93, 108,109,113
M. pneumonia	1-11%	53, 93,108, 113,114
H. influenza	1-13%	93, 115
Coxsackie viruses	15%	116

seropositivity it is very difficult to compare the results from different studies (28, 29, 47). Population-based comparative geographical studies conducted by a central laboratory are therefore needed to address this issue.

Numerous investigators have looked for serological evidence of a *Campylobacter* infection in GBS patients. These studies show a high prevalence of antibodies to *C. jejuni* in the patients with GBS (Table 3). In the first serological study on a larger series of patients, Kaldor and Speed found evidence for a recent infection in 21 (38%) of 56 patients with GBS, but in none of the controls (100). Winer and colleagues found that 14 (14%) of 99 patients with GBS had recent *C. jejuni* infection, compared to only 2% of controls (53). Gruenewald and colleagues found that 3 (18%) of 17 patients with GBS in an uncontrolled population had elevated titers in two or more immunoglobulin classes (99). Kuroki et al. found that 36% of the patients were seropositive for *Campylobacter* in Japan (101).
In a large, blinded, case-control study demonstrate that 36% of the GBS patients were seropositive for *Campylobacter* (104). Rees et al. subsequently made a prospective case-control study, and found the evidence of recent *C. jejuni* infection in 26% of the patients with GBS, compared with 2% of the household controls, and 1% of the age-matched hospital controls (71). In a large comparative and prospective study in the Netherlands, *C. jejuni* infection was found to be predominant (32%) antecedent event in GBS patient. In Northern China, *C. jejuni* infections are more frequent (66%) in patients with AMAN than AIDP (47). *C. jejuni* are strongly associated with the axonal variants of GBS, and some studies concluded that this type of infection is exclusively found in patients with axonal forms of GBS (120). Geographical differences in the incidence of *C. jejuni* infections may therefore also influence the predominant clinical and electrophysiological phenotype. Whether this exclusive association between *C. jejuni* and axonal variants is also present in other geographical areas remains to be determined. Preceding infections with *C. jejuni* are also the predominant type of preceding infections in patients with MFS (121).

Table 3: *Campylobacter jejuni* infections in patients with Guillain-Barré syndrome[a]

Reference	Frequency[b]	Axonal[d]	Clinical association	Poor outcome[e]
Case reports				
Rhodes, (1982) (118)	1[c,f]	+	pure motor	+
Molnar, (1982) (122)	1[c]	+	NT	+
Constant, (1983) (123)	1[c]	NT	MFS	-
Pryor, (1984) (124)	1[c]	-	pure motor	-
Wroe, (1985) (125)	1[c]	-	MFS	-
De Bont, (1986) (126)	1[c,f]	+	severe weakness	-
Kohler, (1987) (127)	1[c]	NT	NT	+
Sovilla, (1988) (128)	3[c,f]	NT	-	+
Ropper, (1988) (102)	1[c]	+	severe pure motor	+
Yuki, (1990) (129)	2[c]	+	severe pure motor	+
Kuroki, (1991) (101)	7[c]	-	-	-
Yuki, (1991) (130)	6[c]	+	pure motor	NT
Duret, (1991) (131)	1[c]	+	pure motor	-
Yuki, (1992) (132)	13[c,f]	NT	NT	NT
Fujimoto, (1992) (133)	4[c]	NT	NT	NT
Frequency studies				
Kaldor, (1984) (100)	21/56 (38%)[f]	NT	severe weakness	-
Speed, (1987) (103)	22/45 (49%)[f]	NT	NT	NT
Winer, (1988a) (53)	14/99 (14%)[f]	NT	NT	-
Walsh, (1991) (134)	14/94 (15%)[f]	+	NT	NT
Gruenewald, (1991) (99)	3/17 (18%)[f,c]	NT	NT	NT
Boucquey, (1991) (135)	6/42 (14%)[f]	NT	-	-
Nobile-Orazio, (1992) (136)	3/16 (19%)[f]	NT	NT	NT
Gregson, (1993) (137)	15/42 (36%)[f]	NT		
Kuroki, (1993) (138)	14/46 (30%)[f]	NT	NT	NT
Enders, (1993) (139)	15/38 (39%)[f]	No	NT	NT
Vriesendorp, (1993) (140)	10/58 (17%)[f]	+	Severe weakness	NT
Mishu, (1993) (104)	43/118 (36%)[f]	NT	NT	NT
Von Wulffen, (1994) (141)	11/42 (26%)[f]	NT	NT	NT
Rees, (1995) (71)	25/96 (26%)[c,s]	+	pure motor	+
Ho, et al. (1995) (47)	25/38 (66%)[f]			
Jacobs, (1996) (142)	46/154 (32%)[f]	NT	severe, pure motor	+ after PE
Hadden, (2001) (143)	53/229 (23%)[f]	+	+	+
Takahashi, (2005) (144)	113/1049 (11%)[c]			
Sivandon, (2005) (145)	58/264 (22%)[f]	-	severe weakness	-
Sinha, (2006) (105)	19/80 (5%)[f]	+	+	-
Nachamkin, (2007) (106))	32/78 (41%)[f]	-	NT	NT
Barzegar, (2008) (146)	23/48 (48%)[f]	+	pure motor	+

[a]NT, not tested; +, present; -, absent; MFS, Miller Fisher syndrome; PE, plasma exchange; IVIg, intravenous immunoglobulins.
[b]Number of patients in case-reports, or number of *C. jejuni* infected patients per number of tested patients in frequency studies.
[c]Determined by stool culture
[d]Association with severe axonal degeneration
[e]Determined at least three months after diagnosis of GBS
[f]Determined by serology

Anti-ganglioside antibodies

The recent literature about GBS and related disorders has been dominated by the discovery of antibodies to a wide variety of gangliosides. Gangliosides were first identified as peripheral nerve targets for antibodies in patients with paraproteinaemic neuropathy (147, 148). Research has been driven by the hypothesis that the different syndromes underlying GBS could be explained by the different patterns of nerve damage caused by antibodies directed against different glycoconjugates shared by infective organisms and the nervous system. Gangliosides are complex glycosphingolipids containing sialic acid (149). Different gangliosides are distinguished by both number and position of sialyl residues attached to a common tetraose backbone (Fig. 4). They are widely distributed in the membranes of many cells and are concentrated in neuronal membranes but occur in low concentrations in glia and myelin. They have multiple functions controlling cell interactions, receptor function and growth (150). Within the nervous system there is marked regional variation in relative abundance of the different molecular species. Behind the search for antibodies that define different clinical syndromes lies the hypothesis that these syndromes are caused by the occurrence of antibodies to different gangliosides which match those regions of the nervous system showing clinical involvement (151).

In 1988, Ilyas et al. first reported the presence of antibodies against gangliosdies in the serum from GBS patients (152). Since then, in about half of patients with GBS, serum antibodies to various gangliosides can be demonstrated in the acute stage of disease (153-155). Antibodies to more than 20 different gangliosides have been identified in various frequencies in serum from GBS patients. The most frequent antibodies in GBS are directed to the gangliosides LM1, GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, and GQ1b (154, 155). These antibodies specifically recognize parts of the oligosaccharide moieties and may bind to various gangliosides with the similar carbohydrate structures. Other antibodies might bind to mixtures or complexes of different gangliosides instead of individual gangliosides (156-159).

The first anti-ganglioside antibodies in GBS described the presence of anti-GM1 antibodies (152). Antibodies against the GM1 have been demonstrated in various frequencies ranging from 9 to 78%. The presence of serum anti-GM1 antibodies was found to be associated with a more severe, pure motor variant of GBS, with more extensive axonal degeneration, and poor recovery (129, 160-163). In most studies, *C. jeuni* infections have been linked to anti-GM1 reactivity (41, 142), although this is not absolute. Anti-ganglioside antibodies in *C. jejuni* infected GBS patients are not exclusively directed against GM1. Several groups have documented the presence of antibody reactivity against GM1-like structures such as GalNAc-GD1a and GM1b (164, 165). Furthermore, *C. jejuni*-infected GBS patients have antibodies against a variety of glycolipids and this could explain the lack of an absolute correlation between anti-GM1 antibodies and *C. jejuni* infection (166). In Chinese and Japanese GBS patients there is a correlation between anti-GD1a antibodies and *C. jejuni* infection but this was not found in studies from Europe and Northern America (93, 132, 167). The presence of anti-GQ1b antibodies is highly associated with MFS variant of GBS (168-171). Antibodies to this ganglioside are present in more than 90% of patients and are absent in other forms of inflammatory neuropathy except for an overlap syndrome in which GBS is associated with ophthalmoplegia (41). Most anti-GQ1b

positive patients with GBS and MFS have additional antibody activity against GT1a, while half of the patients have additional antibody activity against GD1b and GD3 (131, 159, 172).

Molecular mimicry between *C. jejuni* LOS and gangliosides

The original concept of molecular mimicry was based on the structural resemblancees between component of a micro-organism and a host (173). Later this term was also used for the idea that infections because of structural resemblance might result in a cross-reactive antibody or T cells response leading to damage of tissue of the host and causing an autoimmune disease (174).

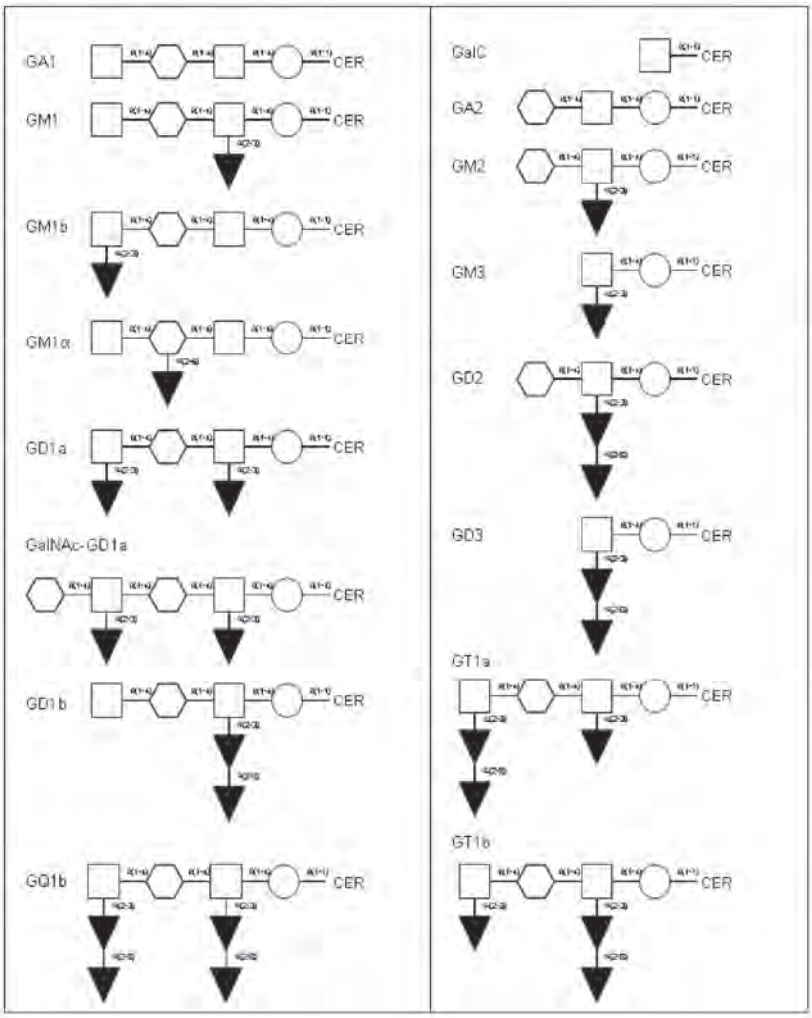

Figure 4. List and schematic representation of ganglioside molecular structures. □ galactose, ▼ sialic acid, ○ glucose, ⬡ N-acetyl- galactosamine, CER ceramide.

The role of molecular mimicry between microbial and autologous antigens in producing a cross-reactive immune response can be established on different level: 1) homology in biochemical structure or linear amino acid sequence, 2) cross-reactivity of antibodies with both structures, 3) induction of cross-reactive antibodies by immunization with the microbial antigen, and 4) induction of cross-reactive antibodies which induce dysfunction or tissue damage. Molecular mimicry between microbial antigens and host tissue is a popular and attractive hypothetical mechanism for the triggering of autoimmune disease by preceding infections (175). According to the mimicry hypothesis, autoantibodies and/or autoreactive T cells that are induced by the infection are initially directed against microbial antigens. Because of the structural resemblance between microbial and particular host antigens, the antibodies and T cells not only destroy the invading pathogen but also attack host tissue. In addition, infections can also lead to activation of auto-reactive immune cells by antigenic non-specific mechanisms, called bystander activation (176). GBS is an autoimmune peripheral neuropathy which has been largely overseen. The crucial contribution of molecular mimicry at the B-cell level in the immunopathogenesis of GBS is overwhelming. GBS is an excellent paradigm for how post-infectious immune-mediated disease in humans can be triggered by molecular mimicry (175).

Molecular mimicry between *C. jejuni* LOS and gangliosides

C. jejuni strains isolated from several GBS and MFS patients showed a similar carbohydrate moiety in LOS and peripheral nerve gangliosides confirmed by mass spectrometry (Fig. 5) (163, 177, 178).

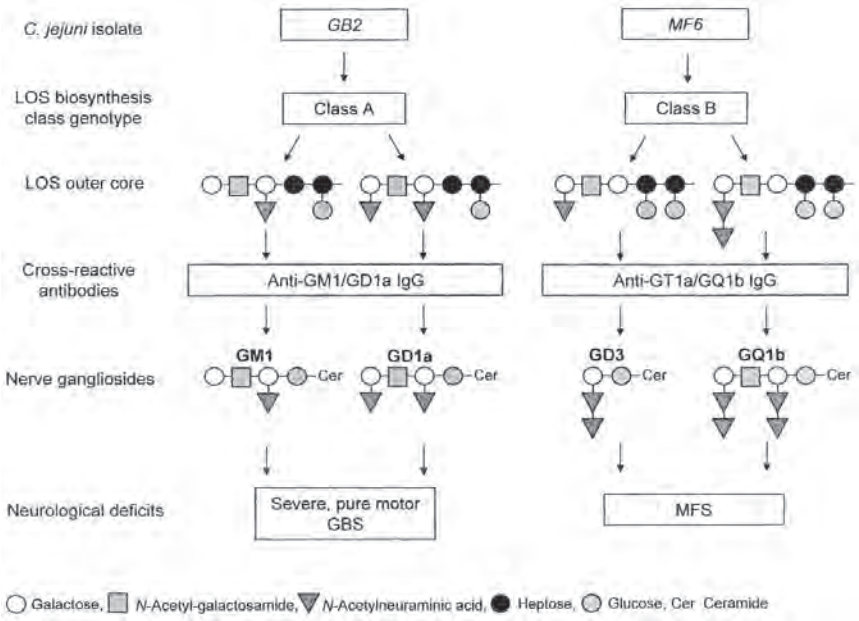

Figure 5. Cross-reactive antibodies to the peripheral nerve gangliosides. Adopted from Jacobs et al. (49).

Certain *C. jejuni* serotypes possess sialylated LOS structures, confined to the outer core of the LOS molecules, which mimic a wide range of peripheral nerve gangliosides including GM1, GA1 (asialo-GM1), GM1b, GalNAc-GM1b, GM2, GD1a, GalNAc-GD1a, GT1a, GQ1b and GD3 (163-165, 177-184). The molecular mimicry in *C. jejuni* has been demonstrated by an immune response in susceptible hosts that results in demyelination or axonal damage of peripheral nerves, partly by the anti-ganglioside antibody response (91). A very recent study reports that GBS- and MFS-related *C. jejuni* strains expressed a ganglioside-like LOS, with up to five identical glycan structures (185).

Most of the strains expressed a heterogeneous LOS, in which the combination of GM1- and GD1a-like structures was most frequently found. The ganglioside mimicry in *C. jejuni* determines the specificity of the anti-ganglioside antibodies and the associated variant of GBS. *C. jejuni* isolates from patients with pure motor or axonal GBS frequently express a GM1-like and GD1a-like LOS, whereas those isolated from patients with ophthalmoplegia or MFS usually express a GD3-like, GT1a-like, or GD1c-like LOS (121, 159, 172). The LOS of some strains contained no sialic acids and showed no mimicry with gangliosides. Some authors report that the ganglioside-like structures in LOS are related to certain HS serotype, which would explain the predominance of certain HS serotypes in GBS (132, 186). Interestingly, LOS from serostrain HS:3 is not sialylated, and HS serotype is not associated with GBS (187). Mass spectrometry analysis indicate that *C. jejuni* strains isolated from GBS and MFS patients more frequently have a ganglioside-like LOS than strains isolated from enteritis controls. However, the association is not absolute. GBS-related strains may not have ganglioside mimicry (188) whereas some strains from enteritis controls do (188). In addition, co-infection with multiple *C. jejuni* strains also occurs in GBS patients. The isolation of only one strain may easily lead to a wrong conclusion with respect to absence or presence of molecular mimicry involved in the pathogenesis of GBS (189).

Though the biochemical analysis of *C. jejuni* LOS clearly identified ganglioside-like structures, it remains to be clarified whether these structures can induce anti-ganglioside antibodies with the specificity found in patients with GBS and MFS. The first study reporting that cross-reactivity of serum anti-GM1 antibodies from GBS patients with the LOS from the autologous *C. jejuni* isolates was published by Yuki et al. (132). Subsequently, serological studies have clearly demonstrated that anti-ganglioside antibodies in serum from patients with GBS and MFS cross-react with the LOS from autologous *C. jejuni* isolate (190-192). Serum antibodies to GM1 and GD1a are specifically adsorbed by *C. jejuni* strains expressing LOS with GM1 and GD1a mimics. Conversely, antibodies to GQ1b are adsorbed by strains expressing a GD3 or GT1a mimics, and antibodies to ganglioside complexes cross-react with *C. jejuni* LOS (159).

Molecular epidemiology of *Campylobacter*

In order to better understand the epidemiology of *Campylobacter* infections in both humans and poultry, reproducible typing methods which can distinguish individual strains are necessary. Several phenotypic methods for typing *C. jejuni* and *C. coli* have been described; these methods include serotyping, phage typing, and biotyping (193-195). A major drawback of this combination of

methods is that it is time-consuming and costly. Some other disadvantages of phenotypic methods are that they have restricted differentiation powers and a high proportion of strains are non-typeable. Since 1980, molecular typing methods have emerged to determine the genetic polymorphisms and possible clonal relationship of *Campylobacter* spp. At present, comparative genotyping methods such as Pulsed-field gel electrophoresis (PFGE), Multilocus sequence typing (MLST), and amplified fragment length polymorphism (AFLP), are currently available to study the population structure of *Campylobacter* (196).

PFGE

PFGE is widely regarded as among the most discriminatory typing methods generally available for bacteria. It involves the digestion of intact genomic DNA with rare-cutting restriction enzymes that cut the DNA into relatively few fragments, in contrast with its predecessor, restriction enzyme analysis (197). Widespread, worldwide adoption of the PulseNet protocol developed at the CDC (198) has significantly improved the compatibility of PFGE. In the case of *C. jejuni*, most researchers have used the enzyme *Sma*I, which typically generates 4 to 10 fragments (199, 200). Comparisons between large numbers of isolates have been facilitated by the widespread availability of software such as BioNumerics (Applied Maths, Ghent, Belgium), which are able to electronically compare electrophoresis patterns generated. Though, this technique has high discriminatory power for subtyping *Campylobacter* (201); however, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies (202).

AFLP

AFLP analysis is based on selective amplification of restriction fragments of chromosomal DNA and has been widely used in a variety of bacterial genera, including *Campylobacter* both for identification and typing purpose (203). This is a reproducible and high discriminatory a whole genome typing method, particularly well suited for computer-assisted data analysis. AFLP schemes were initially developed for typing purpose, incorporating only *C. jejuni* and *C. coli* (203, 204). Subsequently, these observations were extended to other *Campylobacter* species (205, 206) and demonstrated that numerical analysis of AFLP profiles is well suited for identification at the species, subspecies, and strain levels. Numerous subsequent studies have applied AFLP to explore intraspecies diversity (207) and epidemiological relationships (208, 209-212) of *Campylobacter* strains.

MLST

Since the first description of MLST in 1998 (213), major progress has been made in understanding the population biology of *C. jejuni*. Now, this technique has emerged as the state-of-the-art method for the resolution of bacterial population genetics (214) and is recognized as the gold standard typing method for the phylogenetic study of *Campylobacter* genus (215, 216). MLST involves the sequencing of genes, typically those with housekeeping functions, whereby each sequence variant represent a distinct allelic type. Combining the analysis of seven genes alongside appropriate computational analysis is a powerful tool for the study of population biology, epidemiology, and

evolution of microbes that has expanded tremendously (217). The level of genetic divergence among *Campylobacter* housekeeping genes is as high 80.9% (216). *C. jejuni* is highly diverse with a total of 2,243 distinct STs from 3,704 isolates deposited in the pubMLST database (http:// pubmlst.org/campylobactecr) (218).

Sequence typing data provide the resolution and reproducibility necessary to investigate the diversity of *Campylobacter* isolates obtained from farms and environment (219-221). These studies reported that genotypes from human disease are recovered from farm animals (219, 221), while the genotypes from environmental sources seem to be distinct (220). Certain clonal complexes are associated with particular food animal sources, with the ST-61 complex especially associated with cattle (215, 220) and the ST-403 with pigs, and dogs (221-223). Large-scales studies of isolates from a wide variety of sources provide the prospect that in the near future, it will be possible to trace the movement of *Campylobacter* genotypes through the food chain (224) and attribute cases of human infection to particular host sources with reasonable precision (225). MLST has also been used to examine antibiotic-resistant isolates obtained from poultry (226, 227).

***C. jejuni* HS-serotype associated with GBS**

Earlier studies demonstrated that certain Penner HS-serotypes are over-represented among neuropathogenic GBS- and MFS-associated strains in some parts of the world. Studies form different geographical locations such as Japan, South Africa, China, and Mexico revealed that most of the clinical isolates belonged to a restricted set of serotypes including HS:19 and HS:41 (132, 138, 228-231). *C. jejuni* HS:19 and HS:41 are clonal which suggests that these serotypes may have unique and specific virulence properties that trigger GBS (232). Unfortunately, these data were in a sense ambiguous. Subsequent research revealed that the neuropathogenic properties are not restricted to specific HS serotypes as other serotypes (HS:1, HS:2, HS:4, HS:4 complex, HS:5, HS:10, HS:16, HS:23, HS:37, HS:44, and HS:64) commonly isolated from enteritis patients are also found in patients with GBS (71, 138, 177, 190, 233-237). This observation suggested that the ability of strains to cause neuropathological syndrome was more widely disseminated than previously anticipated on the basis of the clonality hypothesis. As GBS- and MFS- associated strains were derived from a variety of different genetic backgrounds, focused experimental searches for non-serotype dependent factors important in the induction of GBS/MFS were facilitated (49).

Comparative genomics of GBS-associated *C. jejuni*

Many researchers have studied collections of GBS- and enteritis- associated strains in search of GBS-specific microbial features. MLST typing and flagellin gene sequencing confirmed the polyclonality among GBS- and MFS-related *Campylobacter* strains (214). AFLP did not contribute substantially for getting the GBS-specific markers. Although AFLP has been used for identifying clonal expansion among clinical, veterinary, and environmental isolates, no specific markers could be identified on the basis of the DNA fingerprints developed for the GBS/MFS isolates (236, 238). Furthermore, no molecular markers specific to GBS were detected after analyzing a highly clonal

HS:41 strains by using a high-throughput AFLP (188). The use of whole genome DNA arrays did not reveal any GBS-specific markers when comparing gene content for strains derived from non-complicated enteritis cases versus GBS (239). Very recently, Taboda et al. determined the gene conservation profile of 1,712 virulence and other genes in GBS-associated and controls strains (240). However, it has been demonstrated that genes involved in the sialylation of LOS are significantly associated with GBS-associated strains (240).

***C. jejuni* LOS and gene specific variation**

Molecular mimicry between *Campylobacter* LOS and gangliosides in human nerves is the causative mechanism for the development of GBS (178, 241). The presence of LOS-associated genes that were identified through genome sequencing revealed that several genes were selectively present or absent, depending on the composition of LOS gene clusters (242). Comparisons of the various *C. jejuni* LOS biosynthesis loci indicate that a variety of genetic mechanisms is responsible for the synthesis of different structures (243). Particularly, the most obvious genetic differences are differences in gene content and organization (Fig. 6). The sequence of LOS biosynthesis genes between Cj1133 (*waaC*) and Cj1148 (*waaF*) are available for *C. jejuni* strains (185, 242, 243, 244). A total of 19 LOS classes (A to S) were identified on the basis of the gene content and organization showing extensive variability in length (244). Some genes are unique to one class, whereas some other genes are present in more than one class but not in all. The significant interest in the LOS biosynthesis gene cluster stems from the potential involvement of the ganglioside-like structure of *C. jejuni* LOSs inducing GBS and MFS (245, 246). Comparison of various *C. jejuni* strains demonstrated that a group of five LOS biosynthesis locus classes (A, B, C, M, and R) (Fig. 6) contain the genes that are necessary for the biosynthesis of ganglioside mimics, in particular genes encoding a sialyltransferase (*cst-II*), a sialic acid synthase (*neuB*), an N-acetylglucosamine-6-phosphatase 2-epimerase (*neuC*), and a CMP-Neu5Ac synthetase (*neuA*) (242, 243).

The presence of certain genes could be associated with a specific clinical symptom and GBS variants. The presence of *cst-II* gene and the presence of GQ1b epitopes on the *Campylobacter* outer LOS surface were intimately linked to the development of MFS (248). Interestingly, specific point mutation with specific genes could also be associated with LOS variation and hence different clinical presentation of the diseases induced (172). The LOS locus classification and some highly specific amino acid changes in, such as the *cstII* gene ultimately determine the LOS biosynthesis capacity. The sialyltransferase gene *cstII* has an Asn/Thr polymorphism at codon 51, which determines substrate specificity; *cstII* (Thr51) has only α-2,3-sialyltransferase activity and is termed monofunctional, whereas *cstII* (Asn51) has both α-2,3-and a-2,8-sialyltransferase activities is termed bifunctional (249). *cstII* (Thr51) is closely associated with GBS and anti-GM1 and anti-GD1a autoantibodies, and *cstII* (Asn51) is closely associated with MFS and anti-GQ1b autoantibodies (121, 232, 250).

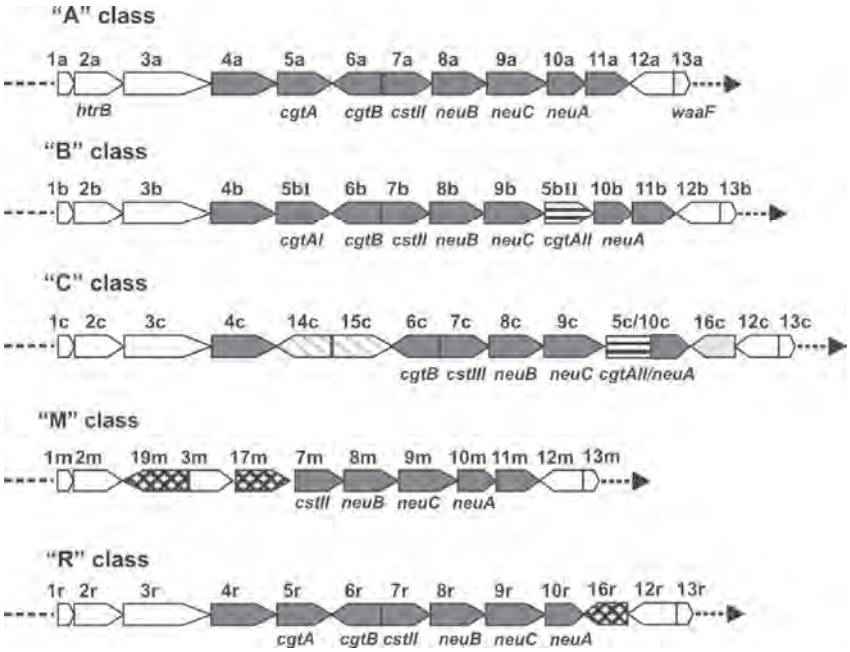

Figure 6. *C. jejuni* LOS classes that posses a sialyltransferase gene. Classes A, B, C, M and R. Adopted from Gilbert et al. (247).

Scope and outline of thesis

The aim of the thesis was to define the clinical epidemiology, preceding infections and antibody response in GBS in Bangladesh. Most knowledge of GBS is currently based on studies in patients from developed countries and very little is known about patients form developing countries. We speculate these disease characteristics may show geographical differences because the type of preceding infections in developing countries may differ from those in developed countries. In developing countries such as Bangladesh, compared to developed countries, there is a much more frequent and repeated exposure to *Campylobacter* occurring already from early childhood. Because of this high exposure in developing countries, *Campylobacter* may more frequently trigger the onset of GBS and in fact may increase the incidence of GBS. These *Campylobacter* from Bangladesh may also differ genetically or biochemically from isolates from other areas. Considering the crucial role of antecedent infections play in inducing cross-reactive anti-ganglioside antibodies, the pathogenesis and neurological deficits and outcome of GBS may differ depending on the geographical region. More specifically, the studies described in this thesis have focused on GBS in Bangladesh, were we have defined the:

- epidemiology in children
- clinical presentation, course and outcome
- spectrum of antecedent infections and anti-ganglioside antibodies

- molecular mimicry of *C. jejuni* and gangliosides
- fine specificity of cross-reactive anti-ganglioside antibodies
- comparative genomics of *C. jejuni* strains from GBS and enteritis patients, and poultry

In Chapter 2 we will describe the crude incidence rates of GBS in Bangladesh among children <15 years of age. A detailed clinical, epidemiological, and neurophysiological data will be described in GBS patients. We will determine the frequency of *C. jejuni* infection in GBS patients compared to family controls and age- and sex-matched controls with other neurological diseases. Also, serum anti-ganglioside antibodies will be determined in patients compared to two controls groups.

In **Chapter 3** we will determine frequency of antecedent infections in GBS patients including those caused by *Campylobacter*, cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumoniae*, and enterovirus.

Chapter 4 will describe the cross-reactivity of anti-ganglioside antibodies with *C. jejuni* isolates from patients with GBS. The cross-reactivity of anti-ganglioside antibodies from GBS and GBS/MFS patients will be determined by inhibition ELISA. In addition, monoclonal mouse antibodies against different gangliosides will be used to determine if ganglioside-mimics were present in the LOS. We will also determine the LOS outer core structures of *C. jejuni* strains associated with GBS/MFS by capillary electrophoresis coupled with electrospray ionization mass spectrometry.

Chapter 5 will describes new *C. jejuni* serotypes and new sequence types overrepresented among isolates from GBS patients in Bangladesh. We will investigate the genetic heterogeneity of *C. jejuni* strains isolated from GBS and enteritis patients. In this comparative genomic analysis, multi-locus sequence typing (MLST), amplified fragment length polymorphism (AFLP), LOS class PCR typing, and pulsed-field gel electrophoresis (PFGE) will be used. In addition, we will study the diversity and clonal relationships among *C. jejuni* from humans and poultry in Bangladesh.

Chapter 6 will discuss the importance of the results described in chapters 2-5. The observations from the studies in chapters 2-5 will be summarized and discussed in relation to current literature, and directions for future research will be given.

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Chapter 2.1

ACUTE FLACCID PARALYSIS SURVEYS TO DETERMINE INCIDENCE RATES FOR GBS

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Bangladesh has achieved remarkable success in its drive to eradicate poliomyelitis as no case has been reported from the year 2000 and onwards. Still, the non-polio incidence rate of paralytic syndromes in Bangladesh is currently 3.25 per 100,000 children <15 year (1). Guillain–Barré syndrome (GBS) is an acute polyradiculoneuropathy and is the most frequent cause of acute flaccid paralysis (AFP) with a worldwide incidence ranging from 1.2 to 2.3 per 100,000 persons per year (2). *Campylobacter jejuni* has been identified as the predominant antecedent infection of GBS in Bangladesh (3). Frequent exposure to enteropathogens in developing countries may lead to an increased incidence of GBS. Our objective was to estimate the crude incidence rates of GBS among children <15 years of age in Bangladesh in 2006 and 2007 on the basis of the ongoing AFP surveillance program.

Methods

An active surveillance program for AFP is conducted by The Government of Bangladesh (GoB) in collaboration with World Health Organization (WHO). AFP is defined as acute onset of focal or general flaccid (hypotonic) weakness without other obvious cause (e.g., trauma) in children <15 years, including GBS. Data on the number of reported AFP cases in Bangladesh in 2006 and 2007 were obtained. Based on the clinical and other information routinely collected through the surveillance system, we defined a GBS case (4). In addition, we considered the absence of injury or birth trauma in our diagnostic criteria. Bangladesh is divided in six divisions and 64 districts. Crude incidence data of GBS were calculated per division and per district, based upon <15 years population reported by WHO/GoB.

Results

The total number of reported AFP cases in children <15 years in 2006 and 2007 was 1619 and 1844, respectively. Among those, the number of cases that fulfilled the case definition of GBS was 608 (37%) and 855 (46%), respectively. The crude incidence of GBS in children <15 years varied from 1.5 to 1.7 per 100,000 populations in the three northern divisions Dhaka, Rajshahi, and Sylhet, and from 2.1 to 2.5 per 100,000 in the three southern divisions Khulna, Barisal and Chittagong of Bangladesh (Fig.). Overall, crude incidence rates of GBS in children <15 years varied from 1.5 to 2.5 per 100,000 per year in the six divisions of Bangladesh. High incidence rates greater than 5.0 per 100,000 were observed in the Meherpur and Barisal district, the southern part of Bangladesh. There was a seasonal fluctuation in the frequency of patients with GBS; the highest number of cases was found in May, with 159 cases, and lowest for February with 84 cases (Fig.). GBS was predominant among boys (59.1% of cases).

Comments

To our knowledge, this is the first report of a population based crude incidence rates of GBS in Bangladesh. A significant proportion of the AFP cases in Bangladesh appear to be caused by GBS. The incidence of GBS in the three northern divisions is much lower than in the three coastal divisions Khulna, Barisal and Chittagong, for which we currently have no explanation. However, differences in incidence rates between northern and southern part of Bangladesh may be explained difference in climate, or epidemiology, further research is required to determine why GBS would occur more frequently in southern part of Bangladesh. The population-based data correlate very well with a smaller scale hospital-based study done in Khulna, Bangladesh (5). The authors reported that 47% of the hospitalized AFP cases were diagnosed with GBS. Based on our population-based and hospital-based study (3), we estimate that the total number of newly diagnosed GBS patients in Bangladesh is approx. 3500 per year– an incidence 3 to 4 times higher than in the rest of the world. A recent review showed that the best estimate of the global incidence of GBS is between 1.1/100,000/year and 1.8/100,000/year with lower rates reported in children (<15 years) of 0.6/100,000/year (6). This review relies mostly on studies from Europe and North America. Rates from other parts of the world were hardly presented. There is a scarcity of GBS incidence data from Asia and Africa.

The association with antecedent viral and upper respiratory infections in GBS has been known for over 100 years. The association of *C. jejuni* and GBS was first reported in 1982 (7). *C. jejuni* is associated in particular with axonal and motor neuron forms of GBS (8, 9). We reported recently a first systematic hospital surveillance of GBS in Bangladesh. In this study, we report a strong association of the acute motor axonal neuropathy variant of GBS in young adults with preceding *C. jejuni* infections (3). The population-based study reported here has some limitations. The study is retrospective and the case definition was based on existing and available clinical data in the AFP surveillance system. Our case definition therefore does not meet the strict NINDS criteria. Future studies should therefore include a validation of the case definition used here.

The possibility of GBS being related to the H1N1 pandemic and its affiliated H1N1 vaccination campaign has resurfaced again (10). For the surveillance of excess cases of GBS after H1N1 influenza and also for post-marketing surveillance of the safety new vaccines in general, background GBS incidence data are critical. This report suggests that the burden of GBS in Bangladesh is substantial, and data obtained through the ongoing AFP surveillance program can be used to obtain crude incidence data of GBS worldwide.

Figure. Crude incidence rate (per 100,000 per year) of GBS in Bangladesh in 2006 and 2007 in children <15 years of age.

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Chapter 2.2

AXONAL VARIANT OF GUILLAIN-BARRÉ SYNDROME ASSOCIATED WITH *CAMPYLOBACTER* INFECTION IN BANGLADESH

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ABSTRACT

Background: *Campylobacter jejuni* enteritis is the predominant bacterial infection preceding Guillain-Barré syndrome (GBS), an acute post-infectious immune-mediated polyradiculoneuropathy. The purpose of this study was to define the clinical phenotype of GBS and the relation with preceding *C. jejuni* infections in Bangladesh.

Methods: We performed a prospective matched case-control hospital surveillance including 100 patients fulfilling the National Institute of Neurological Disorders and Stroke (NINDS) criteria for GBS from 2006 to 2007 in the Dhaka area of Bangladesh. Detailed clinical, electrophysiological, serologic and microbiological data were obtained with a follow-up of six months.

Results: GBS affected predominantly young adult males, living in rural areas. Sixty-nine percent of the patients had clinical evidence of a preceding infection. The most frequent symptom was diarrhea (36%). The majority of patients had a pure motor variant of GBS (92%) with relatively infrequent cranial nerve involvement (30%). Twenty-five percent of patients required respiratory support. Electrophysiological studies showed that 67% of patients had an axonal variant of GBS. Eleven (14%) patients died and 23 (29%) remained severely disabled during the follow-up. Positive *C. jejuni* serology was found in an unprecedented high frequency of 57% as compared to 8% in family controls and 3% in control patients with other neurological diseases ($P<0.001$). *C. jejuni* infection was significantly associated with serum antibodies to the gangliosides GM1 and GD1a, axonal neuropathy, and greater disability.

Conclusions: We report an unusually high frequency of the axonal variant of GBS in Bangladesh, associated with preceding *C. jejuni* infection, severe residual disability, and high mortality.

Introduction

Guillain–Barré syndrome (GBS) is an acute polyradiculoneuropathy and worldwide the most frequent cause of acute flaccid paralysis (AFP) with an incidence of 1.2 to 2.3 per 100,000 persons per year (1). The pathological spectrum of GBS includes acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN) and acute motor sensory axonal neuropathy (AMSAN) (1). GBS is a post-infectious disease and approximately two-third of patients report symptoms suggestive for a preceding infectious illnesses. GBS consists of various phenotypes which are associated with specific types of preceding infection. *Campylobacter jejuni* has been identified as the predominant type of antecedent infections (2-9). *C. jejuni* infections are associated with a severe (10, 11), pure motor (12, 13), axonal variant of GBS (14) and with poor outcome (4, 15). Infection with *C. jejuni* induces the production of cross-reactive antibodies to nerve gangliosides which are related to nerve damage (16, 17).

Considering the crucial role of *C. jejuni* infections in triggering GBS, we hypothesized that this disease may be more frequent in countries with a high incidence of diarrhea and a poor hygienic infrastructure. There is a paucity of data on the incidence and clinical phenotype of GBS in developing countries, including Bangladesh. Data on AFP incidence in Bangladesh are available through the World Health Organization (WHO) global polio eradication initiative and the national polio surveillance. The non-polio AFP incidence rate is currently 3.25 per 100,000 children under 15 years (18, 19). GBS is thought to be responsible for the majority of cases of AFP (20). Our objective was to identify the frequency of preceding *C. jejuni* infections, the clinical phenotype and the prognosis of GBS in a hospital-based prospective study.

Methods

Prospective hospital-based study

Patients and study design

We conducted a prospective case-controlled study with a follow-up of six months including 100 subsequent cases of GBS admitted at the Dhaka Medical College Hospital (DMCH), Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Central Hospital (DCH) in Dhaka between July 2006 and June 2007. DMCH and BSMMU are the largest tertiary medical hospitals in Bangladesh. DCH is one of the largest non-government hospitals. Upon admission of a patient presumptively diagnosed with GBS, a neurologist (M.B.I) from DMCH examined the patient within two days and validated the clinical diagnosis using the National Institute of Neurological Disorders and Stroke (NINDS) criteria for GBS (21). The final diagnosis was confirmed by a senior neurologist (Q.D.M).

Data were collected regarding age, sex, antecedent events, detailed neurological signs and symptoms, treatment, days to nadir, complications, duration of admission, GBS disability score and the Medical Research Council (MRC) sum score at standard points (entry, and 2 weeks, 4 weeks, and

6 months after entry). Both the GBS disability score and MRC sum score give an indication of the severity of disease. The GBS disability score used in this study was defined by Hughes et al. (22). The MRC sum score was defined as the sum of MRC score of six muscles in the upper and lower limbs on both sides, ranging from 60 (normal) to 0 (quadriplegic) (15). The rapidity of progression was indicated by the number of days from onset of weakness to nadir, defined as the lowest MRC sum score. Poor outcome was defined as not reaching a GBS disability score of 2 (independent walking) within six months (15). The study had a follow-up period of six months during which monitoring of disease progression and sampling of serum was performed at predefined standard time points. Two controls were selected for each patient: control 1 was a family member living in the same household (family control, FC); control 2 was an age, sex, and day matched patient hospitalized in the same ward with another neurological disease not related to recent infections (other neurological control, OND).

Standard Protocol Approvals, and Patient Consents

Informed consent was obtained from all patients and controls. The project protocol was reviewed and approved by the institutional review board and the ethical committees at the Dhaka Medical College and Hospital, Dhaka, Bangladesh.

Electrophysiological classification

Standard diagnostic electrophysiology was performed between one and four weeks after onset of weakness. Patients were classified according to predefined criteria as having acute motor axonal neuropathy (AMAN), acute motor sensory axonal neuropathy (AMSAN), and acute inflammatory demyelinating polyneuropathy (AIDP) (23). Patients who did not meet the criteria for one of these subgroups were categorized as unclassified.

***Campylobacter* culture and identification**

Stool samples were cultured according to methods previously described (24). A presumptive identification of *Campylobacter* was done with Gram stain, oxidase and hippurate hydrolysis tests and confirmed with a *C. jejuni* species-specific PCR (25).

***Campylobacter* and anti-ganglioside serology**

Serum obtained from GBS patients at hospital admission and serum from family controls and other neurological disease controls obtained at the same time point were compared for *Campylobacter* and anti-ganglioside serology. Serum antibodies against *C. jejuni* were determined by an indirect enzyme-linked immunosorbent assay (ELISA) for IgG and by antibody class capture ELISA for IgM and IgA antibodies. This method and the criteria for a positive score were previously described (26). Sera were tested for IgM and IgG antibodies against the ganglioside GM1, and GD1a by ELISA according to methods and criteria for a positive score previously described (27).

Statistical analysis

Differences in frequency of various infections between GBS patients and OND controls were tested with the McNemar's test, and between GBS patients and FC with the Chi^2-test without continuity correction or with the Fisher exact test. With respect to the clinical features of the GBS patients, differences in proportion between subgroups were tested with the Chi^2-test or with the Fisher exact test, and differences in medians with Wilcoxon-Mann-Whitney *U* test. Conditional logistic-regression was used to compare the odds ratios for poor outcome between categories of the risk factors. Two-tailed tests were used throughout and $P<0.05$ was regarded as significant. Statistical analysis was performed using Epi Info Version 3.5.1 and SPSS for Windows v.11.5 (SPSS Inc., Chicago, II, USA).

Results

Prospective hospital-based study

Demography

Among the 100 included GBS patients, 57 came from DMCH, 25 from BSMMU, and 18 from DCH, Dhaka. Baseline characteristics are shown in Table 1. In this cohort, patients predominantly were young adult males, living in rural areas. There was a seasonal fluctuation in the frequency of GBS patients included in the study with a peak between January and March (Figure). Clinical symptoms suggestive of an infection in the four weeks preceding onset of weakness was reported in 69% of patients; diarrhea was the most frequent antecedent symptom (36%) (Table 1).

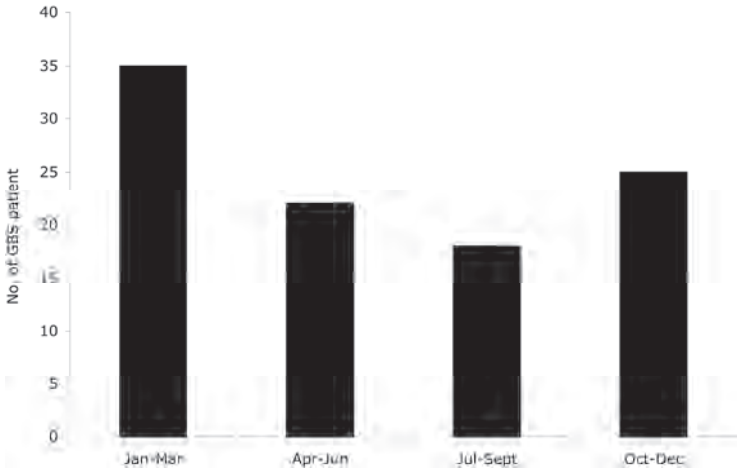

Figure: Seasonal distribution of Guillain–Barré syndrome in Bangladesh

Table 1: Demography, clinical and electrophysiological characteristics of GBS patients[a]

Demography		
Sex (M/F)		72/28
Median age, years (range)		21 (2.0-65.0)
Age categories (years)[b]	<15	26 (26%)
	16-30	47 (47%)
	31-45	19 (19%)
	>45	8 (8%)
Preceding symptoms and signs[c]	Diarrhea	36 (36%)
	Respiratory symptoms	19 (19%)
	Fever	14 (14%)
Cerebrospinal fluid	Protein level >40 mg/dl	77 (99%)
(N=78)	Cell count < 15 cell/µl	78 (100%)
Neurological symptoms		
Cranial nerve impairment	Facial nerve palsy	25 (25%)
	Oculomotor palsy	4 (4%)
	Lower bulbar palsy	10 (10%)
	None	70 (70%)
Sensory deficits		8 (8%)
Pain		10 (10%)
MRC sum score (at entry)[d]	60-51	8 (8%)
	50-41	21 (21%)
	40-31	12 (12%)
	30-21	19 (19%)
	20-0	40 (40%)
GBS disability score (at entry)	1 or 2	11 (11%)
	3	11 (11%)
	4	53 (53%)
	5	25 (25%)
Electrophysiology		
Classification (N=64)[e]	AMAN	36 (56%)
	AMSAN	7 (11%)
	AIDP	14 (22%)
	Unclassified	7 (11%)

Abbreviations:
AIDP = acute inflammatory demyelinating polyneuropathy; AMAN = acute motor axonal neuropathy; AMSAN = acute motor sensory axonal neuropathy; GBS = Guillain-Barré syndrome; MRC = Medical Research Council; unclassified = not fulfilling criteria for AMAN, AMSAN, or AIDP.
[a]Data are expressed as no. (%) unless otherwise indicated.
[b] Age was divided into 4 categories; the first age group was below 15 years based on acute flaccid paralysis surveillance, and others were grouped by 15-year interval.
[c]Symptoms of an infection in the 4 weeks preceding the onset of weakness.
[d] MRC sum score was defined as the sum of 6 muscles in the upper and lower limbs on both sides, ranging from 60 (normal) to 0 (quadriplegic) (15).
[e] Sixty-four patients were available for electrophysiology.

Clinical features

In most patients the disease started as a rapidly progressive symmetrical weakness of the lower extremities. The median interval between onset and maximum of weakness was 8 days with an interquartile range (IQR) of 6-11 days. A large majority of patients had a pure motor variant of GBS without sensory deficits (92%), frequently without cranial nerve involvement (70%). Fifty-three patients were already bed-bound at entry. Twenty-five percent of patients required mechanical ventilation. During follow up, muscle atrophy was observed in 38% of patients which appeared only late in the course of the disease. CSF examination was performed in 78 cases at 2 to 10 days after onset of weakness, showing a mean cell count of 2.6 cell/µl (range 0-15 cells/µl) and a mean protein level of 171 mg/dl (range 45-602 mg/dl, 95% CI). All patients except one had an elevated CSF protein level (>40.0 mg/dl), and all patients had normal cell counts (<15 cell/µl) (Table 1).

Preceding *Campylobacter* infection

Fifty-five GBS patients (57%) fulfilled the predefined serological criteria for a recent *C. jejuni* infection (Table 2). This proportion was higher than the frequency of positive serology in the two control populations: 3% for the OND (P<0.001) and 8% in the FC (P<0.001). Diarrhea in the four weeks preceding GBS was reported in 28 (51%) of the 55 *C. jejuni*-positive patients, which was more frequent compared to the 8 (19%) of 42 *C. jejuni*-negative patients (P<0.01) (Table 3). The association between GBS in Bangladesh and recent *C. jejuni* infections is further supported by the culture of *C. jejuni* in ten GBS patients. Serum anti-ganglioside antibodies (to GM1, and GD1a) were more frequent in GBS patients compared to controls (56 % versus 6% of FC and 1 % of OND, P<0.001) (Table 2). In GBS patients antibodies to GM1 were more frequent (50%) than to GD1a (14%). Anti-ganglioside antibodies were IgG (49%) or IgM (31%) or of both subclasses (20%). Serological evidence for a recent *C. jejuni* infection was more often found in the GBS patients with anti-ganglioside antibodies (78%) as compared to patients without anti-gangliosides antibodies (26%) (P<0.001) (Table 3). There was no association between *C. jejuni* serology and sex, age, degree of overall disability of arms, facial, and bulbar weakness at the peak of the illness.

Electrophysiological classification

Standard electrophysiological examination and classification was performed in 64 of 100 patients with GBS following previously established criteria (23). A majority of 67% of patients had an axonal variant of GBS (56% AMAN and 11% AMSAN), while 22% had AIDP and 11% could not be classified (Table 1). AMAN appeared to occur more frequently in January to March. Preceding diarrhea was more frequent in axonal GBS patients (50%) versus demyelinating cases (28%). AMAN and AMSAN occurred more frequently in the C. jejuni-positive group (81%) than in the C. jejuni-negative group (43%) (P<0.001) (Table 3).

Table 2: *C. jejuni* and anti-ganglioside serology in patients with GBS compared to matched OND and FC[a]

		GBS (N=97)	OND (N=97)	Odds ratio (95% CI)[b]	*p*-value[c]	FC (N=97)	Odds ratio (95% CI)[d]	*p*-value[e]
***C. jejuni* infection**								
Positive serology	IgM	16 (16%)	0 (0)	-	0.002	1 (1%)	19.0 (2.6-391.7)	<0.001
	IgA	25 (25%)	1 (1%)	33.3 (4.6-677)	0.001	2 (2%)	16.5 (3.6-104.2)	<0.001
	IgG	44 (44%)	2 (2%)	39.4 (8.8-245.2)	<0.001	5 (6%)	15.3 (5.3-46.9)	<0.001
	IgM/IgA/Ig	55 (57%)	3 (3%)	41.0 (11.4-174.7)	<0.001	8 (8%)	14.6 (6.0-36.5)	<0.001
Anti-GM1/GD1a antibodies								
	IgM	30 (30%)	1 (%)	72.0 (9.9-1467.0)	0.002	4 (4 %)	10.4 (3.3-36.7)	<0.001
	IgG	48 (49%)	0 (%)	-	<0.001	3 (3%)	30.7 (8.6-130.0)	<0.001
	IgM/IgG	54 (56%)	1 (1%)	120.0 (17.0-2420.0)	<0.001	6 (6%)	19.0 (7.1-53.6)	<0.001

Abbreviations: CI = confidence interval; FC = family controls; GBS = Guillain-Barré syndrome; Ig=immunoglobulin; OND= other neurologic diseases;— = cannot be calculated.

[a]Ninety-seven serum samples were available from each group for serology.

[b]Patients with GBS compared with patients with OND.

[c]Determined by McNemar test.

[d]Patients with GBS compared with FC.

[e] Determined by Chi-square or Fisher exact test.

Table 3: Clinical characteristics of GBS patients related to preceding *C. jejuni* infection[a]

Characteristics	*C. jejuni* positive (n=55)	Negative (n=42)	Odds ratio (95% CI)	P Value[b]
Age (median)	18 (2-65)	23 (5-55)	-	0.375[c]
Sex (M/F)	40/15	30/12	1.07 (0.4-2.86)	0.88
Preceding diarrhea	28 (51%)	8 (19%)	4.4 (1.6-12.6)	<0.01
Ventilation	15 (27%)	8 (19%)	1.6 (0.5-4.7)	0.34
Sensory deficit at entry	2 (4%)	6 (14%)	0.2 (0.03-1.35)	0.06
Cranial nerve impairment	14 (25%)	16 (38%)	0.55 (0.2-1.4)	0.18
Anti-GM1/GD1a antibodies	43 (78%)	11 (26%)	10.1 (3.6-29.1)	<0.001
Electrophysiology (n=64)				
Axonal[d]	34/42 (81%)	9/22 (43%)	6.1 (1.7-23.0)	<0.001
AIDP	7/42 (17%)	7/22 (33%)	0.4 (0.1-1.7)	0.20
Unclassified	1/42 (2%)	6/22 (24%)	0.07(0.0-0.63)	<0.01

Abbreviations: AIDP = acute inflammatory demyelinating polyneuropathy; CI = confidence interval;— = cannot be calculated.
[a]Data are expressed no. (%) of patients unless otherwise indicated.
[b]Determined by Chi-square or Fisher exact test.
[c]Determined by Wilcoxon-Mann-Whitney *U* test.
[d]Axonal variants included acute motor axonal neuropathy and acute motor sensory axonal neuropathy.

Outcome and prognostic factors

Only 23 (23%) of the 100 GBS patients received a specific treatment with intravenous immunoglobulin (IVIg). The outcome analysis was performed in 79 patients as 21 patients were lost to follow-up. Poor outcome, defined as not reaching a GBS disability score of 2 within six months after disease onset, was found in 34 (43%). The patients with poor outcome reached a GBS disability score at six months of 3 in 16 (20%), of 4 in 7 (9%) and 11 (14%) died. Eight of these 11 deceased patients were initially admitted to the intensive care unit due to respiratory failure. The outcome analysis showed that there was no difference in the IVIg versus the non-treated group (Table 2). Poor outcome was significantly associated with the GBS disability score at entry, presence of preceding diarrhea, positive serology for recent *C. jejuni* infection and anti-GM1/GD1a antibodies (Table 4).

Discussion

To obtain a detailed description of the clinical and electrophysiological phenotype of GBS in relation to preceding infections we performed a prospective case-control hospital surveillance in the Dhaka area of Bangladesh. The majority of the patients were men, less than 30 years old, and suffering from a severe, pure motor and axonal variant of GBS for which they did not receive any specific treatment. After six months of follow-up, 43% of the patients were either unable to walk or had died. We found a strong association with preceding *C. jejuni* infection, in particular in those cases diagnosed with AMAN. Our study indicates that *C. jejuni* frequently induces a severe form of axonal

GBS in young adults in Bangladesh. The demography and phenotype of GBS varies considerably between different geographical areas.

In Australia, Europe and the USA, GBS occurs slightly more frequent in men. In addition, the median age in these areas is approximately 50 years, and the incidence shows no seasonal fluctuation (28-30). The predominant phenotype in those countries is a sensory-motor form of AIDP present in at least 80% of patients (28). In contrast, patients from China usually are young men from rural areas (31). In China, the disease has a seasonal distribution with peaks in the summer months. According to these studies, the predominant variant is AMAN (32). Large studies in northern China, Japan, Pakistan, and Central and South America show that axonal forms of the syndrome constitute 30-47% of cases (9, 14, 32-34). In our study, the majority of the GBS cases are pure motor variants (92%) with predominant axonal degeneration (67%). In Bangladesh, the incidence of GBS appears to peak between January and March, especially in the AMAN subgroup. The geographical differences in demography and phenotype may relate to differences in exposure to specific infectious agents and/or to host-related or environmental factors.

There are various arguments in support of the hypothesis that *C. jejuni* plays a major role in the pathogenesis of GBS in Bangladesh. First, diarrhea was the most frequent symptom of preceding infection. Second, 57% of GBS patients had serological evidence for a recent *C. jejuni* infection using an assay that has been reported to be very sensitive and specific in GBS patients (26). Third, *C. jejuni* was isolated in stool specimens of 10% of GBS patients as compared to none in the control groups. Fourth, *C. jejuni* related patients usually had serum anti-gangliosides antibodies, reflecting the well known cross-reactive immune response by which *C. jejuni* infections may lead to peripheral nerve damage (35). Fifth, the high association with *Campylobacter* may also explain the high frequency of pure motor and axonal variants of GBS. These data clearly indicate that more than half of GBS patients in Bangladesh are triggered by a *C. jejuni* infection. Studies from the Netherlands, UK, Mexico and Japan report preceding *C. jejuni* infections in 26% to 41% of the GBS patients (3, 4, 6, 9). Infections with *C. jejuni* were reported to be frequent in Chinese GSB patients, although if stringent criteria for seropositivity, comparable to those described in our study, were used, only 24% of GBS cases were related to *Campylobacter* (5). A recent study from Egypt also reported higher levels of antibody production against *Campylobacter* and gangliosides in GBS patients (36) but these investigators did not use a validated cut off level of positivity. Difference in diagnostic assays used in these various studies hampers the comparison of the frequency of *C. jejuni* related patients in these countries.

The observed mean age of 21 years preponderance of men in our study is of particular interest. In larger studies from the UK, and The Netherlands, the mean age is around 50 years of age and men are slightly more frequently involved then women (4, 28). Our data confirm the outcome of a retrospective analysis of 175 GBS patients from Pakistan. The mean age in that study was 23 year and 68% of the patients were men (34). This retrospective study from Pakistan and our prospective surveillance in Bangladesh are both hospital-based studies, and selection bias could possibly have been introduced as preferential selection of men in hospitalization is not an unknown phenomenon in developing countries.

After a follow-up of six months a very large proportion of the patients in our cohort had either died (14%) or remained severely disabled (29%). This suggests that GBS has a much poorer prognosis in Bangladesh as compared to developed countries, where this proportion is usually less than 20% and where young age is usually associated with a good prognosis (15). The poor prognosis may be explained in part by the high frequency of *Campylobacter* diarrhea and the disease severity at entry, two known independent predictors for a poor prognosis (4, 15) and by the high frequency of patients that did not receive specific treatment. However, 23 (23%) of 100 GBS patients received a specific treatment with intravenous immunoglobulin (IVIg). With respect to outcome at six months, there was no difference in the IVIg versus the non-treated group, and IVIg treatment was also not a prognostic factor (15). It is important to notice that, as this study was not designed to compare treatment outcome significant bias due to variable access to treatment may have occurred. Also, the analysis of treatment outcome lacked sufficient power. In our cohort of patients we confirmed that *C. jejuni* infections and disease severity both are associated with poor outcome. Nutrition status and poor health are other factors that might interfere with outcome.

Table 4: Factors related to a poor outcome after 6 months (not reaching disability score of 2 at six months)

Factors	Number of patients	Number (%) of patients with poor outcome	Odds ratio (95% CI)	P-Value
Total	79	34 (43%)		
Age (years)				
≤ 15	25	8 (32%)	1.0	-
16-30	32	14 (44%)	1.6 (0.5-5.7)	0.52
> 30	22	12 (55%)	2.5 (0.7-10.0)	0.20
Preceding symptoms				
Non-diarrhea	26	7 (27%)	1.0	
Diarrhea	30	18 (60%)	4.0 (1.1-15.0)	0.02
GBS disability score at entry				
3-4	58	18 (31%)	1.0	
5	21	16 (76%)	7.1 (2.0-26.6)	<0.001
Serology				
C. jejuni negative	32	7 (21%)	1.0	
C. jejuni positive	47	24 (51%)	3.7 (1.2-11.7)	0.01
Anti-GM1/GD1a negative	34	8 (23%)	1.0	
Anti-GM1/GD1a positive	45	23 (51%)	3.4 (1.2-10.3)	0.02
Treatment				
IVIg	23	8 (34%)	1.0	
Non-treated	56	26 (46%)	1.6 (0.5-5.0)	0.48

Our data strongly indicate that infections with *C. jejuni* are the most important trigger for AMAN variants of GBS in Bangladesh and are associated with significant morbidity and mortality. Population-based studies are needed to determine the incidence of GBS. *C. jejuni* related GBS appear to be a dominant cause of AFP after the eradication of poliomyelitis in Bangladesh. The identification of associated host and microbial risk factors that contribute to the high endemicity of GBS in Bangladesh is imperative. Further studies should also allow the impact of the disease and disease burden to be estimated.

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Chapter 3

ANTECEDENT INFECTIONS IN GUILLAIN-BARRÉ SYNDROME IN BANGLADESH: A CASE CONTROL STUDY

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Abstract

***Background*:** Guillain-Barré syndrome (GBS) is an acute polyneuropathy and immune-mediated flaccid paralysis usually preceded by an infection. *Campylobacter jejuni* has recently been identified as a major cause of antecedent infection in GBS patients in Bangladesh. We here present antecedent infections including *C. jejuni,* cytomegalovirus (CMV), Epstein-Barr virus (EBV), *Mycoplasma pneumoniae*, and enterovirus of GBS patients compared with controls.

Methodology/Principle Findings: We performed a prospective matched case-control study including 100 patients fulfilling the National Institute of Neurological Disorders and Stroke (NINDS) criteria for GBS. Enzyme-linked immunosorbent assay (ELISA) was used for detecting antibodies against above mentioned infectious agents. Recent infections with CMV, EBV, and *M. pneumoniae* were demonstrated in GBS patients, but comparison with controls did not revealed statistical significance. The seroprevalence of Coxsackie B virus (CvB) (36%) was significantly associated with GBS ($p<0.01$). *C. jejuni* (57%) was the most common causes of recent infection of GBS patients in Bangladesh. In this study, 64% of CvB-associated GBS patients have a positive serology for *C. jejuni* infection.

Conclusion/Significance: No significant association with CMV, EBV, and *M. pneumonia* was observed among GBS patients**.** We conclude that antecedent *Campylobacter* infections play a prominent role for the pathogenesis of GBS patients in Bangladesh. Our findings on CvB-associated GBS must be confirmed in prospective large case-control studies using sensitive and specific serological assays and new molecular detection tools.

Introduction

Guillain–Barre´ syndrome (GBS) is the most common form of acute neuromuscular paralysis in countries where poliomyelitis has been eradicated (1). GBS represents patients with a wide clinical spectrum (2, 3); including variations in the clinical pattern and differences in immunologic, electrophysiological and pathologic findings (4, 5). Two-thirds of patients with GBS report symptoms of a preceding gastrointestinal or respiratory infectious illness (6, 7, 8). A wide variety of infectious agents have been reported as possible triggering factors in patients with GBS. *Campylobacter jejuni* has been identified as the predominant type of antecedent infections (1, 9). *C. jejuni* infections are associated with a severe (10, 11), pure motor, axonal variant of GBS and with poor outcome (12, 13). Infection with *C. jejuni* induces the production of cross-reactive antibodies to nerve gangliosides which triggers nerve damage (14). Other infections associated with GBS were cytomegalovirus (CMV), Epstein-Barr virus (EBV), and *Mycoplasma pneumonia* (7, 12, 15-19). A recent prospective multicentre study in children with GBS demonstrated that coxsackie viruses were associated as antecedent infection (20).

We performed a prospective matched case-control study on patients with GBS admitted at the tertiary medical college hospital in Dhaka area of Bangladesh between 2006 and 2007. Recently, we reported that *C. jejuni* was the predominant cause of GBS in Bangladesh compared with controls (21). However, we do not know the status of other infectious agents whether responsible for the development of GBS. The aim of the current study was to determine the spectrum of antecedent infections including *C. jejuni,* CMV, EBV, *M. pneumoniae*, and enterovirus in a large group of GBS patients and to investigate whether these infections are more common in GBS than in a matched control group.

Materials and Methods

Patients and methods

We prospectively included all GBS cases admitted in Dhaka Medical College Hospital (DMCH), Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Central Hospital (DCH) in Dhaka between July 2006 and June 2007. All patients fulfilled NINCDS diagnosis criteria for GBS (2). Data were collected on age, sex, antecedent events, detailed neurological signs and symptoms, treatment, days to nadir, complications, duration of admission, GBS disability score and the Medical Research Council (MRC) sum score at standard points (entry, and 2 weeks, 4 weeks, and 6 months later). Both the GBS disability score and MRC sum score give an indication of the severity of disease. The MRC sum score was defined the sum of MRC score of six muscles in the upper and lower limbs on both sides, ranging from 60 (normal) to 0 (quadriplegic) (13). The rapidity of progression was indicated by the number of days from onset of weakness to nadir, defined as the lowest MRC sum score. Pre-treatment serum samples obtained within 2 weeks of neurological onset were available from 97 GBS patients.

Controls: Two controls were selected for each patient: control one was a family member living in the same household (family control, FC); control two was an age and sex matched patient hospitalized in the same ward with an other neurological disease (other neurological control, OND). Blood and up to three stool samples were collected from 194 controls. All studies were approved by appropriate institutional review boards and all patients gave informed consent.

Infection serology

Serum samples from GBS patients were tested to determine recent infections with viruses or bacteria frequently reported in GBS. Serum antibodies against *C. jejuni* were determined by an indirect enzyme-linked immunosorbent assay (ELISA) for IgG and by antibody class capture ELISA for IgM and IgA antibodies and criteria for positivity previously described (22). EBV early antigen and heterophile antibodies were qualitatively detected with the Plexus™ Multi-Analyte Diagnostic serology test kits (Focus Diagnostics). CMV-IgG were qualitatively determined by Microparticle Enzyme Immunoassay, MEIA, Abbott Diagnostics, USA. IgG were quantitatively detected with SERION ELISA classic Enterovirus (EV) IgG and SERION ELISA classic *M. pneumoniae* IgG (Virion Serion), respectively.

Anti-ganglioside antibodies

Serum samples from all GBS patients were tested for IgM and IgG antibodies against GM1, GD1a, and GQ1b by ELISA according to methods and criteria for positivity previously described (23).

Result

Infectious serology

Fifty-five GBS patients (57%) had a recent *C. jejuni* infection. This proportion was significantly higher than the frequency of positive serology in the two control populations: 3% for the OND ($p<0.001$) and 8% in the FC ($p<0.001$). Seroprevalences of EBV VCA IgG and IgM, and EBV-EA IgG were not increased in GBS patients compared with controls. In contrast, the prevalence of EB-NA antibodies was significantly higher in GBS patients ($P<0.05$) (Fig. 1). However EB-NA IgG titers were similar for GBS and controls. No patients or controls had detectable heterophile antibodies. CMV-IgG seroprevalence was nearly 100% in GBS patients and 100% in controls. CMV-IgG titers were significantly higher in GBS patients compared to OND and family controls (CO ratio 4.74 vs 4.35 and 4.13 respectively; $P<0.005$). Enterovirus IgG prevalence was not increased in GBS patients compared to controls. However, EV IgG titers were significantly increased in GBS patients compared to OND and family controls (161.7 vs 139.4 and 118.9 U/ml; $P<0,05$ and $P<0,01$). Furthermore, echovirus IgG levels were significantly increased in GBS patients compared to family controls (168.4 vs 126.6; $P<0.05$) but not compared to OND controls. Seroprevalence Coxsackie B virus (CvB) was significantly increased in GBS patients compared to family controls (36.1 vs 19.0%, OR 2,5, $P<0,01$). In addition, antibody levels of CvB were higher in GBS vs controls (c/o ratios 181.3 vs 110.4; $P<0.01$). However,

seroprevalence for *M. pneumoniae* was not increased in GBS patients. The IgG titers of *M. pneumoniae* were significantly higher in GBS patients compared to OND controls (54.5 U/ml vs 36.9 U/ml (P<0.005), but not compared to family controls.

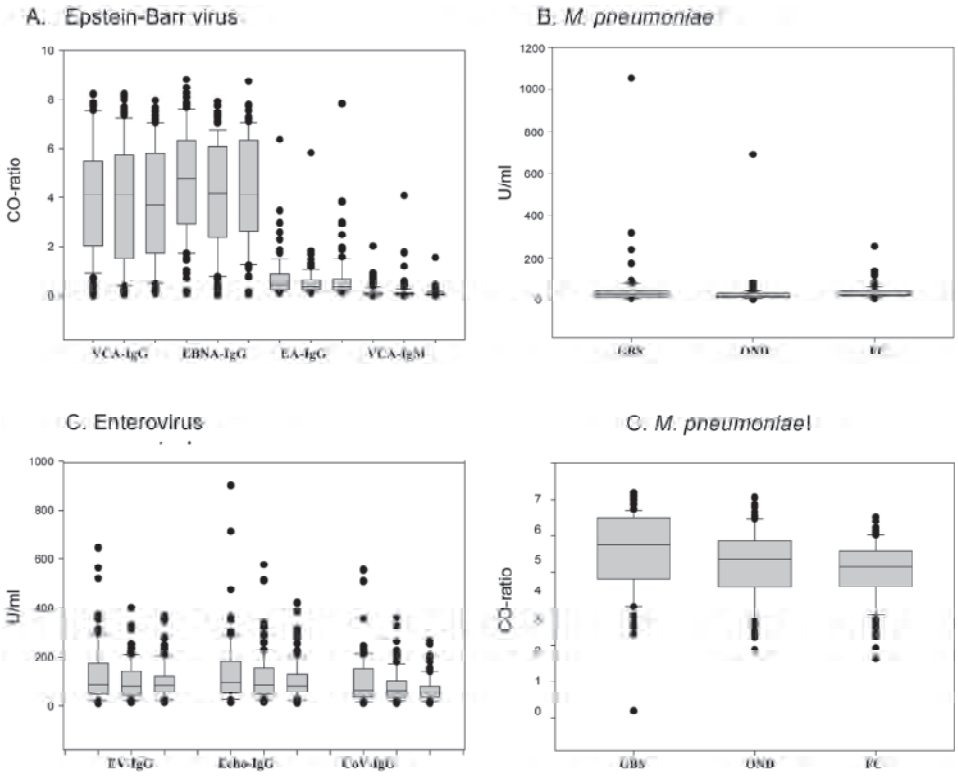

Figure 1. Serum samples from patients with GBS and from family controls and other neurological disease controls was tested to define the frequency of recent infections.

Anti-ganglioside antibodies

Serum anti-ganglioside antibodies (to GM1, GD1a and GQ1b) were more frequent in GBS patients compared with controls (56 % versus 6% of FC and 1 % of OND, p< 0.001). Anti-ganglioside antibodies were IgG (49%) or IgM (31%) or of both subclasses (20%). Serological evidence for a recent *C. jejuni* infection was more often found in the GBS patients with anti-ganglioside antibodies (80%) as compared to patients without anti-gangliosides antibodies (26%) (p<0.001) (Fig 2).

Discussion

To reveal the aetiology of the antecedent infections in patients with GBS in Bangladesh, we performed a prospective case control study, and tested predominant infectious agents using standard microbiological tests and diagnostic criteria. In the present study, *C. jejuni* and CvB were identified as the most common causes of antecedent infections in GBS in Bangladesh. These infections were more frequently found in GBS patients than family controls and the age- and sex- matched controls with OND. Infections with CMV, EBV, and *M. pneumoniae* were also identified, but their frequency was not significantly higher than in controls (Table).

Our finding is in accordance with earlier case-control studies demonstrated that *C. jejuni* infections in 14% to 66% of GBS patients, and 1% to 24% of controls (7, 12, 17, 24-26). This present study strongly support the previous data that *C. jejuni* is the predominant cause of antecedent infection in GBS. However, *C. jejuni* is also one of the predominant causes of acute bacterial enteritis in Bangladesh (27). Early eighties, Blaser et al. demonstrated that *C. jejuni* is more common in healthy individual among Bangladeshi population (28). Therefore, one may raise question that GBS is a post-infectious disease not related to specific infections, in which the predominance of *C. jejuni* simply reflects the high frequency of this infection in the community. However, *C. jejuni* infections in GBS patients are related to distinct severe and pure motor variants of GBS (12, 11, 29). The high incidence of *C. jejuni* preceding GBS has given rise to a number of studies on the pathophysiological link between the infection and neurological disease. These have demonstrated a close association between *C. jejuni* aetiology, the axonal variants of GBS and elevated antibody titres against different ganglioisdes (11, 12, 30, 31). Common epitopes have been demonstrated between *C. jejuni* antigens and a number of gangliosides, predominantly GM1, thereby supporting the hypothesis of molecular mimicry (11, 31). Recently, we reported that *C. jejuni* infection was significantly associated with serum antibodies to the gangliosides GM1 and GD1a, axonal neuropathy, and greater disability (24).

Of interest, the results for CvB revealed that both seroprevalence and IgG titers against CvB were significantly increased in GBS patients compared to controls. Our results therefore provide evidence for an association between GBS and acute CvB infection. More detailed analysis showed an increased CvB sero-prevalence mainly in the *Campylobacter*-positive GBS subgroup. This would either indicate that co-infection with CvB and *Campylobacter* in GBS patients or, alternatively, immunologic cross reactivity or cross reactivation could play a role. Positive serology for more than one infectious agent was not uncommon, as was also found in other serological studies (17, 32). This may indicate that dual-antigen induced immune responses play a role in a subgroup of GBS patients, as was suggested for other immune mediated neuropathies (17, 33). Our findings on CvB-associated GBS must be confirmed in prospective large case-control studies using sensitive and specific serological assays and new molecular detection tools. Our data are in accordance with a recent prospective multicentre study in children with GBS demonstrated that CvB are associated as antecedent infection (23).

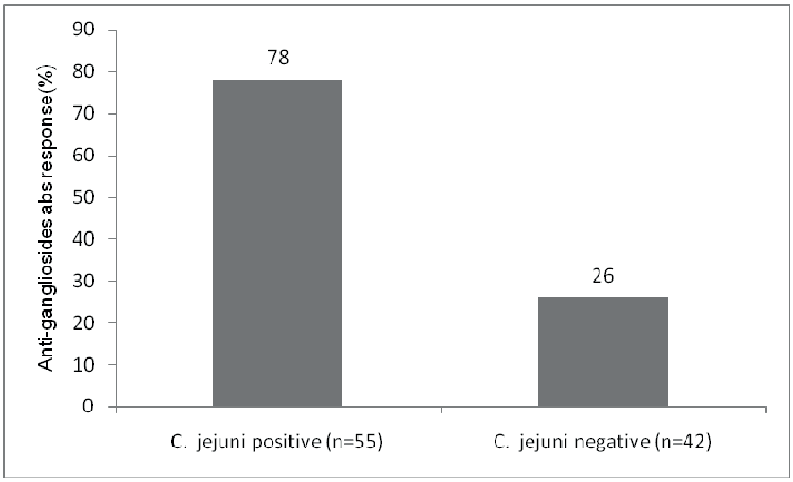

Figure 2. Anti-ganglioside antibodies response among *C. jejuni* related Guillain-Barré syndrome in Bangladesh.

Many case control studies demonstrated that CMV, EBV and *M. pneumonia* specifically associated with GBS (7, 17, 32). In this study, no associations in prevalence or titers of EBV antibodies were found. Moreover EB-NA titers were not increased in GBS patients. Therefore, our results argue against a role for EBV in the pathogenesis of GBS. The finding of a slightly enhanced seroprevalence of EB-NA IgG antibodies in GBS patients in fact indicates latent EBV, therefore has no relevance in the context of GBS pathogenesis. The very high seroprevalence for CMV in GBS patients and in the population makes a role of primary CMV infection in the pathogenesis of GBS patients unlikely. However, IgG titers were significantly increased in GBS patients which could indicate reactivation of CMV or up-regulation of anti CMV IgG by some immunologic reaction. The results for *M. pneumonia* showed a slight but significant increase of IgG titers in OND but not in family controls. Furthermore, seroprevalence was not increased in GBS patients compared with controls. Our data therefore, could not confirm an association of *M. pneumonia* infection and GBS.

Based on our prospective case control study, we conclude that antecedent *Campylobacter* infections play a prominent role in GBS in Bangladesh, but that in addition, several other agents may be involved may play an important role as well. Our data strongly indicate that infections with *C. jejuni* are the most important trigger for axonal variants of GBS in Bangladesh and are associated with significant morbidity and mortality. *C. jejuni* related GBS appears to be a dominant cause of acute flaccid paralysis after the eradication of poliomyelitis in Bangladesh.

Table. Incidence of recent infections in GBS patients compared to matched OND controls and to family controls[a]

Infection serology	GBS patients (n=97)	OND controls (n=97)	Odds ratio (95% CI)[b]	p-value[c]	Family controls (n=97)	Odds ratio (95% CI)[d]	p-value[e]
C. jejuni	55	3	41.0 (11.4-174.7)	<0.001	8	19.0 (2.6-391.7)	<0.001
Cytomegalovirus	97	97	-		97	-	
Epstein-Barr virus	88	80	2.1 (0.8-5.4)	0.140	82	1.8 (0.7-4.7)	0.27
M. pneumoniae	37	26	1.7 (0.9-3.2)		29	1.4(0.8-1.9)	0.288
Coxsackie B virus	36	25	1.70 (0.88-.3)		18	2.6 (1.3-5.3)	<0.01

a. Abbreviations: CI = confidence interval; FC = family controls; GBS = Guillain-Barré syndrome; Ig=immunoglobulin; OND=other neurologic diseases; — = cannot be calculated.
b. GBS patients compared to OND patients.
c. Determined by McNemars's test
d. GBS patients compared to FC.
e. Determined by Chi-square or Fisher's test.

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Chapter 4

MOLECULAR MIMICRY OF AND CROSS-REACTIVE ANTIBODIES AGAINST *CAMPYLOBACTER JEJUNI* LIPO-OLIGOSACCHARIDES IN PATIENTS WITH GUILLAIN-BARRÉ SYNDROME IN BANGLADESH

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Abstract

Background. Molecular mimicry between lipo-oligosaccharides (LOS) of *Campylobacter jejuni* and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of *C. jejuni*-related Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS).

Methods: We determined the molecular mimicry and the role of *C. jejuni* infections in GBS in Bangladesh, with focus on the role of cross-reactive antibodies to *C. jejuni* LOS and gangliosides. We also determined the LOS outer core structures of *C. jejuni* strains associated with GBS/MFS by capillary electrophoresis coupled with electrospray ionization mass spectrometry.

Results: Anti-LOS *C. jejuni* BD-07, BD-39, BD-10, and BD-67 IgG antibodies were found in serum from 56%, 58%, 14% and 15% of GBS patients respectively, as compared to a very low frequency (<3%) in controls (p<0.001). Monoclonal antibodies specific for GM1, GM1/GD1b/GA1 and GD1a reacted strongly with LOS BD-07 and LOS BD-39. Mass spectrometry analysis are in agreement with the fact that both BD-07 and BD-39 expressed GM1 and GD1a ganglioside mimic. Both BD-10 and BD-67 express the same LOS outer core, which appears to be a novel structure of GA2 and GD3. Up to 90-100% of serum reactivity to gangliosides in two patients (DK-07 and DK-39) was inhibited by 50 µg/ml of LOS from the autologous *C. jejuni* isolates. However, patient DK-07 developed an anti-GD1a immune response while patient DK-39 developed an anti-GM1 immune response.

Conclusion: This is the first report to demonstrate that cross-reactive antibody responses to *C. jejuni* LOS and gangliosides precipitate the majority of GBS cases in Bangladesh. Our results strongly support the hypothesis that anti-GM1 antibodies in GBS patients are induced during antecedent infections with GM1-mimicking *C. jejuni* strains. We conclude that molecular mimicry between gangliosides and *C. jejuni* LOS is the pathogenic mechanism involved in the majority of cases of *C. jejuni*-related GBS in Bangladesh.

Introduction

Campylobacter jejuni is recognized as the most common pathogen associated with Guillain–Barré syndrome (GBS) and Miller Fisher syndrome (MFS), an acute autoimmune-mediated disease affecting the peripheral nerve system (1-3). The pathogenesis of *Campylobacter*-induced GBS is complex and involves bacterial virulence factors as well as host susceptibility factors (4-7). Certain *C. jejuni* serotypes possess sialylated LOS structures, confined to the outer core of the LOS molecules, which mimic a wide range of peripheral nerve gangliosides including GM1, GA1 (asialo-GM1), GM1b, GalNAc-GM1b, GM2, GD1a, GalNAc-GD1a, GT1a, GQ1b and GD3 (4, 6, 8-16). This molecular mimicry in *C. jejuni* is hypothesized to elicit an immune response in susceptible hosts that results in demyelination or axonal damage of peripheral nerves, partly by the anti-ganglioside antibody response (17).

The exact pathogenesis of post-*Campylobacter* neuropathy is not clearly understood, but molecular mimicry between bacterial glycoconjugates and peripheral nerve gangliosides has been implicated (13, 18). Cross-reactive antibodies between *C. jejuni* lipopolysaccharides (LPS) and gangliosides have been identified in GBS and MFS patients (19-21). The specificity of this cross-reactive antiganglioside-LPS response is associated with different disease profiles. Antibody reactivity against GM1, GM1b, and GalNAc-GD1a is associated with pure motor GBS (22), and anti-GQ1b antibody reactivity has a strong association with oculomotor symptoms and ataxia (23). Mass spectrometry showed that the oligosaccharide core of the lipo-oligosaccharide (LOS) molecules expressed by *C. jejuni* can structurally resemble the oligosaccharide core of certain molecules present in neural tissue (24). Neuropathic-associated *C. jejuni* strains more frequently express ganglioside-mimics compared to *C. jejuni* associated with uncomplicated enteritis (25). Many studies have been carried out in the developed world to establish the pathogenesis of *Campylobacter*-induced GBS but little information is available concerning the expression of ganglioside mimics in neuropathy-associated strains from developing countries such as Bangladesh.

Recently, we reported an unusually high frequency of acute motor axonal neuropathy (AMAN) variant of GBS in Bangladesh, associated with preceding *Campylobacter* infections and the presence of serum antibodies against GD1a and GM1 (26). The aim of the present study is to investigate the pathogenic mechanism of the endemic GBS in Bangladesh. We hypothesize that *C. jejuni* infection causes GBS in these patients by inducing cross-reactive antibodies to nerve gangliosides by expressing gangliosides-like LOS. In order to evaluate the role of molecular mimicry as the pathogenic mechanism of the GBS endemic in Bangladesh, we screened *C. jejuni* LOS for ganglioside-mimics and sera of GBS patients were screened for cross-reactive antibodies against gangliosides and *C. jejuni* LOS. Furthermore, we determined the LOS outer core structure of 4 new GBS- and MFS-associated *C. jejuni* strains isolated in Bangladesh.

Materials and Methods

Patient and control population

Pre-treatment blood samples were obtained from all consecutive GBS patients admitted to Dhaka Medical College Hospital (DMCH), Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Central Hospital (DCH) between July 2006 and June 2007. All patients fulfilled the diagnostic criteria for GBS (27), as evaluated by a neurologist and a senior neurologist (28). Data were collected on age, sex, antecedent events, detailed neurological signs and symptoms, treatment, days to nadir, complications, duration of admission, GBS disability score and the Medical Research Council (MRC) sum score at standard points (entry, and 2 weeks, 4 weeks, and 6 months later). Two controls were selected for each patient: control one was a family member living in the same household (family control, FC); control two was an age and sex matched patient hospitalized in the same ward with an other neurological disease (other neurological control, OND). Blood and up to three stool samples were collected from all patients and controls. All studies were approved by appropriate institutional review boards and all patients gave informed consent.

Anti-ganglioside antibodies

The first blood sample obtained upon hospitalization was used in this analysis. Sera were tested for IgM and IgG antibodies against the gangliosides GM1, GD1a and GQ1b by enzyme-linked immunosorbent assay (ELISA) according to methods and criteria for positivity previously described (29).

C. jejuni and lipo-oligosaccharides

We isolated *C. jejuni* from the stools of 10 patients with GBS/or MFS (30). Then *C. jejuni* isolates from the stools of five patients of the above mentioned GBS cohort were selected for studies on molecular mimicry and cross-reactivity. The clinical features and laboratory findings of these patients are given in Table 1. The *C. jejuni* isolates, BD-07, BD-10, BD-39, BD-67 and BD-94 (30), were classified according to the heat-stable (HS) seroytyping system described by Penner (31). The LOS fraction from all *C. jejuni* strains were isolated by hot phenol-water extraction and processed as described before (21, 32).

Antibody reactivity to *C. jejuni* LOS

Sera from 97 GBS patients, including the 5 patients from whom *C. jejuni* was isolated, were tested for IgG activity against LOS from *C. jejuni* (BD-07, BD-10, BD-39, BD-67 and BD-94) in ELISA. LOS from the *C. jejuni* serostrain (HS:3), lacking gangliosides mimicry, was included for control studies. Serum samples from 60 family control (FC) and 60 other neurological disease controls (OND) were used as controls.

A 96 wells plate (Nunc Maxisorb) was coated with 1 µg/well LOS diluted in 50 µl PBS (pH=7.8). After incubation overnight, PBS containing 1% BSA (Sigma) was added to all wells to block

non-specific binding sites. The wells with PBS-1% BSA were incubated for 2 hours at room temperature and for another 2 hours at 4ºC. The pre-treatment sera were diluted 1:1000 for IgG in PBS-1%BSA (pH=7.8) for antibody determination. Each serum was tested in duplicate in LOS-coated wells and non-coated wells. The plates were incubated at 4ºC overnight. After the wells were washed with PBS (pH=7.8), 100 µl peroxidase-conjugated rabbit anti-human IgG antibody (Sanbio) diluted 1:2500 in PBS-1% BSA was added to the wells. After the plates were incubated for 90 minutes at room temperature and subsequently washed, 100 µl citrate buffer (pH=5.1) containing the substrate ortho-phenyl diamine (Sigma) was added to the wells for 10 minutes at room temperature. The extinctions of the wells were measured in an ELISA-reader at 492 nm.

The corrected optical densities (OD) were calculated by subtracting the extinctions of non-coated wells from the extinctions of LOS-coated wells. A serum was considered positive for anti-LOS reactivity when the corrected OD was higher than the cut-off value. The cut-off value was determined by the mean corrected OD of all controls plus 3 times the standard deviation. A cut-off value for serum IgG activity to LOS was determined for each *C. jejuni*. Serology was performed without knowledge of the clinical data. We also determined the cut-off value for IgM and IgA to LOS BD-07 and BD-39.

Determination of cross-reactivity

Cross-reactivity of anti-ganglioside antibodies to *C. jejuni* LOS was determined by pre-incubation of serum with LOS from the *C. jejuni* isolated from the autologous patients and with LOS from the *C. jejuni* HS:3 serostrain as a control, according to methods previously described (33, 34). To detect cross-reactive antibodies, serum DK-07 (diluted 1:100) and DK-39 (diluted 1:200) were pre-incubated with LOS from their autologous *C. jejuni* isolate and, serostrain HS:03. *C. jejuni* LOS concentrations of 200, 50, 12.5, 3.1, 0.8 and 0.2 µg/ml were incubated with serum for 3 hours at 4ºC. After the incubation with LOS, the sera were centrifuged at 3000 rpm for 5 minutes at 4ºC. The supernatants were tested for residual anti-ganglioside IgG reactivity in a ganglioside ELISA. The percentage of inhibition was defined as:

$$\frac{\text{OD (serum without LOS)} - \text{OD (serum with LOS)}}{\text{OD (serum without LOS)}} \times 100\%$$

Mass spectrometry analysis

Samples were prepared for LOS mass spectrometric analysis by overnight growth of *C. jejuni* strains (BD-07. BD-10, BD-39, BD-67 and BD-94) at 37°C on Butzler agar plates in a microaerobic atmosphere. Material from one confluent agar plate was harvested and treated with proteinase K at 60 µg/ml, RNase A at 200 µg/ml, and DNase I at 100 µg/ml (Promega, Leiden, The Netherlands). O-deacylated LOS samples were prepared and analyzed by capillary electrophoresis coupled to electrospray ionization mass spectrometry (24).

Statistical analysis

Differences in median values were tested with the Mann-Whitney *U* test. Differences in proportions were tested with the chi square test or Fisher's exact test. Differences were considered

significant at P<0.05 after two-sided testing. Statistical analysis was performed using InStat version 4.0 (Graphpad Software, San Diego, CA).

Results

Antibodies to *C. jejuni* LOS

The IgG reactivity in serum from the patients with GBS, and the FC and OND control groups to *C. jejuni* LOS is shown in Fig. 1. Anti-LOS BD-07, BD-39, BD-10, and BD-67 IgG antibodies were found in 56%, 58%, 14% and 15% of GBS patients respectively, compared with very low frequency (<3%) of controls (p <0.001). Except two, anti-LOS BD-94 IgG antibodies were not found in GBS patients as well as controls. The IgG activity to LOS from the serostrain HS:3 in serum from the GBS patients did not differ from that in the two control groups. Anti-LOS BD-07 and anti-LOS BD-39 IgA antibodies were found in 50% and 48% of GBS patients, respectively, compared with low frequency (0% in OND and 4% in FC) in controls (p<0.001) (data not shown). Antibodies of the IgM isotype against LOS BD-07 were found in 22% of GBS patients, compared to 4% of OND controls (P=0.052) and 0% of family controls (P=0.012). Anti-LOS IgM BD-39 antibodies were found in 20% of GBS patients, in 4% of OND controls (p=0.088) and in 0% of family controls (P=0.025). The IgG activity to *C. jejuni* LOS was significantly higher in patients with a positive *C. jejuni* serology than in patients with a negative *C. jejuni* serology (P<0.005). In addition, diarrhea in the four weeks preceding GBS was reported in 26 (48%) of the 54 patients with IgG to *C. jejuni* LOS compared to 10 (23%) of 43 of the without anti-LOS patients (P=0.02).

Antibodies to *C. jejuni* LOS and gangliosides

Serum anti-ganglioside antibodies (to GM1, GD1a and GQ1b) were more frequent in GBS patients compared with controls (56% versus 6% of FC and 1 % of OND, P<0.001) (26). The GBS patients were divided into two subgroups according to their anti-ganglioside immune response: one group which was positive for anti-GM1 and/or anti-GD1a antibodies and another group which was negative for both anti-GM1 and anti-GD1a antibodies.

Anti-LOS reactivity was significantly associated with the presence of anti-ganglioside antibodies (p<0.001) (Fig. 2). Diarrhea in the four weeks preceding GBS was reported in 26 (48%) of the 54 ganglioside-positive patients, which was more frequent compared to the 10 (19%) of 43 of the ganglioside-negative patients (P<0.01) (Table 2). There was no association between anti-GM1/GD1a serology and sex, age, degree of overall disability of arms, facial, and bulbar weakness at the peak of the illness.

LOS characterization

Monoclonal mouse antibodies against different gangliosides were used to determine if ganglioside-mimics were present in the LOS. Monoclonal antibodies DG-1 (binding to GM1), DG-2 (binding to GM1/GD1b/GA1) and TBG-3 (binding to GD1a) reacted strongly with LOS BD-07 and LOS BD-39 as shown in Fig. 3. These serological data indicate that *C. jejuni* BD-07 and BD-39 both have a GM1 and/or GD1a mimicking LOS. Control studies with monoclonal antibodies EG-7 (binding to

GD1b), EG-3 (binding to GQ1b/GT1a) and EG-1 (binding to GQ1b) did not bind to the LOS, demonstrating that BD-07 and BD-39 have no GD1b, GQ1b and GQ1b/GT1a mimicry. The monoclonal antibodies bound to positive control *C. jejuni* LOS with known ganglioside-mimics (data not shown). None of the antibodies were bound by BD-10, BD-67, BD-94 and negative control *C. jejuni* LOS P:03.

Cross-reactive antibodies

Serum DK-07 and serum DK-39 showed reduced anti-ganglioside IgG antibody reactivity after pre-incubation with LOS from their autologous *C. jejuni* isolates (Fig. 4). Serum DK-07 was tested for residual anti-GD1a IgG antibodies, whereas serum DK-39 was tested for anti-GM1 IgG antibodies. The percentage inhibition of anti-ganglioside reactivity was dose-dependent with the LOS concentration. About 50% of inhibition was seen after pre-incubation with 3.1 µg/ml of LOS BD-07 and with 12.5 µg/ml of LOS BD-39. In control studies, LOS from *C. jejuni* serostrain HS:3 did not inhibit the ganglioside antibodies of serum DK-07 and DK-39, indicating that the cross-reactive anti-ganglioside antibodies show no specific binding to *C. jejuni* LOS (Fig.4).

Expression of ganglioside mimics in the LOS.

We used CE-ESI-MS on O-deacylated LOS to propose LOS outer core structures for five strains (Fig. 5 and supplemental Table 1). The CE-ESI-MS did not provide linkage information but provided information about the sugar composition of the LOS outer core. The glycosyltransferase variants present in the LOS locus of each strain (Table 3) and the comparison with strains of known LOS outer core structures were used to propose LOS outer core structures for four strains (BD-07, BD-10, BD-39 and BD-67). The CE-ESI-MS data were not sufficient to propose a structure in the case of BD-94. However, we can conclude that the LOS outer core of BD-94 does not show ganglioside mimicry because no major ion was observed in a precursor ion mass spectrum at *m/z* 290.2 (data not shown), confirming that there is no sialic acid present. The CE-ESI-MS data obtained with BD-07 and BD-39 showed mass species with either one or two sialic acids (supplemental Table 1) which are proposed to be derived from GM1 and GD1a mimicry (Fig. 5). Tandem mass spectrometry of the triple charged ion at *m/z* 1289.3 revealed a fragment ion at *m/z* 290.2 (NeuAc) and none at 581.3 (NeuAc-NeuAc). Since the composition of the triply charged ion at m/z 1289.3 contains two NeuAc, we conclude that these two residues are present on different Gal residues, which is consistent with GD1a mimicry. The glycosyltransferase variants in the LOS biosynthesis locus of BD-07 and BD-39 are consistent with GM1/GD1a mimicry (Table 3). They both contain a single domain glucosyltransferase (Cj1135) and an active β-1,3-galactosyltransferase (Cj1136) which suggests that the inner core will be extended with a Galβ-β1,3-linked residue on HepII. The Gal residue will be modified with a single NeuAc since the *Cst-II* variant in BD-07 and BD-39 is mono-functional (Thr51). The outer core is further extended with GalNAc and Gal residues by variants of *CgtA* and *CgtB*, respectively, which are specific for sialylated acceptors. The terminal Gal residue is partially substituted by the mono-functional *Cst-II*, which results in a mixture of GM1 and GD1a mimicry.

CE-ESI-MS on O-deacylated LOS from strains BD-10 and BD-67 resulted in spectra with similar mass species (supplemental Table1). These two strains also have LOS biosynthesis loci that are 100% identical to each other (GenBank accession numbers GQ249164 and GQ249165). We thus propose that they both express the same LOS outer core, which appears to be a novel structure with two extension sites from the inner core and two branches mimicking gangliosides GA2 and GD3 (Fig. 5). The presence of di-NeuAc on one of the branches is confirmed by the fragment ion at *m/z* 581.3 when tandem mass spectrometry was carried out on the triply charged ion at *m/z* 1302.3 (data not shown). The *Cst-II* variant in these two strains is bi-functional (Asn51) which further supports the presence of di-NeuAc. The absence of a terminal Galβ-1,3-linked residue is consistent with a *cgtB* gene that has a frame-shift mutation.

Table 1. Clinical and laboratory findings in five patients with Guillain-Barré syndrome from whom a *Campylobacter jejuni* was isolated used in our study[a]

Patients	BD-39	BD-07	BD-10	BD-67	BD-94
Clinical characteristics					
Age, years	30	40	15	40	10
Sex	F	M	M	M	F
Diarrhoea	+	+	+	+	-
Days to nadir	12	2	5	6	3
Ophthalmoplegia	-	-	+	+	-
Sensory sign	-	-	-	-	-
Motor involvement	+	+	+	+	+
GBS disability score					
At nadir	5	4	5	4	5
At 26 weeks	4	0	3	3	4
Serum IgG antibody					
GM1	+	-	-	+	-
GD1a	-	+	+	+	-
GQ1b	-	-	+	+	-
C. jejuni serology ELISA	+	+	+	+	+
C. jejuni Penner serotype	HS:19	HS:19	HS:23	HS:23	HS:21

a. Abbreviations: M, Male; F, Female; +, present; -, absent; N, normal, GBS disability score,

Table 2. Clinical characteristics of GBS patients associated with presence of serum anti-gangliosides antibodies.*

Characteristics	Gangliosides (GM1, GD1a and GQ1b)			
	positive (n=54)	Negative (n=43)	Odds ratio (95% CI)	P Value†
Age (median)	18 (2-52)	21 (5-65)	-	0.375¶
Sex	41M/13F	29M/14F	1.07 (0.4-2.86)	0.88
Preceding diarrhea	26 (48%)	10 (19%)	3.06 (1.6-8.2)	<0.02
Ventilation	10 (18%)	13 (30%)	0.56 (0.2-1.5)	0.26
Sensory deficit at entry	0 (0%)	8 (14%)	-	0.001
Positive *C. jejuni* serology	43 (80%)	12 (28%)	10.1 (3.6-29.1)	<0.001
Anti-LOS serology	47 (87%)	10 (23%)	22.1 (6.9-75.5)	<0.001
Electrophysiology (N=64)				
Axonal‡	28/42 (67%)	15/22 (68%)	0.9 (0.3-3.2)	0.28
AIDP§	8/42 (19%)	6/22 (27%)	0.8 (0.5-1.4)	0.60
Unclassified	6/42 (14%)	1/22 (5%)	3.5(0.4-82.5)	0.42

*Data were expressed as median or number of patients (percentage); M, male; F, female; CI, confidence interval; - cannot be calculated.

†Determined by Chi-square or Fisher's exact test.

¶ Determined by Wilcoxon-Mann-Whitney *U* test.

‡Axonal variants included acute motor axonal neuropathy (AMAN) and acute motor sensory axonal neuropathy (AMSAN).

§AIDP, acute inflammatory demyelinating polyneuropathy.

Table 3. Variants of the glycosyltransferases involved in the synthesis of the LOS outer core structures in *C. jejuni* strains BD-07, BD-10, BD-39 and BD-67. [a]

Strain	BD-07	BD-10	BD-39	BD-67
Class	A	B	A	B
GenBank Accession No.	GU289927	GQ249164	GU289928	GQ249165
Cj1135	One-domain	Two-domain	One-domain	Two-domain
Cj1136	on [b]	On	On	On
CgtAI	Mono-sialyl. [c]	Non-sialyl. [d]	Mono-sialyl.	Non-sialyl.
CgtAII	Absent	off [e]	Absent	Off
CgtB	Mono-sialyl.	Off	Mono-sialyl.	Off
Cst-II	Mono- [f]	Bi-	Mono-	Bi-
GalT	Absent	Present	Absent	Present

[a] Assignment of the glycosyltransferase variants is based on amino acid sequence comparisons with variants of known specificities.
[b] on: indicates that a gene has no frame-shift mutation.
[c] Mono-sialyl.: the glycosyltransferase is specific for mono-sialylated acceptors.
[d] Non-sialyl.: the glycosyltransferase is specific for non-sialylated acceptors.
[e] off: indicates that a gene is inactive because of a frame-shift mutation.
[f] *Cst-II* variants: mono-: monofunctional, *Cst-II* has α-2,3-sialyltransferase activity
bi-: bifunctional, *Cst-II* has both α-2,3-sialyltransferase and α-2,8-sialyltransferase activity

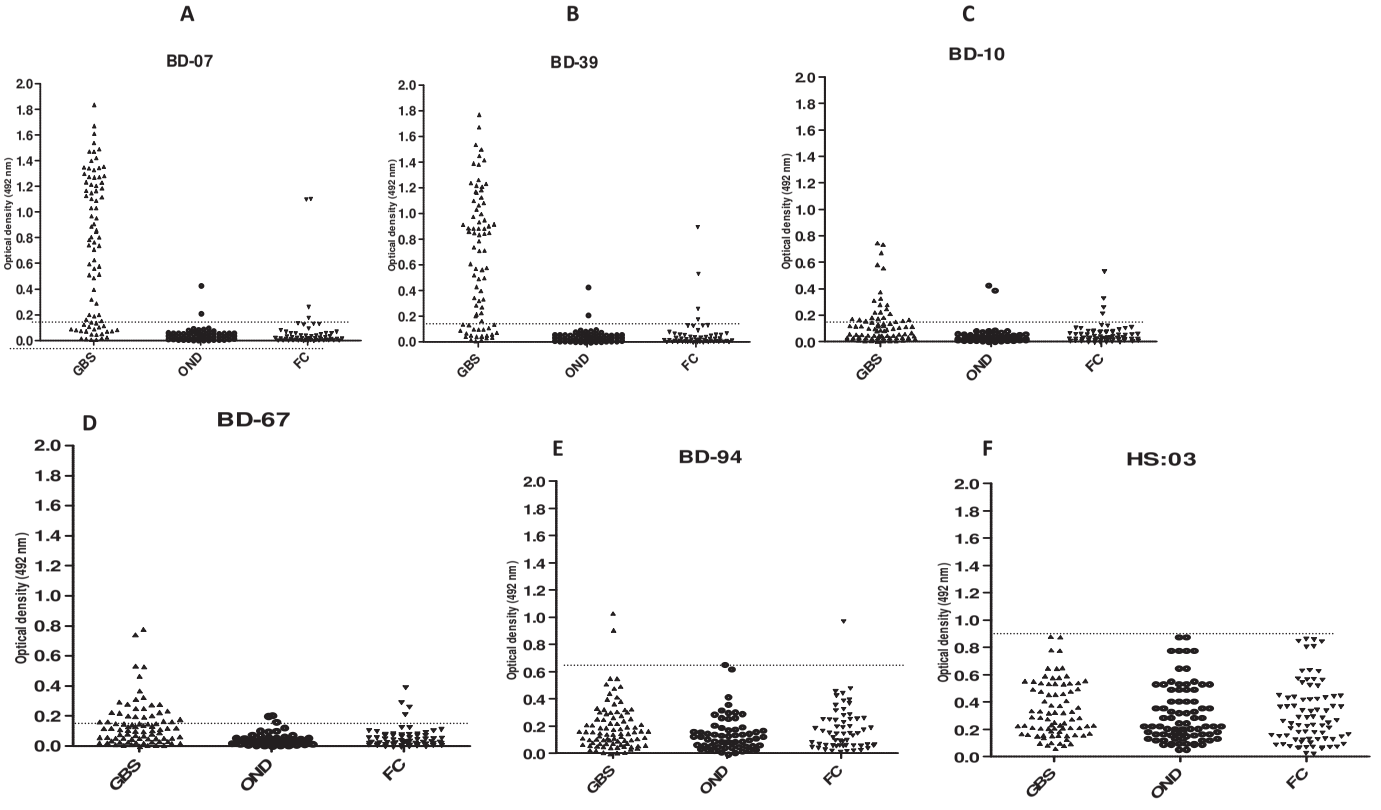

Figure 1. Serum samples from patients with GBS and from family controls and other neurological disease controls was tested for IgG activity to purified *C. jejuni* lipo-oligosaccharides (LOSs). **A, B**) LOS from GBS patient BD-07 and BD-39 reacted predominantly with sera from GBS patients. **C, D)** LOS from BD-10, and BD-67 who have an overlap between GBS and MFS, react with sera from GBS patients. **E**) LOS from BD-94 except two does not react with the sera of GBS patients. **F)** LOS from HS:3 serostrain, which does not contain a GM1 or GD1a/GQ1b-like structure, did not react with any of the sera. Dashed lines indicate cut-off value. GBS, Guillain-Barré syndrome; OND, other neurological disease; FC, family controls; LOS, lipo-oligosaccharides.

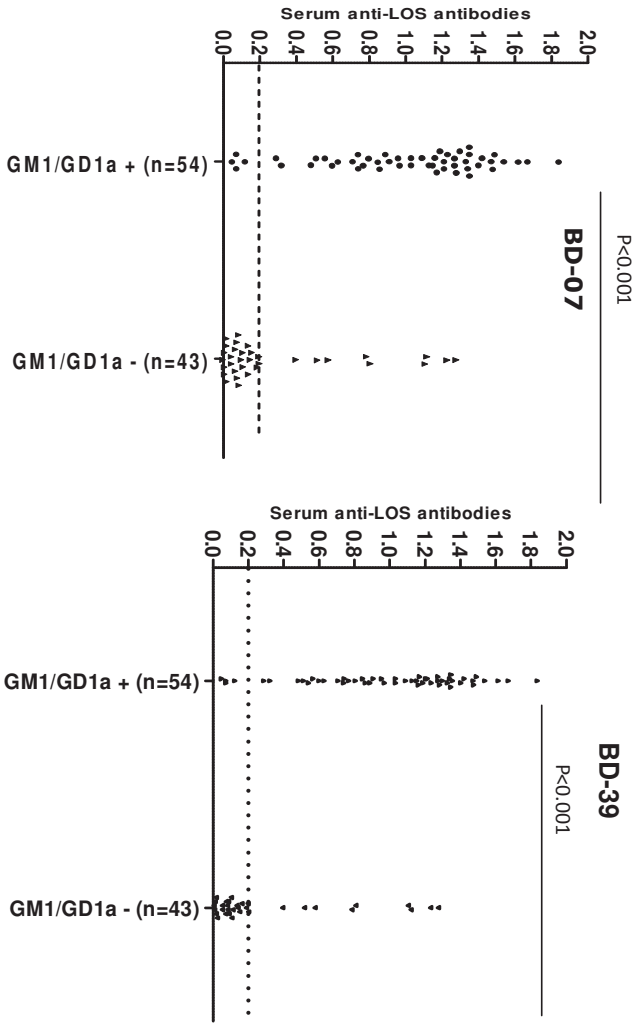

Figure 2. Serum IgG to LOS from *C. jejuni* BD-39 and BD-07 in relation to IgG reactivity to gangliosides GM1 and GD1a. LOS from BD-07 and BD-39 strongly reacted with sera of the anti-GM1/GD1a positive group.

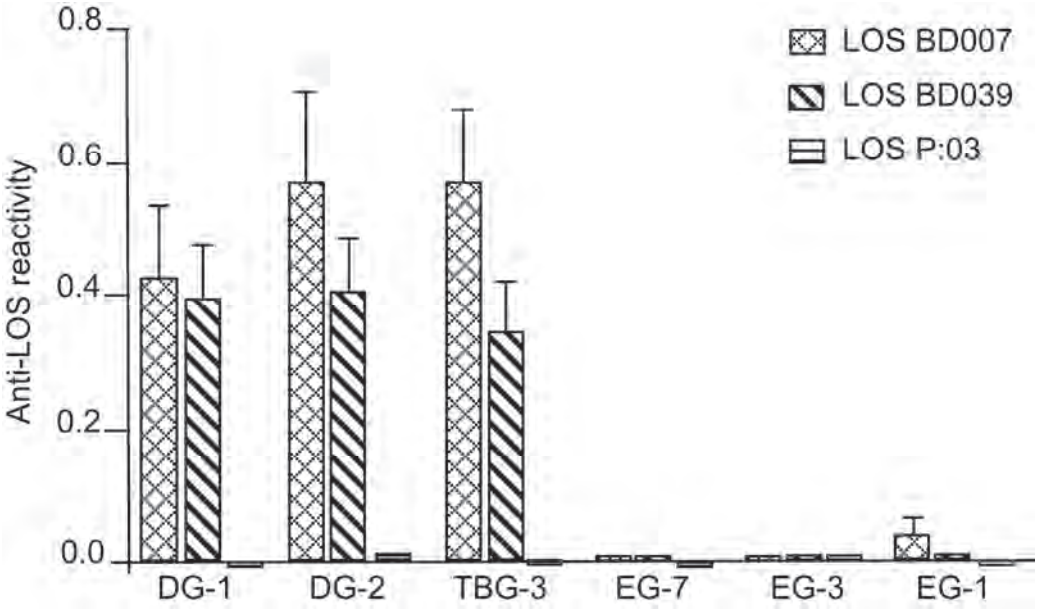

Figure 3 The binding of mouse monoclonal antibodies against LOS BD-07 and BD-39. DG-1 (binding to GM1), DG-2 (binding to GM1/GD1b/GA1) and TBG-3 (binding to GD1a) showed reactivity to LOS BD-07 and BD-39, whereas monoclonal antibodies EG-7 (binding to GD1b), EG-3 (binding to GQ1b) and EG-1 (binding to GQ1b/GT1a) showed no reactivity to LOS BD-07 or LOS BD-39. LOS HS:03 bound none of the monoclonal antibodies.

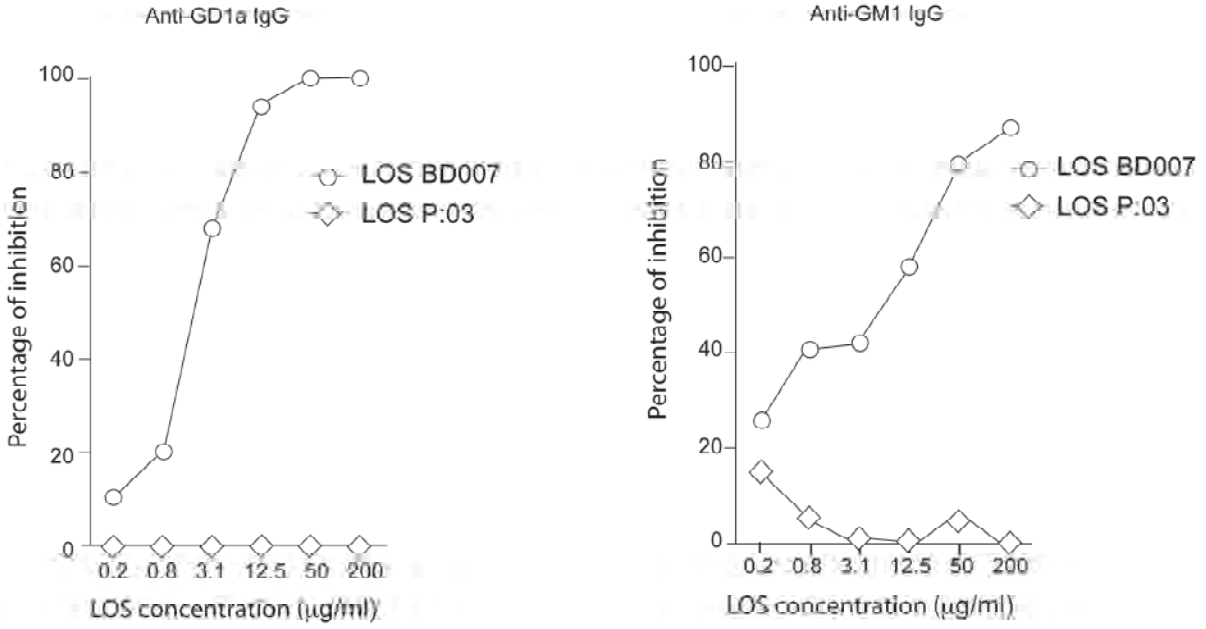

Figure 4: Cross-reactivity of GBS patient serum anti-ganglioside antibodies to LOS from the autologous *C. jejuni* isolate, demonstrated by inhibition ELISA. Serum antibodies to ganglioside cross-reacted with LOS from the autologous *C. jejuni* strains (circles) but not with LOS from the control HS:3 strain lacking ganglioside mimicry (triangles). **A)** Inhibition of IgG anti-GD1a reactivity in serum from patients DK-07 by pre-incubation with LOS from the autologous *C. jejuni* BD-07 strain and from Penner serostrain HS:03. **B)** BD-07, DK-39 by pre-incubation with LOS from the autologous *C. jejuni* BD-39 strain and from Penner sero-strain HS:03.

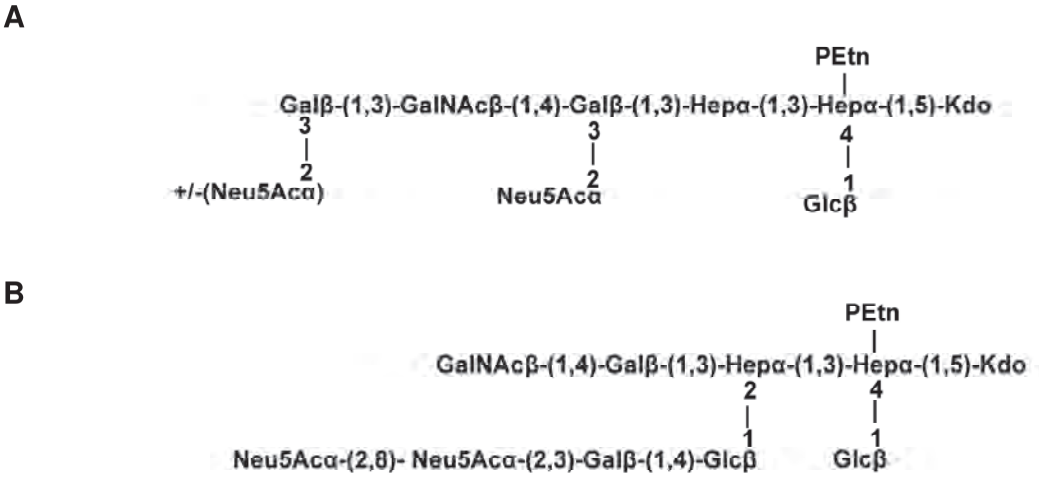

Figure 5: Proposed LOS outer core structures based on capillary-electrophoresis electrospray ionization mass spectrometry analysis of *O*-deacylated LOS samples (see supplemental Table 1). A) strains BD-07 and BD-39 show mimicry with GM1 and GD1a; B) strains BD-10 and BD-67 show mimicry with GA2 and GD3.

Supplemental Table 1. Negative ion ESI-MS data and proposed compositions for *O*-deacylated LOS from *Campylobacter jejuni* BD-07, BD-10, BD-39, BD-67 and BD-94.

Strain	Observed ions (*m/z*)			Molecular mass (Da)		Proposed compositions		
	$[M-4H]^{4-}$	$[M-3H]^{3-}$	$[M-2H]^{2-}$	Observed	Calculated [1]	Core oligosaccharide	Phosphorylation in lipid A	Acylation in lipid A
BD-07	806.8	1075.9		3231.0	3230.1	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
	837.6	1117.1		3354.8	3353.1	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	894.0	1192.0		3579.5	3578.5	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
	966.8	1289.3		3871.1	3870.7	$Neu5Ac_2•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
BD-10		979.1	1468.7	2939.9	2938.8	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
		1020.0	1530.1	3062.6	3061.9	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
		1054.1	1581.1	3164.8	3164.2	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	829.0	1105.4		3319.6	3318.1	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
	859.8	1146.7		3443.2	3441.2	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	885.4	1180.7		3545.4	3544.5	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	920.4	1227.4		3685.4	3683.5	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
	951.3	1268.4		3808.7	3806.5	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	976.8	1302.3		3910.6	3909.8	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	1007.5	1343.3		4033.5	4032.9	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
BD-39	806.9	1076.0		3231.3	3230.1	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
	837.6	1117.1		3354.8	3353.1	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	893.9	1192.2		3579.6	3578.5	$Neu5Ac_1•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
	966.8	1289.3		3871.1	3870.7	$Neu5Ac_2•Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
BD-67		978.8	1468.5	2939.6	2938.8	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
		1019.8	1530.0	3062.3	3061.9	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
		1053.9	1581.0	3164.4	3164.2	$Hex_3•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	828.9	1105.6		3319.4	3318.1	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)

	859.7	1146.5	3442.9	3441.2	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	885.4	1180.7	3545.2	3544.5	$Neu5Ac_2•Hex_3•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	920.2	1227.2	3684.7	3683.5	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	3 *N*-(3-OH 14:0)
	950.9	1268.3	3807.8	3806.5	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	3 *N*-(3-OH 14:0)
	976.6	1302.1	3909.9	3909.8	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *P*	4 *N*-(3-OH 14:0)
	1007.5	1343.1	4033.2	4032.9	$Neu5Ac_2•Hex_4•HexNAc_1•Hep_2•PEtn_1•KDO_2$	*PP*Etn, *PP*Etn	4 *N*-(3-OH 14:0)
BD-94	918.8		3979.2		Unknown		
	975.1	1300.1	3903.9		Unknown		

[1] Isotope-average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Hex,162.14; HexNAc, 203.19; Hep, 192.17; KDO, 220.18; *P*, 79.98; *P*Etn, 123.05; Neu5Ac, 291.26;

Discussion

In this study we provide biophysical and serological evidence for the involvement of molecular mimicry in the pathogenesis of *C. jejuni*-related GBS in Bangladesh. The IgG response against *C. jejuni* LOS and gangliosides are closely associated in patients with GBS. We determined that serum ganglioside antibodies cross-reacted specifically with LOS from autologous *C. jejuni* LOS. To our knowledge, this is the first report in which MS is combined with DNA sequence data to determine the LOS outer core structures of neuropathy-associated *C. jejuni* strains isolated in South Asia. We found that strains with class A LOS gene loci express both GM1 and GD1a epitopes on their LOSs. Expression of GM1 and GD1a was enhanced in *C. jejuni* HS:19, and this expression was dependent on the predominance of the *cstII* (Thr51) gene. Our data confirm that ganglioside mimicry is the most likely pathogenic mechanism underlying the majority of *C. jejuni*-associated GBS cases. Our data further support the hypothesis that antecedent *C. jejuni* infections in GBS trigger the production of antibodies that cross-react with gangliosides.

Molecular mimicry between gangliosides and LOS has been demonstrated with *C. jejuni* isolates from GBS and MFS patients (13, 19, 20, 35-38). Several findings in the current study support the hypothesis that cross-reactive antibodies to ganglioside in these patients contributed to the development of GBS in Bangladesh. Anti-LOS antibody levels are significantly associated with GBS as compared with data from two control groups ($p<0.001$). In the present study, we provide strong evidence that in GBS patients in Bangladesh anti-LOS antibody levels are strongly associated with recent *C. jejuni* infection. *C. jejuni* is a frequent antecedent pathogen associated with GBS in Bangladesh (26). The association between anti-LOS and anti-ganglioside antibodies is significant but not absolute. In some patients with anti-ganglioside antibodies we did not find a high IgG activity against LOS. This may result from relatively low titres or different fine-specificities of the anti-ganglioside antibodies in these patients. In previous studies, Bart et al. found no binding of anti-GM1 antibodies with LPS of *C. jejuni* from MFS and GBS patients (21). There are some GBS patients without anti-ganglioside antibodies but with anti-LOS antibodies. Patients with a *C. jejuni* infection may have antibodies against complexes of gangliosides (32). It is interesting to note that our preliminary data showed that patients with anti-LOS reactivity but without reactivity against single gangliosides can have antibodies against complexes gangliosides (data not shown). Our results are in agreement with recent observations that ganglioside complexes are important target antigens in GBS as well as in MFS (39, 40).

Monoclonal antibodies against gangliosides were bound by LOS BD-07 and LOS BD-39 in a similar pattern. Serum DK-07 and DK-39 showed a dramatic reduction in anti- ganglioside reactivity after incubation with the autologous LOSs. Incubation with LOS that lacked ganglioside-mimics did not inhibit ganglioside-reactivity, demonstrating that serum DK-07 and serum DK-39 were specifically deprived of their ganglioside antibodies by the incubation with the autologous LOSs. This finding indicates that in these two GBS patients the antibodies against GM1 and GD1a were induced by LOS during the preceding *C. jejuni* infection. Immune response against *C. jejuni* involved in the pathogenesis of GBS by cross-reactivity with neural tissue (19, 32).

Various ganglioside mimics were found in the LOS of neuropathy-associated strains in Bangladesh. GM1/GD1a was the most prevalent ganglioside mimic in GBS-associated strains, and it was predominantly found in LOS class A strains. This finding is consistent with the results previously described (24, 41), but in contrast with those of Nachamkin et al., who reported previously that the expression of GD1a and not GM1 is associated with GBS (16). MS analysis has not demonstrated true GQ1b-like structures in *C. jejuni* LOS. The detection of structures with a terminal di-NeuAc-Gal in both strains (BD-10 and BD-67) from Bangladesh associated with ophthalmoplegia suggests that in these patients, pathogenic antibodies are raised against GA2- and GD3-like LOS. Both express the same LOS outer core, which appears to be a novel structure with two extension sites from the inner core and two branches mimicking gangliosides. There are alternative explanations for the observation that one GBS-associated strain (BD-94) did not express ganglioside mimic in its LOS. It has been demonstrated previously that GBS patients can occasionally be infected with two different *C. jejuni* strains. In such cases only one of the strains could be linked to GBS (24).

LOS structure variability is defined by genetic polymorphism of *C. jejuni*. Hence also the specificity of the anti-ganglioside antibody response and clinical features of GBS is affected by mutability of the bacteria (37, 42, 43). The presence of, and polymorphism within the *cstII* gene has been associated with the expression of ganglioside mimics and with clinical features of GBS (25, 44). The *Thr51* variant was associated with monosialylated LOS and seemed to occur more frequently in class A strains and in GBS-related strains (BD-07 and BD-39). We found that the *CstII* Asn51 variant was associated with the expression of disialylated LOS and seemed to occur more frequently in class B strains, and strains related with clinical symptoms of MFS or GBS with ophthalmoplegia. Our findings are concordant with recent data reported earlier (24, 25, 41).

Patients DK-07 and DK-39 have developed interesting immune responses. Bacterial genotypying showed that these patients were infected with clonal *C. jejuni* strains (30). Mass spectrometry results and our LOS characterization data demonstrated that LOS BD-07 and BD-39 share the same ganglioside-mimics. However, patient DK-07 developed an anti-GD1a immune response while patient DK-39 developed an anti-GM1 immune response. These results suggest that the immune response against gangliosides is not only determined by ganglioside mimicry present in the *C. jejuni* LOS but may also be determined by genetic host factors.

In conclusion, our study further supports the hypothesis that infections with specific *C. jeuni* strains induce cross-reactive IgG antibodies against LOS and gangliosides in GBS patients. The conformation of the oligosaccharide moieties in *C. jejuni* LOS and the recognition of the anti-ganglioside antibodies by the adaptive immune system cannot be predicted by the biochemical structure only. Further research is necessary to elucidate the mechanism by which *C. jejuni* determine the fine-specificity of the anti-ganglioside antibodies.

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Chapter 5.1

CAMPYLOBACTER JEJUNI HS:23 AND GUILLAIN-BARRÉ SYNDROME, BANGLADESH

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Guillain-Barré syndrome (GBS) is an acute peripheral neuropathy triggered by a preceding infectious illness. Gastroenteritis caused by *Campylobacter jejuni* is the most frequently reported antecedent event (1). In Japan, South Africa, China, and Mexico, *Campylobacter* strains with certain Penner heat-stable (HS) serotypes, including HS:19 and HS:41, are overrepresented among isolates from GBS case-patients, compared with isolates from enteritis case-patients (2,3). Several studies indicate that *C. jejuni* HS:19 and HS:41 have a clonal population structure and suggest that these serotypes might have unique virulence properties that are intricately linked to development of GBS (4). However, data from the United Kingdom and the Netherlands suggest that such virulence properties may not be restricted to specific HS serotypes because many other serotypes can be cultured from patients with GBS (5*)*. We report a non-HS:19 and non-HS:41 *C. jejuni* serotype and sequence type (ST)–3219 that are overrepresented among isolates from GBS patients in Bangladesh.

We conducted a prospective case-control study of the serotype and genotype of *C. jejuni* associated with GBS in Bangladesh. Case-patients were 97 persons with GBS admitted to Dhaka Medical College Hospital, Bangabandhu Sheikh Mujib Medical University, and Dhaka Central Hospital during July 2006–June 2007. All fulfilled the diagnostic criteria for GBS of the National Institute of Neurological Disorders and Stroke of the US National Institutes of Health (Bethesda, MD, USA) (6). The control group comprised 97 patients with other neurologic diseases, matched with case-patients by sex, age, and date of admission to the hospital. A second control group comprised 97 healthy family members of case-patients. Up to 3 stool samples were cultured from each case-patient and control. *Campylobacter* strains were presumptively identified with Gram stain, oxidase, and hippurate hydrolysis tests and confirmed with a *C. jejuni* species-specific PCR. Serotyping was performed at the National Laboratory for Enteric Pathogens, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada. All strains were serotyped according to the HS serotyping schemes of Penner et al. (7). To determine the class of lipooligosaccharides (LOS) locus in each of the *C. jejuni* strains, genomic DNA was isolated by using the DNeasy tissue kit (QIAGEN, Venlo, the Netherlands). PCR analysis was performed with primer sets specific for classes A, B, C, D, and E (8).

We isolated *C. jejuni* from fecal samples of 10 case-patients. *Campylobacter* strains were not isolated from the control groups ($p<0.001$). Serotyping of the 10 GBS-related strains showed 4 different HS serotypes. *C. jejuni* HS:23 was found in 5 (50%) strains; HS:19, in 2 (20%); HS:55 and HS:21, in 1 strain each. One strain was untypeable according to the HS typing scheme. In a collection of clinical *C. jejuni* isolated during the same period from patients with enteritis, HS:23 was encountered in 9 (28%) of 32 patients. Serotypes previously associated with GBS were HS:1, HS:2, HS:4, HS:4/50, HS:5, HS:10, HS:13/65, HS:16, HS:19, HS:23, HS:35, HS:37, HS:41, HS:44, and HS:64 (5,9).

Nine (90%) of the *C. jejuni* isolates from the case-patients had the class A or class B LOS, which are highly associated with the presence of ganglioside-mimicking structures in LOS (10). Godschalk et al. found that 14 (82%) of 17 GBS-associated isolates possessed a class A/B/C locus

(8). Parker et al. (10) found that all GBS related strains and 64% of the other clinical and environmental isolates belonged to LOS class A/B/C loci. The expression of ganglioside-mimicking structures in *Campylobacter*, LOS is considered essential for the induction of autoantibodies that lead to GBS. Godschalk et al. (8) demonstrated that specific genes involved in *C. jejuni* LOS biosynthesis are crucial for the induction of antiganglioside antibodies that lead to GBS.

We performed multilocus sequence typing to examine the overall genomic variation among 10 GBS-related *C. jejuni* strains. We identified 6 different STs among the GBS-related *C. jejuni* strains (Table). However, ST-3219 has a new combination of alleles and was identified in 4 strains. Concordantly, the analysis demonstrated that *C. jejuni* isolates with serotype HS:23 were all ST-3219. Of particular interest, ST-985 (BD-67) shared 5 alleles (aspA, uncA, glnA, glyA, pgm) with ST-3219 (Table).

Our findings of a *C. jejuni* HS:23 serotype and ST-3219 that is highly prevalent among GBS-related *C. jejuni* strains from Bangladesh are consistent with previous observations that specific LOS types and serotypes are overrepresented among GBS-related *C. jejuni* strains. These observations support the hypothesis that, although a great variety of *C. jejuni* serotypes can be isolated from GBS patients in some geographic areas, specific clonal serotypes and multilocus types are prevalent in GBS patients in other places. The association of GBS with *C. jejuni* LOS class A/B/C is the only consistent finding when universal collections of GBS- associated strains are considered.

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Table: Serotyping and MLST analysis of *C. jejuni* strains associated with Guillain-Barré syndrome from Bangladesh

Strains	Year	Disease *	LOS Class	Penner type(s) †	ST‡	Allele number						
						aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
BD-07	2006	GBS	A	HS:19	22	1	3	6	4	3	3	3
BD-10	2006	GBS/MFS	B	HS:23	3219	10	27	33	19	10	5	7
BD-22	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7
BD-27	2006	GBS	A	UT	587	1	2	42	4	90	25	8
BD-34	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7
BD-39	2006	GBS	A	HS:19	660	1	3	6	4	54	91	3
BD-67	2007	GBS/MFS	B	HS:23	985	10	27	89	19	10	132	7
BD-74	2007	GBS/MFS	B	HS:23	3219	10	27	33	19	10	5	7
BD-75	2007	GBS	A	HS:55	587	1	2	42	4	90	25	8
BD-94	2007	GBS	E	HS:21	2109	4	7	10	4	10	7	1

*GBS, Guillain-Barré syndrome; MFS, Miller Fisher syndrome.

†Penner heat-stable (HS) serotypes; UT, untypeable. ‡ST, Sequence type.

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Chapter 5.2

COMPARATIVE GENOTYPING OF *CAMPYLOBACTER JEJUNI* STRAINS FROM PATIENTS WITH GUILLAIN-BARRÉ SYNDROME IN BANGLADESH

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Abstract

Background: *Campylobacter jejuni* is a common cause of acute gastroenteritis and is associated with post-infectious neuropathies such as the Guillain-Barré syndrome (GBS) and the Miller Fisher syndrome (MFS). We here present comparative genotyping of 49 *C. jejuni* strains from Bangladesh that were recovered from patients with enteritis or GBS. All strains were serotyped and analyzed by lipo-oligosaccharide (LOS) genotyping, amplified fragment length polymorphism (AFLP) analysis, multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE).

Methodology/Principle Findings: *C. jejuni* HS:23 was a predominant serotype among GBS patients (50%), and no specific serotype was significantly associated with GBS compared to enteritis. PCR screening showed that 38/49 (78%) of strains could be assigned to LOS classes A, B, C, or E. The class A locus (4/7 vs 3/39; $p<0.01$) was significantly associated in the GBS-related strains as compared to enteritis strains. All GBS/oculomotor related strains contained the class B locus; which was also detected in 46% of control strains. Overlapping clonal groups were defined by MLST, AFLP and PFGE for strains from patients with gastroenteritis and GBS. MLST defined 22 sequence types (STs) and 7 clonal complexes including 7 STs not previously identified (ST-3742, ST-3741, ST-3743, ST-3748, ST-3968, ST-3969 and ST-3970). *C. jejuni* HS:23 strains from patients with GBS or enteritis were clonal and all strains belonged to ST-403 complex. Concordance between LOS class B and ST-403 complex was revealed. AFLP defined 25 different types at 90% similarity. The predominant AFLP type AF-20 coincided with the *C. jejuni* HS:23 and ST-403 complex.

Conclusion/Significance: LOS genotyping, MLST, AFLP and PFGE helped to identify the HS:23 strains from GBS or enteritis patients as clonal. Overall, genotypes exclusive for enteritis or for GBS-related strains were not obtained although LOS class A was significantly associated with GBS strains. Particularly, the presence of a clonal and putative neuropathogenic *C. jejuni* HS:23 serotype may contribute to the high prevalence of *C. jejuni* related GBS in Bangladesh.

Introduction

Campylobacter jejuni is the most significant bacterial cause of human gastroenteritis (1-4). Clinical syndromes vary from mild to severe and from gastroenteritis to extraintestinal diseases. This latter category includes acute autoimmune neuromuscular complications such as the Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (5). The pathogenesis of *Campylobacter*-induced GBS is complex and involves host susceptibility factors as well as bacterial virulence factors (6-8). The development of these autoimmune neuropathies after *C. jejuni* infection is thought to be primarily related to sialylated lipooligosaccharides (LOS) on the cell surface of *C. jejuni*. These exhibit significant molecular mimicry with gangliosides on human peripheral nerves (9-13). Most patients who develop GBS after *C. jejuni* enteritis have IgG autoantibodies that react with gangliosides (such as GM1, GD1a, and GQ1b) (14). Comparison of the LOS loci of various *C. jejuni* strains has demonstrated that only the class A, B and C LOS loci contain the genes that are necessary for the biosynthesis of ganglioside mimics (15).

Extensive effort has been put into the identification of novel determinants of *C. jejuni* associated with the development of GBS (16, 17). In Japan, South Africa, China, and Mexico, *Campylobacter* strains with specific Penner heat-stabile (HS) serotypes, including HS:19 and HS:41, were overrepresented among strains isolated from GBS patients (5, 18, 19). *C. jejuni* HS:19 and HS:41 are clonal which suggests that these serotypes may have unique and specific virulence properties that trigger GBS (20). However, more recent data has shown that these neuropathogenic properties are not restricted to specific HS serotypes as other serotypes commonly isolated from enteritis patients (HS:1, HS:2, and HS:4 complex) are also found in patients with GBS (21). We recently reported non-HS:19 and non-HS-41 *C. jejuni* serotypes that are overrepresented among strains from GBS patients in Bangladesh (22). Recently, we reported a high frequency of *Campylobacter*-related GBS from Bangladesh (12th ASCODD).

The aim of the present study was to investigate the genetic heterogeneity of *C. jejuni* strains isolated from GBS and enteritis patients between 2006 and 2007 in Bangladesh. In this comparative genomic analysis, multi-locus sequence typing (MLST), amplified fragment length polymorphism (AFLP), LOS class PCR typing, and pulsed-field gel electrophoresis (PFGE) were employed to define detailed strain specific genotypes.

Materials and Methods

Bacterial strains

A systematic hospital-based study has been carried out among GBS patients in Dhaka, Bangladesh between 2006 and 2007. During this period, we isolated 10 *C. jejuni* strains from stool specimens of GBS patients and 39 *C. jejuni* from enteritis patients (22). All GBS patients fulfilled the diagnostic of GBS criteria (23). Bacteria were grown on blood agar plates with 5% sheep blood, at 37°C for 48 h under micro-aerobic conditions, with 6% O2, 7% CO2, 80% N2, and 7% H2 using the

Anoxomat system (AnoxomatTM Mart II, Drachten, The Netherlands). Bacteria were stored at -80°C in 15% glycerol in brain heart infusion broth. All strains were serotyped with the heat-stable (HS) serotyping schemes of Penner at the National Laboratory for Enteric Pathogens, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada (24). The project protocol was reviewed and approved by the institutional review board and the ethical committees at the Dhaka Medical College and Hospital, Dhaka, Bangladesh. Verbal informed consent was obtained from all patients and controls.

Bacterial DNA isolation

Genomic DNA was isolated with the Qiagen Genomic DNA purification kit according to the manufacturer's instructions (Qiagen, Venlo, The Netherlands).

Determination of the LOS locus class

To determine the LOS class in *C. jejuni* strains, we used specific primer sets for the classes A/B, C, D, and E, based on the DNA sequence of genes unique to the respective LOS locus class(es) described earlier (9). To discern between classes A and B, we used a primer set that was based on the DNA sequence of *orf5-II* (9). PCR assays were performed using a Thermocycler 60 (Biomed GmbH) with a program consisting of 40 cycles of 1 minute at 94°C, 1 minute at 52°C, and 2 minutes at 74°C. Per reaction, approximately 50 ng of template DNA was used in a buffer system consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM of each of the deoxyribonucleotide triphosphates (Promega Corp.), and 0.2 U Super Taq polymerase (HT Biotechnology Ltd.).

Multilocus sequence typing of *C. jejuni* strains

Nucleotide sequence analysis of internal fragments of seven housekeeping genes (aspartase A, a*spA*; glutamine synthetase, *glnA*; citrate synthase, *gltA*; serine hydroxymethyl transferase, *glyA*; phosphoglucomutase, *pgm*; transketolase, *tkt* and ATP synthase α subunit, *uncA*) was performed as described by Dingle et al. (25). Where no amplification product was observed on agarose gel electrophoresis, the reaction was repeated substituting primers described by Miller et al. (26). The same primers used to obtain each amplicon were used for nucleotide sequencing, which was carried out at least once on each DNA strand using BigDyeTM Ready Reaction Mix (Version 3, Applied Biosystems, Foster City, CA) at a concentration of 1/32 of that described in the manufacturer's instructions. Existing and new alleles, sequence types (ST) and clonal complexes were assigned using the MLST database located at http://pubmlst.org/*Campylobacter*/). Sequence types (STs) were assigned to clonal complexes as described by Dingle et al. (25) by identification of central genotypes and the assignment of variants that differed at one, two, or three loci (25, 27). The data were used to draw an unweighted pair group mean average (UPGMA) dendrogram by using the program START (http://outbreak.ceid.ox.ac.uk/software.htm) (28).

AFLP analysis and data processing

Strains were typed by AFLP (29). In short, chromosomal DNA was digested with *HindIII* and HhaI and simultaneously ligated with restriction site-specific adapters for 2 h at 37°C. This was followed by a preselective PCR using adapter-specific primers with *HindIII* (5'-GACTG*CGTA*CCAGCTT) and HhaI (5'-GATGAGTCCTGATCGC-3'). Next, an aliquot was subjected to a selective PCR using a fluorescently labelled *HindIII* primer that contained an additional A nucleotide at the 3' end (5'-GACTG*CGTA*CCAGC TTA) and a HhaI primer with an A extension (59-GATGAGTCCTGATCGCA). The final products were run on a 7.3% denaturing acrylamide gel for 5 h using a ABI 373A automated DNA sequencer. Fingerprints were collected by fluorography and interpreted with ABI Genescan software (PE Applied Biosystems). Gels were normalized using an internal ROX-labeled size standard included in each lane. Densitometric curves were processed with the GelCompar version 4.1 software (Applied Maths, Kortrijk, Belgium). After normalization and background subtraction, the levels of genetic similarity between AFLP patterns were calculated with the Pearson product-moment correlation coefficient (r).

PFGE

PFGE was performed as previously described (30). In short, samples of genomic DNA extracted from overnight cultures of the strains were digested with *SmaI* (Boehringer GmbH, Mannheim, Germany). Electrophoresis was performed in 1% SeaKem agarose in 0.53 Tris-borate-EDTA buffer by using a Bio-Rad CHEF Mapper programmed in the auto-algorithm mode (run time, 18 h; switch time, 6.76 to 35.38 s). Gels were stained with ethidium bromide for 30 min, destained in distilled water for 1 h; images of ethidium bromide-stained gels were captured under UV illumination by a video system (Gel DOC 1000; Bio-Rad).

Data analysis

Electrophoretic patterns from PFGE were compared by means of BioNumerics, version 4.01 (Applied Maths, Sint-Martens-Latem, Belgium). Analysis was based on band position and derived by the Dice coefficient with a maximum position tolerance of 1%. Strains were clustered by the unweighted pair group method using arithmetic averages. Statistical analysis was performed with EpiInfo (version 3.0) using 2 × 2 contingency tables. Fisher's exact tests were executed and 2-sided P values determined. Associations were considered significant at $P < 0.05$.

Results

Serotyping

Serotyping of the 10 GBS-related strains revealed 4 different HS serotypes (22). *C. jejuni* HS:19 was encountered in 2/10 (20%) patients. *C. jejuni* HS:23 was found in 5/10 (50%), a predominant serotype in GBS patients. Serotyping of enteritis strains revealed 15 HS-serotypes (Table 1). *C. jejuni* HS:23 was predominant serotype (28%) among enteritis strains.

LOS locus class

The results presented in Table 2 indicate that 38/49 (78%) of the *C. jejuni* strains characterized in this study could be assigned to one of the five LOS locus classes (A-E) screened by the class-specific PCR. The class A LOS locus was significantly associated with GBS-associated strains compared to controls strains (57% versus 8%, $p<0.01$; Table 2). In contrast, the three strains isolated in GBS patients with oculomotor symptoms contained a class B locus. LOS class B was detected in 18/39 (46%) of control strains (Table 2). No LOS locus classes C or D were found in GBS strains. LOS classes C and D were absent in enteritis strains with one exception; strain CZ-32 which had class C. The class E locus was rare in GBS and enteritis (1/10 vs 6/39 respectively).

Multilocus sequence typing of *C. jejuni* strains

A total of 22 different STs were found belonging to 7 clonal complexes, and a further 18 STs remained unassigned (Table 1). Seven new STs not previously registered in the *Campylobacter* pubMLST database were identified (ST-3742, ST-3741, ST-3743, ST-3748, ST-3968, ST-3969 and ST-3970). The most prevalent lineage was the ST-403 complex (Fig. 1), which included 5 of the 10 (50%) GBS-related strains studied and 10 of the 38 (26%) enteritis strains. The ST-362 complex included 2 of 10 (20%) GBS-related strains studied and 5 of 38 (13%) enteritis strains (Fig. 1). All Penner serotypes HS:23 strains belong to the ST-403 complex. ST-22 and ST-660, which both belong to the ST-22 complex, were found only in GBS-related strains and both strains belong to Penner serotype HS:19 (Table 1). UPGMA clustering of MLST data for *C. jejuni* strains isolated from GBS and enteritis patients yielded 4 major clonal groups (A, B, C and D) consisting 13 STs, and 8 STs were singletons (Fig. 2).

Table 1: MLST analysis of the *C. jejuni* strains from GBS and enteritis patients from Bangladesh

Strains	Year	Disease[a]	LOS[b] Class	Penner type(s)[c]	Allele number[e]								
					ST[d]	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*Tkt*	*uncA*	CC[f]
BD-07	2006	GBS	A	HS:19	22	1	3	6	4	3	3	3	22
BD-10	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7	403
BD-22	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7	403
BD-27	2006	GBS	A	UT	587	1	2	42	4	90	25	8	362
BD-34	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7	403
BD-39	2006	GBS	A	HS:19	660	1	3	6	4	54	91	3	22
BD-67	2007	GBS	B	HS:23	985	10	27	89	19	10	132	7	403
BD-74	2007	GBS	B	HS:23	3219	10	27	33	19	10	5	7	403
BD-75	2007	GBS	A	HS:55	587	1	2	42	4	90	25	8	362
BD-94	2007	GBS	E	HS:21	2109	4	7	10	4	10	7	1	45
CZ-02	2006	Enteritis	ND	NT	3632	91	2	42	4	169	25	8	UA
CZ-5	2006	Enteritis	ND	HS:15	27	1	2	42	85	11	9	8	362
CZ-9	2006	Enteritis	ND	HS:15	27	1	2	42	85	11	9	8	362
CZ-10	2006	Enteritis	A	HS:41	587	1	2	42	4	90	25	8	362
CZ-12	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-13	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-14	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-16	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-17	2007	Enteritis	ND	HS:12	3632	91	2	42	4	169	25	8	UA
CZ-19	2007	Enteritis	B	NT	3219	10	27	33	19	10	5	7	403
CZ-20	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-21	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-22	2007	Enteritis	B	HS:4	1374	24	2	5	72	2	5	6	UA
CZ-23	2007	Enteritis	ND	NT	3632	91	2	42	4	169	25	8	UA
CZ-26	2007	Enteritis	E	HS:21	2109	4	7	10	4	10	7	1	45
CZ-27	2007	Enteritis	B	HS:23	3219	10	27	33	19	10	5	7	403
CZ-29	2007	Enteritis	B	HS:21	1374	24	2	5	72	2	5	6	UA
CZ-31	2007	Enteritis	B	HS:13	1374	24	2	5	72	2	5	6	UA
CZ-32	2007	Enteritis	C	HS:8	**3968**	8	2	52	68	11	5	7	UA

Table 1: MLST analysis of the *C. jejuni* strains from GBS and enteritis patients from Bangladesh (cont.)

CZ-33	2007	Enteritis	E	NT	NT								NT
CZ-36	2007	Enteritis	B	HS:53	588	1	82	5	90	2	88	1	UA
CZ-37	2007	Enteritis	E	HS:3,4	**3969**	7	2	33	2	10	3	6	UA
CZ-39	2007	Enteritis	A	HS:10	**3742**	1	**308**	95	49	**436**	**353**	**258**	UA
CZ-46	2007	Enteritis	ND	HS:45	2993	1	2	42	4	11	9	8	362
CZ-51	2007	Enteritis	B	NT	3219	10	27	33	19	10	5	7	403
CZ-54	2007	Enteritis	E	NT	**3970**	4	7	10	249	3	7	1	UA
CZ-57	2007	Enteritis	E	NT	5	7	2	5	2	10	3	6	353
CZ-60	2007	Enteritis	A	HS:10	**3742**	1	**308**	95	49	**436**	**353**	**258**	UA
CZ-69	2007	Enteritis	B	HS:23	985	10	27	89	19	10	132	7	403
CZ-75	2007	Enteritis	ND	HS:45	2993	1	2	42	4	11	9	8	362
CZ-77	2007	Enteritis	ND	UT	**3743**	**233**	2	42	4	90	25	8	UA
CZ-81	2007	Enteritis	B	HS:1	1323	7	17	5	10	11	3	6	353
CZ-85	2007	Enteritis	ND	HS:53	**3741**	**234**	10	2	2	67	12	6	UA
CZ-93	2007	Enteritis	E	HS:53	**3741**	**234**	10	2	2	67	12	6	UA
CZ-94	2007	Enteritis	ND	HS:53	354	8	10	2	2	11	12	6	354
CZ-96	2007	Enteritis	B	HS:40	1377	1	2	42	4	153	9	8	42
CZ-98	2007	Enteritis	ND	HS:53	**3741**	**234**	10	2	2	67	12	6	UA
CZ-99	2007	Enteritis	B	NT	**3748**	**235**	2	42	62	11	9	8	UA
CZ-100	2007	Enteritis	B	HS:66	**3748**	**235**	2	42	62	11	9	8	UA

[a]GBS, Guillain-Barré syndrome (Islam et al. 2009)
[b]ND, not belong to LOS class A-E
[c]Penner heat-stable (HS) serotypes; UT, untypeable; NT, not typed
[d]ST, Sequence type; The MLST ST first reported in this study are indicated in boldface
[e]New alleles identified in this study are in boldface
[f]UA, unassigned

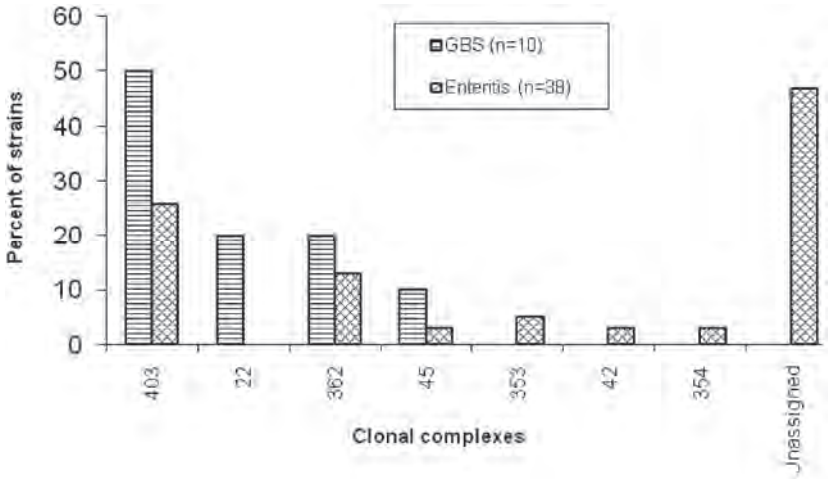


Figure 1. Frequency distribution of *C. jejuni* clonal complexes isolated from GBS and enteritis patients

AFLP

AFLP fingerprints were identified as distinct types when band patterns shared less than 90% similarity according to Duim et al. [29]. Among 49 *C. jejuni* strains, 25 different AFLP types were found. Twelve AFLP types were encountered in more than one strain (Fig. 3). The predominant AFLP type AF-20 was observed in 9 (18%) strains and type AF-3 in 3 (6%) and AF-4 in 4 (8%) strains each (Fig. 3). Seven distinct AFLP fingerprints were found in GBS-related *C. jejuni* strains. The predominant AFLP type AF-20 consisted of two GBS-related strains (BD-22 and BD-34) and 7 enteritis strains; this AFLP type correlated with Penner serotype HS:23 and clonal complex ST-403 (Fig. 3). Strains BD-07 and BD-39 displayed identical fingerprints (AF-9) and belong to the same Penner serotype HS:19. AFLP subdivided PFGE type A11 strains (BD-10 and BD-67) into two sub-clusters (AF-22 and AF-23). Twelve AFLP fingerprints of gastroenteritis strains (CZ-31, CZ-32, CZ-33, CZ-36, CZ-51, CZ-54, CZ-77, CZ-81, CZ-94, CZ-96, BD-67 and BD-74) were unique (Fig. 3).

PFGE

The *Sma*I-PFGE fingerprints contained six to nine bands and resolved into 4 major lineages at 60% similarity [31] (data not shown). These results correlate well with both the MLST clonal complexes and the different sub-clusters as defined by AFLP (Fig. 3). The 4 PFGE lineages could be subdivided in 33 subclusters at 90% similarity (Fig. 3). The majority of the strains belonging to a given clonal complex (ST-403) were also included in the same PFGE group (A). PFGE subdivided AFLP type AF-20 into several subtypes. PFGE analysis of 10 GBS related *C. jejuni* revealed the presence of 7 distinct types. Alhough AFLP and serotyping suggested that BD-07 and BD-39 were clonal (AF-9) these two strains were distinguishable by PFGE (A12 and A13).

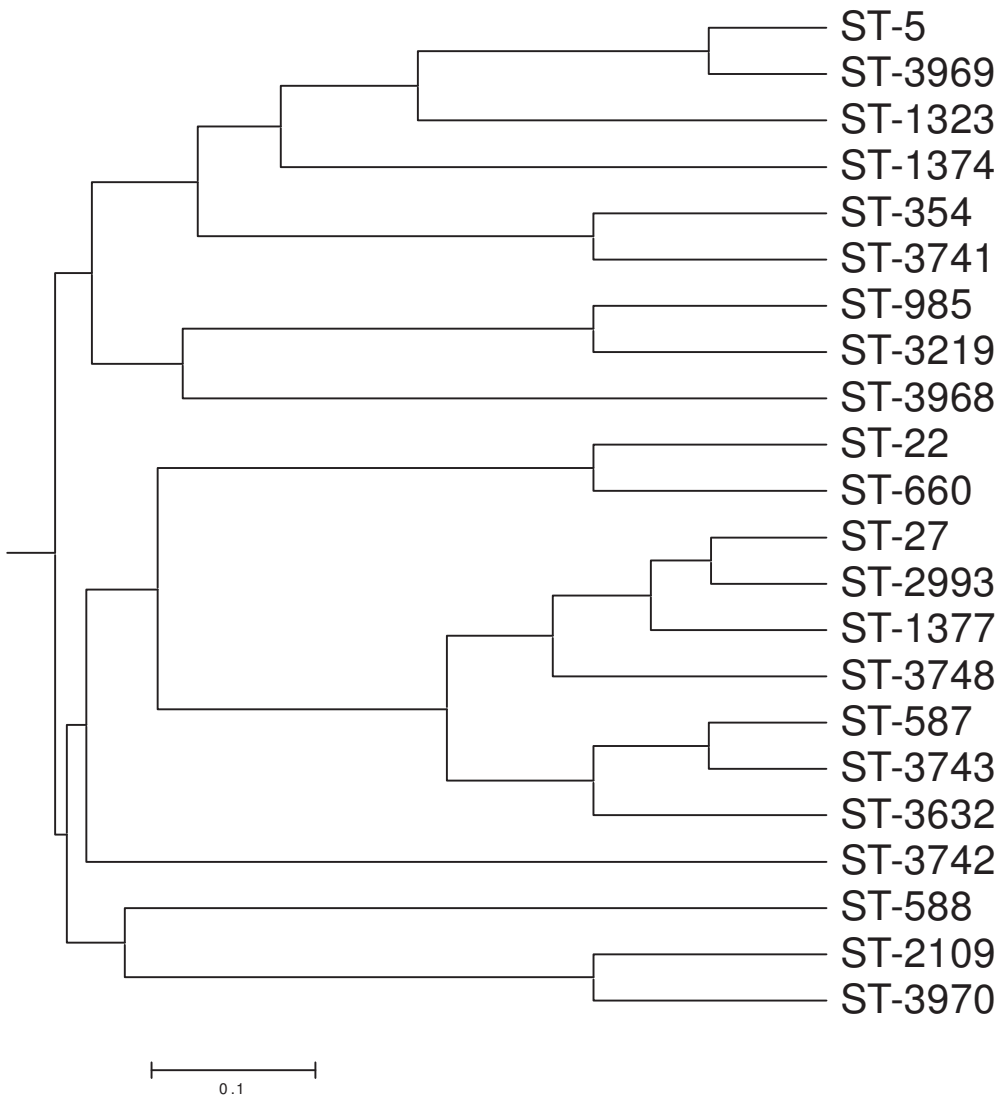
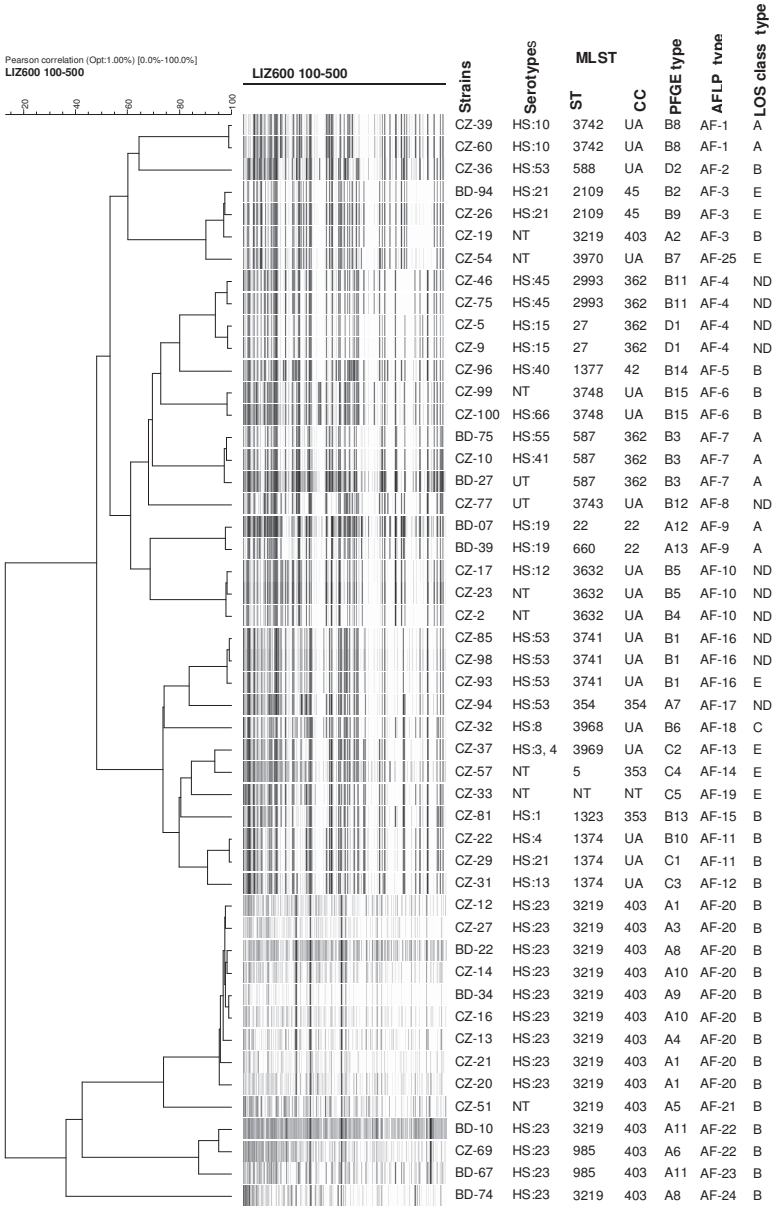


Figure 2. Dendrogram of *Campylobacter jejuni* sequence types, including GBS and enteritis related strains from Bangladesh. The dendrogram was constructed by using UPGMA.



Strains	Serotypes	MLST		PFGE type	AFLP type	LOS class type
		ST	CC			
CZ-39	HS:10	3742	UA	B8	AF-1	A
CZ-60	HS:10	3742	UA	B8	AF-1	A
CZ-36	HS:53	588	UA	D2	AF-2	B
BD-94	HS:21	2109	45	B2	AF-3	E
CZ-26	HS:21	2109	45	B9	AF-3	E
CZ-19	NT	3219	403	A2	AF-3	B
CZ-54	NT	3970	UA	B7	AF-25	E
CZ-46	HS:45	2993	362	B11	AF-4	ND
CZ-75	HS:45	2993	362	B11	AF-4	ND
CZ-5	HS:15	27	362	D1	AF-4	ND
CZ-9	HS:15	27	362	D1	AF-4	ND
CZ-96	HS:40	1377	42	B14	AF-5	B
CZ-99	NT	3748	UA	B15	AF-6	B
CZ-100	HS:66	3748	UA	B15	AF-6	B
BD-75	HS:55	587	362	B3	AF-7	A
CZ-10	HS:41	587	362	B3	AF-7	A
BD-27	UT	587	362	B3	AF-7	A
CZ-77	UT	3743	UA	B12	AF-8	ND
BD-07	HS:19	22	22	A12	AF-9	A
BD-39	HS:19	660	22	A13	AF-9	A
CZ-17	HS:12	3632	UA	B5	AF-10	ND
CZ-23	NT	3632	UA	B5	AF-10	ND
CZ-2	NT	3632	UA	B4	AF-10	ND
CZ-85	HS:53	3741	UA	B1	AF-16	ND
CZ-98	HS:53	3741	UA	B1	AF-16	ND
CZ-93	HS:53	3741	UA	B1	AF-16	E
CZ-94	HS:53	354	354	A7	AF-17	ND
CZ-32	HS:8	3968	UA	B6	AF-18	C
CZ-37	HS:3, 4	3969	UA	C2	AF-13	E
CZ-57	NT	5	353	C4	AF-14	E
CZ-33	NT	NT	NT	C5	AF-19	E
CZ-81	HS:1	1323	353	B13	AF-15	B
CZ-22	HS:4	1374	UA	B10	AF-11	B
CZ-29	HS:21	1374	UA	C1	AF-11	B
CZ-31	HS:13	1374	UA	C3	AF-12	B
CZ-12	HS:23	3219	403	A1	AF-20	B
CZ-27	HS:23	3219	403	A3	AF-20	B
BD-22	HS:23	3219	403	A8	AF-20	B
CZ-14	HS:23	3219	403	A10	AF-20	B
BD-34	HS:23	3219	403	A9	AF-20	B
CZ-16	HS:23	3219	403	A10	AF-20	B
CZ-13	HS:23	3219	403	A4	AF-20	B
CZ-21	HS:23	3219	403	A1	AF-20	B
CZ-20	HS:23	3219	403	A1	AF-20	B
CZ-51	NT	3219	403	A5	AF-21	B
BD-10	HS:23	3219	403	A11	AF-22	B
CZ-69	HS:23	985	403	A6	AF-22	B
BD-67	HS:23	985	403	A11	AF-23	B
BD-74	HS:23	3219	403	A8	AF-24	B

Figure 3. UPGMA dendrogram of AFLP fingerprints from 40 gastroenteritis and 10 strains related to patients with GBS. The percentage of genetic homology between banding patterns is indicated. Serotypes, LOS type, MLST, PFGE and AFLP types are plotted next to dendrogram. UA, unassigned; NT not typed; UT untypeable.

Congruence between typing methods

After cluster analysis of the data obtained by the respective methods and construction of a composite data set comprising the LOS typing, MLST, PFGE and AFLP, a similar clustering of the strains was observed. All ST-403 complex strains belonged to LOS locus class B. The correlation between LOS class B and ST-403 complex was evident in both GBS and enteritis collections (Table 1). Upon analysis of clustering of the sequence types by MLST, 81% showed overlap with AFLP types (Fig. 3).

Discussion

We performed comparative genomics of a set of 49 *C. jejuni* strains isolated from GBS and enteritis patients in Bangladesh by MLST, AFLP, LOS typing and PFGE fingerprinting. This is the first report on molecular characterization of GBS and enteritis related *C. jejuni* strains from Bangladesh. Cluster analysis of LOS typing, AFLP, PFGE and MLST showed significant overlaps. The LOS class A was significantly over-represented in the GBS-associated strains compared to the enteritis strains. Our MLST analysis demonstrated that all of the Bangladeshi strains with HS:23 serotype are clonal and clearly distinct from the non-HS:23 strains. The clonal complex ST-403 was overlapped by LOS typing, AFLP and PFGE. We recently reported that *C. jejuni* HS:23 serotype is prevalent among GBS and enteritis-related *C. jejuni* strains from Bangladesh (22). Our comparative genotyping analysis supported that *C. jejuni* HS:23 strains are clonal. However, comparison of a worldwide non-HS:19 associated with GBS and enteritis showed heterogeneity (32).

In the present study, we targeted only five specific classes (A-E) of LOS loci, despite recent increases in the number of LOS locus classes identified (33). LOS class A, B and C have been associated previously with GBS (9). We identified LOS A or B in 90% of GBS associated strains and in 46% of enteritis strains. Interestingly, we found that the class A locus is significantly associated with GBS without oculomotor symptoms whereas the class B locus associated with GBS with oculomotor symptoms. Previously Nachamkin et al. (34) reported a strong association between GBS-associated *C. jejuni* strains and the simultaneous presence of three LOS biosynthesis genes, *cst-II*, *cgtA* and *cgtB*. Our data confirm these findings, as the combination of *cstII*, *cgtA* and *cgtB* only exist in class LOS A and B. Other studies have demonstrated that the class A, B and C LOS loci contain the specific genes involved in the biosynthesis of ganglioside mimics (9, 15). Molecular mimicry between *Campylobacter* LOS and gangliosides in human peripheral nerves is thought to be the mechanism involved in the development of GBS (35).

We have used a variety of molecular techniques to demonstrate the genomic differences or similarities among the *C. jejuni* strains. In this study, we identified 7 distinct clonal complexes with 22 different STs. The most common Bangladeshi lineage was the ST-403 complex (Fig. 2). This predominant clonal complex is corroborated by LOS class B loci. In addition, distribution of STs showed a good concordance between GBS and enteritis related strains (Fig. 2). No representatives of ST-21 were present among GBS and enteritis related strains from Bangladesh, whereas ST-21 is the

prevalent complex in the general population structure of *C. jejuni (*25), it is widespread in multiple hosts and has previously been described to be associated with infections in humans, and with livestock and environmental sources; as in chicken, cattle, contaminated milk and water (25, 36). Molecular epidemiological evidence suggests that this clonal complex is frequently associated with environmental and food borne transmission (36, 37). Recently, Habib et al. (31) demonstrated that ST-21 complex strongly correlated with class LOS C loci. Both ST-21 and LOS C appear to be rare in Bangladesh. The GBS-associated strains were assigned to different clonal complexes (22), which correlated with earlier data describing heterogeneity among neuropathogenic *C. jejuni* strains (21, 25). ST-22 and ST-660 (both ST-22 complex) belong to Penner serotype HS:19 and were only found in GBS-related strains; ST-362 complex was the second most prevalent complex found in both GBS and enteritis strains; corroborated with LOS class A or B loci. A number of new STs were identified for the first time in this study (ST-3442, ST-3741, ST-3743, ST-3748, ST-3968, ST-3969 and ST-3970), but were not assigned to any known complex. To date, these unassigned STs have only been found only in enteritis strains from Bangladesh.

Cluster analysis of AFLP data in this study supports previous reports that no distinct subpopulation of *C. jejuni* strains is associated with GBS or enteritis (29). AFLP analysis revealed that HS:23 strains are clonal but substantial heterogeneity was found among non-HS:23 strains. PFGE and AFLP analysis were shown to have a high level of discriminatory power, although in some cases AFLP was able to distinguish further patterns. In some cases AFLP patterns of the strains were highly similar, whereas PFGE patterns showed differences (Fig. 2). Our PFGE and AFLP data also support those reported in a previous study carried out on Finnish *C. jejuni* strains (38). The genetic diversity of *C. jejuni* is well recognised and is attributed to a number of distinct phenomena, including genomic rearrangements and horizontal gene transfer (39). A study carried out in England by Owen et al. (39) showed that *C. jejuni* strains from human strains were highly diverse by PFGE analysis. Previous studies also described that MLST, AFLP, PFGE and DNA microarrays could not identify GBS-specific genetic markers by comparing the genomes of *C. jejuni* strains (16, 21, 25, 29). Furthermore, no molecular markers specific to GBS were detected after analyzing a highly clonal HS:41 population from South African patients by using a high-throughput AFLP (40). However, the LOS class typing significantly differentiated GBS-related strains from enteritis in Bangladesh.

In conclusion, our results support *C. jejuni* HS:23 are over represented among GBS related strains in Bangladesh and appear to be clonally related; LOS class A is significantly associated with GBS. The present study revealed a correlation between MLST clonal complex (ST-403) and certain LOS locus class B. Particularly, putative neuropathogenic *C. jejuni* HS:23 serotype may circulate at an elevated prevalence in Bangladesh.

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Chapter 5.3

COMPARATIVE POPULATION STRUCTURE ANALYSIS OF *CAMPYLOBACTER JEJUNI* FROM HUMAN AND POULTRY ORIGIN IN BANGLADESH

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ABSTRACT

Campylobacter jejuni is one of the most important causes of human diarrhea worldwide. The main objective was to understand the possible role of poultry in the transmission of *Campylobacter* to humans in Bangladesh. We used a comparative genotyping approach to determine the population structure of 66 *C. jejuni* isolated from poultry, and to determine the similarities with *C. jejuni* isolated from enteritis (n=39) and Guillain-Barré syndrome (GBS) (n=10) patients. MLST defined 16 sequence types (STs) and 5 clonal complexes (CCs) among poultry isolates, including 6 STs not previously documented in the public database. The most commonly identified clonal complex in chicken was ST-353 (n=13), followed by ST-354 (9%), and ST-574 (8%). MLST data suggested that there was significant genetic diversity between chicken and human isolates. The predominant clonal complex (ST-403) in humans was not found in chicken. The profiles obtained by AFLP and PFGE were largely in concordance with the MLST results, and showed a similar level of genetic diversity. AFLP defined 15 different types in chicken isolates at 90% similarity. The predominant AFLP type AF-1 mostly coincided with the ST-353 complex. The AFLP types observed in GBS-related *C. jejuni* were not found among the chicken isolates. The diversity of *C. jejuni* found among humans suggests that transmission occurs from various sources. The dissimilarity of the sequence types/or genotypes of *C. jejuni* from humans and poultry suggests the presence of additional sources other than poultry in Bangladesh.

INTRODUCTION

Campylobacter jejuni is the most bacterial cause of human gastroenteritis in developed countries (1-4). *C. jejuni* infection in humans is associated with the sudden onset of fever, abdominal cramps, and bloody diarrhea (2). In addition, *C. jejuni* has been identified as an important trigger for Guillain-Barré syndrome, the most common cause of acute flaccid paralysis in polio-free regions (5). *Campylobacter spp*. are widespread in the environment and constitute part of the natural intestinal flora of many mammalian species and birds (6). This includes not only domestic farm animals such as cattle, sheep, and pigs but also pet animals such as cats and dogs (7). Poultry meat is considered to be the main vector of *C. jejuni* infection, and transmission is thought to occur either as a result of cross-contamination due to improper handling of raw meat or consumption of undercooked food (8).

Molecular typing is an important tool to describe the diversity of *Campylobacter* and understand the transmission through the food chain. Several international molecular epidemiological studies indicate that genotypes of *C. jejuni* isolated from humans are shared with strains from poultry origin, indicating that poultry is an important source of *Campylobacter* (9, 10). Various molecular typing methods are currently available to study the population structure of *Campylobacter* (11). Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory technique that has been effectively applied for subtyping *Campylobacter* (12). However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies (13). Despite the efforts to standardize PFGE protocols and interpretation criteria of the data, comparison of inter-laboratory results remains difficult (14, 15). Multilocus sequence typing (MLST) has emerged as the state-of-the-art method for the resolution of bacterial population genetics (16) and is now recognized as the gold standard typing method for the phylogenetic study of the *Campylobacter* genus (17). MLST is not as discriminatory as PFGE for distinguishing between isolates but it does provide a direct measure of bacterial relationship. This technique is highly reproducible, easy to interpret, and results can be shared through a publicly-accessible online database (18, 19). Conversely, amplified fragment length polymorphism (AFLP), a whole genome typing method, also documents the contribution of accessory genetic elements next to the core genome polymorphisms as defined by MLST (7, 20). AFLP fingerprinting has been shown to have high potential for strain identification (7, 21-24).

In many low-income developing countries, hygienic conditions are severely compromised, and living in close contact with poultry and domestic animals within the same premises is common in both rural and urban areas. Recently, we reported an unusually high frequency of the axonal variant of GBS in Bangladesh, associated with preceding *C. jejuni* infection (57%) (25). We also reported *C. jejuni* MLST type ST-403 as being over-represented among GBS and enteritis related isolates in Bangladesh (26, 27). No information is available regarding the population structure of *Campylobacter* isolated from poultry in this country. To determine the role of poultry in the epidemiology of *Campylobacter* infections and their sequelae, we studied the diversity and clonal relationships among *C. jejuni* from humans and chicken with MLST, AFLP and pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial isolates

Sixty six *C. jejuni* isolated between January and June 2007 from chicken cloacae in the Dhaka area of Bangladesh, were included in the present study. The chickens originated from diverse geographic locales in the Dhaka region and were randomly selected. All isolates were cultured and identified to the genus and species levels as previously described (28). In addition, 10 *C. jejuni* strains isolated from stool specimens of GBS patients and 39 *C. jejuni* from enteritis patients were included (26). All GBS patients fulfilled the diagnostic criteria of the National Institute of Neurological Disorders and Stroke (NINDS) (29). Bacteria were grown on blood agar plates with 5% sheep blood at 37°C for 48 h under micro-aerophilic conditions, with 6% O_2, 7% CO_2, 80% N_2, and 7% H_2 using the Anoxomat system (Anoxomat™ Mark II, Drachten, The Netherlands). Bacteria were stored at -80°C in 15% glycerol in brain heart infusion broth.

Bacterial DNA isolation

Genomic DNA was isolated with the Qiagen Genomic DNA purification kit according to the manufacturer's instructions (Qiagen, Venlo, The Netherlands).

Multilocus sequence typing of *C. jejuni* isolates

Nucleotide sequence analysis of internal fragments of seven housekeeping genes (aspartase A, *aspA*; glutamine synthetase, *glnA*; citrate synthase, *gltA*; serine hydroxymethyl transferase, *glyA*; phosphoglucomutase, *pgm*; transketolase, *tkt* and ATP synthase α subunit, *uncA*) was performed as described by Dingle et al. (16). Where no amplification product was observed on agarose gel electrophoresis, the reaction was repeated while substituting the primers by those described by Miller et al. (18). The same primers were used for nucleotide sequencing, which was carried out at least once on each DNA strand using BigDye™ Ready Reaction Mix (Version 3, Applied Biosystems, Foster City, CA) at a concentration of $^1/_{32}$ of that described in the manufacturer's instructions. Nucleotide sequences were deposited in the Internet-accessible pubMLST *Campylobacter* database (http://*Campylobacter*.mlst.net), enabling the identification and assignment of sequence types (STs) and clonal complexes (CC). Related STs were assigned to clonal complexes as described by Dingle et al. (16) by identification of central genotypes and the sequential assignment of variants that differed at one, two, or three loci (16, 17). Alleles already present in the database were assigned those numbers; novel alleles and STs were submitted to the *C. jejuni* MLST database and assigned new numbers.

Phylogeny analysis

The 7-allele profiles were concatenated and used to construct genealogies using two methods for inferring evolutionary relationships among *C. jejuni* STs. First, relatedness of isolates was represented by a dendrogram constructed by cluster analysis using the unweighted pair group method with arithmetic means (UPGMA) in the programme Start2, available at http://www.pubmlst.org (30). The second phylogenetic analysis estimated the clonal genealogy of STs using the model-based approach to determine bacterial microevolution (31). This is a model that calculates clonal relationships with improved accuracy as it distinguishes point mutations from chromosomal recombination events. The latter comprise the source of the majority of allelic polymorphisms in *C. jejuni*. Analysis was carried out on concatenated sequences representing 35 STs, from 102 isolates from chicken, GBS patients and gastroenteritis patients. The programme was run with 50,000 burn-in followed by 50,000 subsequent iterations. The consensus trees represent combined data from three independent runs with 50% majority rule consensus required for inference of relatedness.

AFLP analysis and data processing

Isolates were typed by AFLP (20). In short, chromosomal DNA was digested with *Hin*dIII and *Hha*I and simultaneously ligated with restriction site-specific adapters for 2 h at 37°C. This was followed by a preselective PCR using adapter-specific primers with *Hin*dIII (5'-GACTGCGTACCAGCTT) and *Hha*I (5'-GATGAGTCCTGATCGC). Next, an aliquot was subjected to a selective PCR using a fluorescently labelled *Hin*dIII-specific primer that contained an additional A nucleotide at the 3' end (5'-GACTGCGTACCAGC TTA) and a *Hha*I-specific primer with an A extension (59-GATGAGTCCTGATCGCA). The final products were run on a 7.3% denaturing polyacrylamide gel for 5 h using an ABI 373A automated DNA sequencer. Fingerprints were collected by fluorography and interpreted with ABI Genescan software (PE Applied Biosystems). Gels were normalized using an internal ROX-labeled size standard included in each lane. Densitometric curves were processed with the GelCompar version 4.1 software (Applied Maths, Kortrijk, Belgium). After normalization and background subtraction, the levels of genetic similarity between AFLP patterns were calculated with the Pearson product-moment correlation coefficient (*r*).

PFGE and Data analysis

PFGE was performed as previously described (27, 32). In short, samples of genomic DNA extracted from overnight cultures of the isolates were digested with *Sma*I (Boehringer GmbH, Mannheim, Germany). Electrophoresis was performed in 1% SeaKem agarose in 0.53 Tris-borate-EDTA buffer by using a Bio-Rad CHEF Mapper programmed in the auto-algorithm mode (run time, 18 h; switch time, 6.76 to 35.38 s). Gels were stained with ethidium bromide for 30 min, destained in distilled water for 1 h; images of ethidium bromide-stained gels were captured under UV illumination by a video system (Gel DOC 1000; Bio-Rad).

Electrophoretic patterns from PFGE were compared by means of BioNumerics, version 4.01 (Applied Maths, Sint-Martens-Latem, Belgium). Analysis was based on band positions and derived by the Dice coefficient with a maximum position tolerance of 1%. Isolates were clustered by the unweighted pair group method using arithmetic averages. Statistical analysis was performed with EpiInfo (version 3.0) using 2 × 2 contingency tables. Fisher's exact tests were executed and 2-sided *P* values determined. Associations were considered significant at $P < 0.05$.

RESULTS

Diversity of MLST sequence types (ST) and clonal complexes (CC)

Sixteen STs were identified among *C. jejuni* isolated from chicken cloacae in Bangladesh. Six (37.5%) of the STs were single isolates, while 10 STs included between 2 and 13 isolates (Fig. 1).

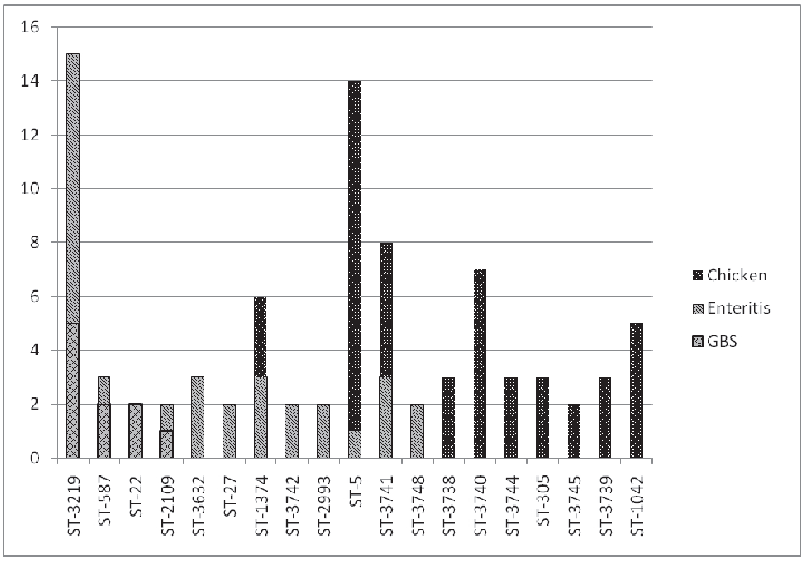


Figure 1. Distribution of sequence types (ST) among *C. jejuni* isolated GBS/enteritis patients and chicken in Bangladesh. Ten STs contained one isolate each, and are not included in the figure.

ST-5 was the most frequent MLST type among poultry isolates (n=13; 24.5%), followed by ST-3740 and ST-354, indicating 7 (13%) and 5 (9%) isolates respectively. ST-3738, ST-3739, ST-3744, ST-1374 and ST-305, covered 3 isolates each (Fig. 1). Overall, 6 STs representing 19 (36%) isolates were not reported previously (Table 1). The 16 STs were grouped into 5 previously defined clonal complexes (CCs). However, 9 STs featured in 28 isolates could not yet be grouped into defined clonal complexes. ST-353 complex encompassed the largest number of isolates (24.5%), followed by ST-354 (9%), ST-574 (8%) and the remaining STs (6%; ST-828, ST-257, and ST-1374). Thus, these CCs encompass almost half (48%) of the *C. jejuni* isolates characterized in the present study.

Clonal population structure and comparative analysis

The distribution of the *C. jejuni* isolated from enteritis, GBS and poultry were investigated by clonal frame tree demonstrating the genetic relationship of sequence types (Fig.2).

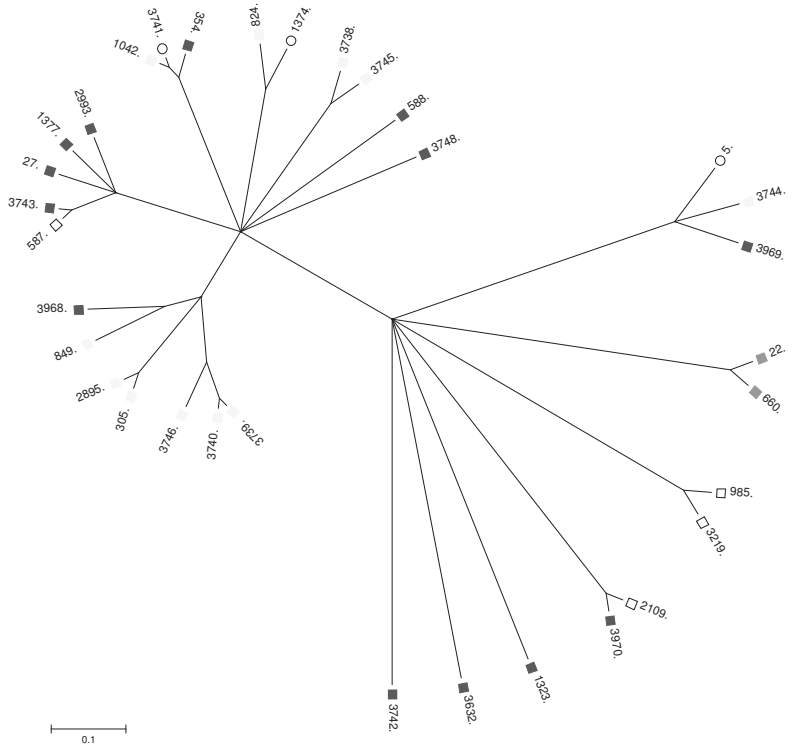


Figure 2. Clonal frame tree demonstrating the genetic relationship of sequence types between *Campylobacter jejuni* isolated from chicken, patients with gastroenteritis (GI) and Guillain-Barré syndrome (GBS) in Bangladesh. Chickens are shown as yellow boxes, GI blue boxes and GBS red boxes. Sequence types found associated with both GI and GBS patients are indicated by a white box, those found associated with both chicken and GI as white circle and those associated with both chicken and GBS patients as white triangle.

GBS isolates were found in ST-22, 403, and 362 complexes. The isolates from enteritis were most frequently represented with the ST-403 (28%), and ST-362 complexes (13%) (2009b). The most frequent ST-353 in poultry isolates was infrequently found among human isolates (4%). The ST-403 complex, the predominant ST in humans (GBS and enteritis) was not found among the isolates from poultry (Fig. 2). However, ST-22, which was only found among the isolates from GBS patients. We also compared 5 predominant STs from human and poultry; among them 22% isolates from human overlapped with poultry isolates (data not shown). Fisher exact test demonstrates a significant association between CC and the sources ($p<0.02$). UPGMA clustering of MLST data for *C. jejuni*

isolated from chicken and humans yielded 9 major clonal groups (A, B, C, D, E, F, G, H and I) consisting 25 of STs. Eight STs were singletons (Fig. 3).

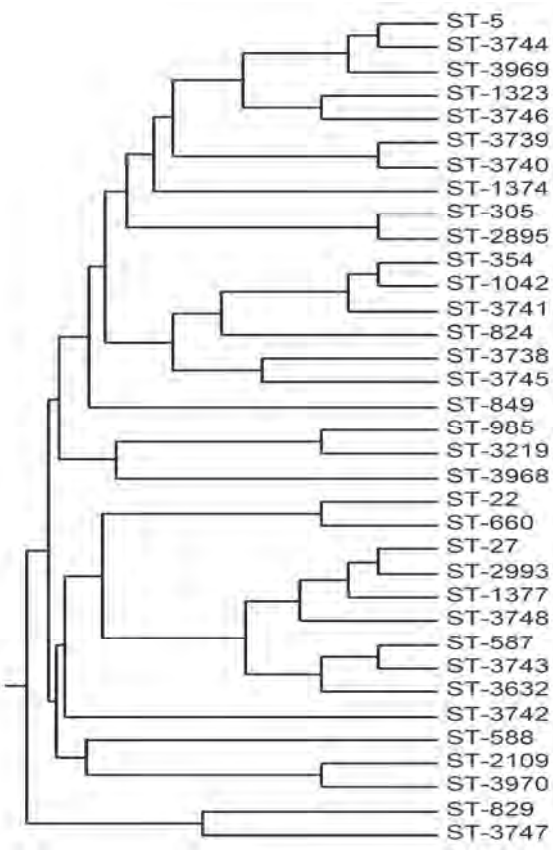
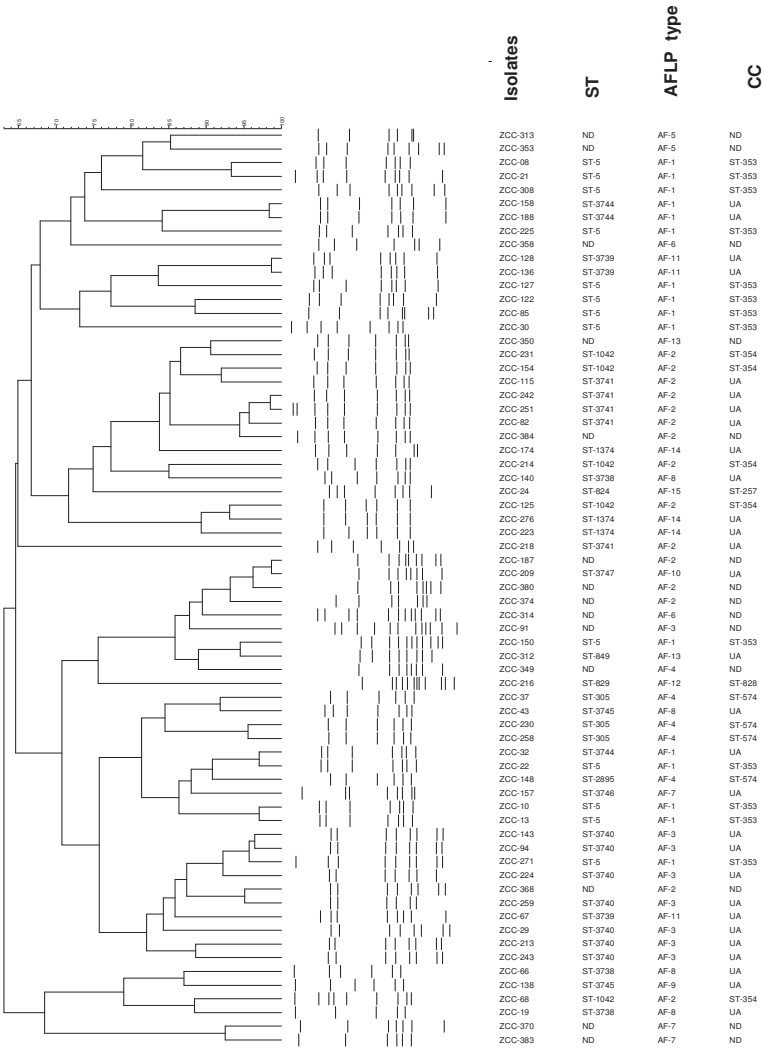


Figure 3. Dendrogram of *Campylobacter jejuni* sequence types, including GBS/enteritis and chicken related isolates from Bangladesh. The dendrogram was constructed by using UPGMA.

AFLP

AFLP fingerprints were identified as distinct types when band patterns shared less than 90% similarity (20). At a cutoff of 90%, clustering yielded 15 different AFLP types among the 66 *C. jejuni* isolated from poultry (Fig. 5). The largest cluster (AF-1) of AFLP patterns in chickens with similarities of 90% consisted of 16 (24%) isolates; other predominant clusters were AF-2 (21%) and AF-3 (14%). In addition, there were nine small clusters that each contained two to five isolates isolated from poultry. Three AFLP fingerprints of poultry isolates (ZCC-24, ZCC-324, & ZCC-382) were unique (Fig.

5). In a further effort to determine whether the AFLP patterns could be separated into host-specific



Isolates	ST	AFLP type	CC
ZCC-313	ND	AF-5	ND
ZCC-353	ND	AF-5	ND
ZCC-08	ST-5	AF-1	ST-353
ZCC-21	ST-5	AF-1	ST-353
ZCC-308	ST-5	AF-1	ST-353
ZCC-158	ST-3744	AF-1	UA
ZCC-188	ST-3744	AF-1	UA
ZCC-225	ST-5	AF-1	ST-353
ZCC-358	ND	AF-6	ND
ZCC-128	ST-3739	AF-11	UA
ZCC-136	ST-3739	AF-11	UA
ZCC-127	ST-5	AF-1	ST-353
ZCC-122	ST-5	AF-1	ST-353
ZCC-85	ST-5	AF-1	ST-353
ZCC-30	ST-5	AF-1	ST-353
ZCC-350	ND	AF-13	ND
ZCC-231	ST-1042	AF-2	ST-354
ZCC-154	ST-1042	AF-2	ST-354
ZCC-115	ST-3741	AF-2	UA
ZCC-242	ST-3741	AF-2	UA
ZCC-251	ST-3741	AF-2	UA
ZCC-82	ST-3741	AF-2	UA
ZCC-384	ND	AF-2	ND
ZCC-174	ST-1374	AF-14	UA
ZCC-214	ST-1042	AF-2	ST-354
ZCC-140	ST-3738	AF-8	UA
ZCC-24	ST-824	AF-15	ST-257
ZCC-125	ST-1042	AF-2	ST-354
ZCC-276	ST-1374	AF-14	UA
ZCC-223	ST-1374	AF-14	UA
ZCC-218	ST-3741	AF-2	UA
ZCC-187	ND	AF-2	ND
ZCC-209	ST-3747	AF-10	UA
ZCC-380	ND	AF-2	ND
ZCC-374	ND	AF-2	ND
ZCC-314	ND	AF-6	ND
ZCC-91	ND	AF-3	ND
ZCC-150	ST-5	AF-1	ST-353
ZCC-312	ST-849	AF-13	UA
ZCC-349	ND	AF-4	ND
ZCC-216	ST-829	AF-12	ST-828
ZCC-37	ST-305	AF-4	ST-574
ZCC-43	ST-3745	AF-8	UA
ZCC-230	ST-305	AF-4	ST-574
ZCC-258	ST-305	AF-4	ST-574
ZCC-32	ST-3744	AF-1	UA
ZCC-22	ST-5	AF-1	ST-353
ZCC-148	ST-2895	AF-4	ST-574
ZCC-157	ST-3746	AF-7	UA
ZCC-10	ST-5	AF-1	ST-353
ZCC-13	ST-5	AF-1	ST-353
ZCC-143	ST-3740	AF-3	UA
ZCC-94	ST-3740	AF-3	UA
ZCC-271	ST-5	AF-1	ST-353
ZCC-224	ST-3740	AF-3	UA
ZCC-368	ND	AF-2	ND
ZCC-259	ST-3740	AF-3	UA
ZCC-67	ST-3739	AF-11	UA
ZCC-29	ST-3740	AF-3	UA
ZCC-213	ST-3740	AF-3	UA
ZCC-243	ST-3740	AF-3	UA
ZCC-66	ST-3738	AF-8	UA
ZCC-138	ST-3745	AF-9	UA
ZCC-68	ST-1042	AF-2	ST-354
ZCC-19	ST-3738	AF-8	UA
ZCC-370	ND	AF-7	ND
ZCC-383	ND	AF-7	ND

Figure 4. UPGMA dendrogram of PFGE fingerprints from 66 *C. jejuni* isolates related with chicken. The percentage of genetic homology between banding patterns is indicated. MLST and AFLP types are plotted next to dendrogram. UA, unassigned; ND not done.

Table 1. Characteristics of the novel sequence types identified among *C. jejuni* in Bangladeshi chicken. New alleles are indicated in boldface type

Sequence types	Clonal complex	Frequency	MLST Allelic profile						
			aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
ST-3740	UA	7	7	4	5	68	11	1	**257**
ST-3744	UA	3	7	2	5	**341**	10	3	6
ST-3745	UA	2	24	30	**255**	2	89	59	6
ST-3741	UA	5	**234**	10	2	2	67	12	6
ST-3746	UA	1	7	84	5	10	**437**	3	6
ST-3747	UA	1	124	39	30	79	**438**	47	17

groups, we compared all poultry isolates with GBS and enteritis isolates. After clustering, each pattern within the three groups was compared by calculating the maximum similarities with the patterns of the members of each group. The analysis showed that only four enteritis isolates were related to the members of the predominant AFLP type (AF-1 and AF-2) from poultry (supplementary file). No GBS related isolates were found among the AFLP type of poultry isolates. However, 22% of human isolates overlapped with chicken isolates. Fisher exact test demonstrates a significant association between AFLP types and the sources ($p<0.001$) (data not shown).

Correlation between PFGE and MLST

The *SmaI*-PFGE fingerprints contained six to twelve bands and resolved 3 major lineages at 65% similarity. A significant degree of agreement between different typing methods was evident (Fig. 4). However, a one-to-one correlation between PFGE types and STs did not always exist. Some isolates with the same PFGE type had different STs, and on the contrary, isolates with the same ST had distinct PFGE types.

Congruence between typing methods

After cluster analysis by the respective methods and construction of a composite data set comprising the MLST, PFGE, and AFLP, a similar clustering of the isolates was observed. Categorical clustering of the MLST and AFLP profiles according to the Pearson product-moment correlation coefficient resulted in 91% congruence (Fig. 4).

DISCUSSION

We performed comparative genotyping of *C. jejuni* isolated from poultry and patients with enteritis and GBS, respectively in Bangladesh by MLST, AFLP, and PFGE fingerprinting. Although the number of poultry isolates was relatively small, remarkable diversity was observed by identification of various genotypes and clonal complexes. PFGE analysis identified 3 major lineages, which were essentially similar to the major lineages as defined by MLST and AFLP. We observed discrepancies between the results of the three typing methods, although limited in number. In comparison with MLST, 5 of the 13 ST-5 isolates are clustered separately by PFGE, but not by AFLP. A study carried out in England by Owen et al. (33) showed that *C. jejuni* isolates were highly diverse by PFGE analysis. A recent study characterizing Australian isolates by MLST and PFGE (34) reported that PFGE profiles are not good predictors of CC. Overall, *C. jejuni* genotypes found in GBS patients partially overlap with those found in enteritis patients. There seems to be a similar partial overlap between genotypes isolated from chicken and enteritis patients. Interestingly, no overlap was documented between *C. jejuni* genotypes encountered in GBS patients and in poultry. However, the number of GBS-related *C. jejuni* is notably too small to allow for definite conclusion in this respect.

In the present study, we identified five distinct clonal complexes with 16 STs in *C. jejuni* isolated from poultry. Consistent with our results, Dingle et al. 16 identified six clonal complexes in 34 isolates obtained from poultry. Kinana et al. (35) found seven clonal complexes in 46 isolates from poultry in Senegal. In contrast to previous studies in which the ST-21 complex was the largest complex (16, 17, 36), this complex appears to be uncommon among *Camylobacter* poultry isolates from Bangladesh. However, the ST-21 complex is widespread in multiple hosts and has previously been described to be associated with infections in humans, and with livestock and environmental sources (poultry, cattle, contaminated milk, and water) (16, 37). The ST-353 complex, the most

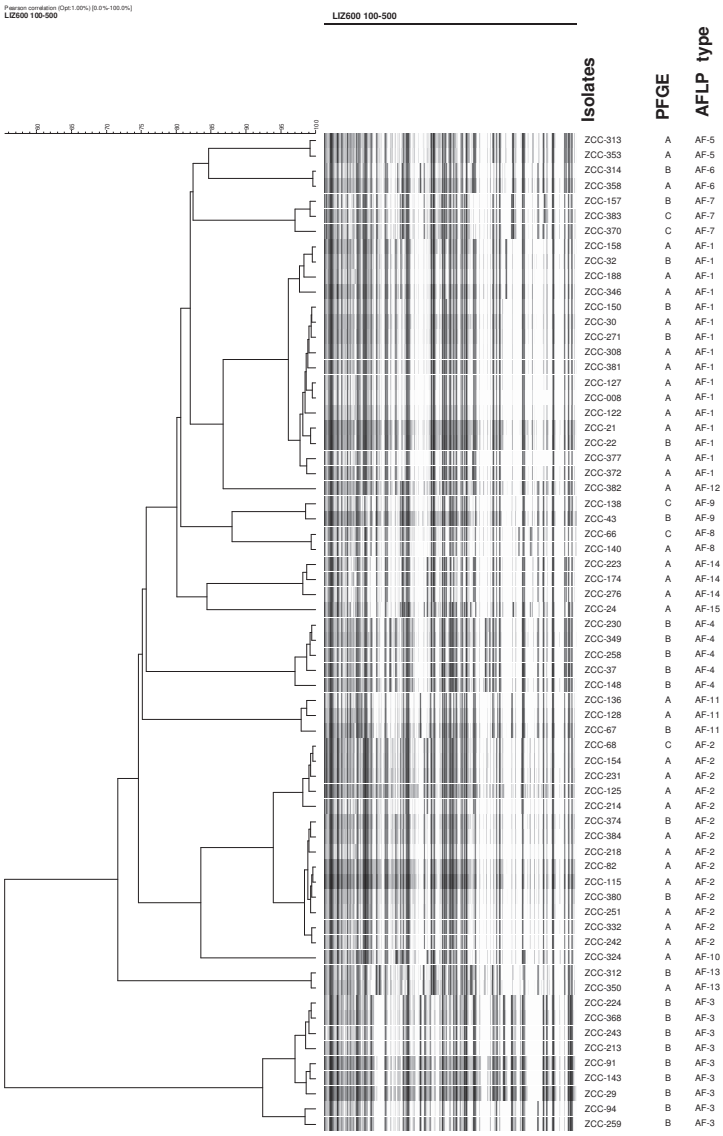


Figure 5. UPGMA dendrogram of AFLP fingerprints from 66 *C. jejuni* isolates related with chicken. The percentage of genetic homology between banding patterns is indicated. AFLP types and PFGE clonal groups plotted next to dendrogram.

common complex in this present study, and the ST-574 and ST-354 complexes have all been found previously in poultry (38). A study carried out in Senegal by Kinana et al. (35) found that the ST-353 complex is the most common complex among Senegalese poultry in accordance with our findings.

We recently reported that ST-403 is prevalent among GBS and enteritis-related *C. jejuni* from Bangladesh (26, 27). Our comparative genotyping analysis suggests that these ST-403 isolates are uncommon or absent in poultry in Bangladeshi chicken. Larger studies and nationwide studies will be necessary to confirm this preliminary observation. Recently, ST-403 has been demonstrated in *C. jejuni* isolated from dogs in England (39); this clonal complex has also been found in porcine isolates and also occasionally in cattle (40, 41,10, 42). This ST complex has also been observed in humans previously, and was reported to be the dominating genotype in a study in Curacao (16, 17, 43). In a large study carried out in New Zealand, McTavish SM et al. concluded that ST-403 strains were predominantly linked to cattle but not to poultry (44). A number of new STs from chicken isolates were identified in this study (ST-3740, ST-3741, ST-3744, ST-3745, ST-3746, and ST-3747), but were not assigned to any known complex.

Cluster analysis of AFLP data in this study supports previous reports that no distinct subpopulation of *C. jejuni* is associated with poultry, enteritis, or GBS (43). The AFLP fingerprints of all *C. jejuni* appeared to be highly heterogeneous, and no characteristic pattern of isolates infecting either chickens or humans was identified. Also, no separate grouping of GBS related isolates was obtained. The high diversity of AFLP fingerprints may be a reflection of the normal genetic diversity among *C. jejuni* as several other studies also have identified significant diversity among both human and chicken isolates (43).

A leading risk factor for human *Campylobacter*iosis is thought to be the consumption of contaminated poultry meat or cross-contaminated food (6). Only 11 (22%) out of 50 *C. jejuni* isolated from humans shared genotypes with poultry isolates. This suggests that sources other than poultry are likely to exist. A study carried out in Denmark by Siemer et al. (45) also recommend that poultry might not be the sole source of *Campylobacter* infection in human, and other sources should be considered as sources of human infection. Additional AFLP analysis of *C. jejuni* from other sources, including domestic pets, other animals and environmental samples, is needed.

This study has several limitations: first, the number of isolates from chicken was relatively small and the resuting loss of power may leave true associations undetected; second, although we sampled poultry from different geographical area, the precise origin was unknown. Larger, follow-up studies should include GPS mapping of all poultry and sample locations; third, as we identified only one bacterial colony per chicken sample, we cannot exclude the presence of more than one *Campylobacter* genotype/phenotype in poultry.

In conclusion, there appears to be considerable genetic diversity between *C. jejuni* obtained from poultry and humans in Bangladesh. Our data provide an insight into the complex population structure of *C. jejuni* in chickens. The dissimilarity of the sequence types/or genotypes of *C. jejuni* from human and poultry origin suggests the presence of sources other than poultry in Bangladesh.

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Chapter 6

General Discussion

Guillain-Barré syndrome (GBS) is an acute post-infectious immune-mediated peripheral neuropathy with a marked patient-to-patient variation in pathology, clinical presentation and prognosis, and worldwide the most frequent cause of acute flaccid paralysis (AFP) with an incidence of 1.2 to 2.3 per 100,000 persons per year (1). Bangladesh has achieved a remarkable success in its drive to eradicate poliomyelitis, however, non-polio AFP still exist. *Campylobacter jejuni* has been identified as the predominant cause of antecedent infection in GBS and its rare variant called Miller Fisher syndrome (MFS). Considering the crucial role of *C. jejuni* infections in triggering GBS, we hypothesized that this disease may be more frequent in countries with a high incidence of diarrhea and a poor hygienic infrastructure. To test this hypothesis, we determined the crude incidence of GBS in Bangladesh among children <15 years of age (Chapter 2.1); and we investigated the frequency of *C. jejuni* infection and anti-gangliosides antibodies in GBS patients, family controls and age- and sex-matched controls with other neurological diseases (Chapter 2.2). Molecular mimicry between *Campylobacter* LOS and gangliosides in human nerves is thought to be the underlying pathophysiological mechanism. We investigated the cross-reactivity of anti-ganglioside antibodies with *C. jejuni* strains from patients with GBS. We also determined the LOS outer core structures of *C. jejuni* strains associated with GBS/MFS (Chapter 4). In addition, to investigate whether specific bacterial determinants are related to GBS, we analysed the genetic heterogeneity among *C. jejuni* strains isolated from GBS, enteritis patients and poultry using various comparative microbial genotyping methods (Chapter 5.1, 5.2 and 5.3).

In this Chapter, the results of previous Chapters will be outlined and discussed, followed by concluding remarks and suggestions for further research.

Guillain–Barré syndrome in Bangladesh

Bangladesh has made an impressive progress towards the eradication of poliomyelitis, and no new cases have been reported since 2000. However, non-polio AFP cases are frequently diagnosed, and the incidence rate is currently 3.25 per 100,000 children less than 15 year of age. In Chapter 2.1, we aimed to determine the crude incidence rate of GBS in Bangladesh among children <15 years of age. As a part of South East Asia Region's polio eradication strategies, the Government of Bangladesh, in collaboration with World Health Organization, is conducting an active surveillance program for AFP. We obtained clinical and demographic data on the reported AFP cases in Bangladesh, and defined a case definition of GBS based on clinical data that is routinely collected as part of the AFP surveillance. The analysis indicates that poliomyelitis is absent, but that a great proportion of the AFP cases in Bangladesh are diagnosed as GBS. In this study, we calculated the

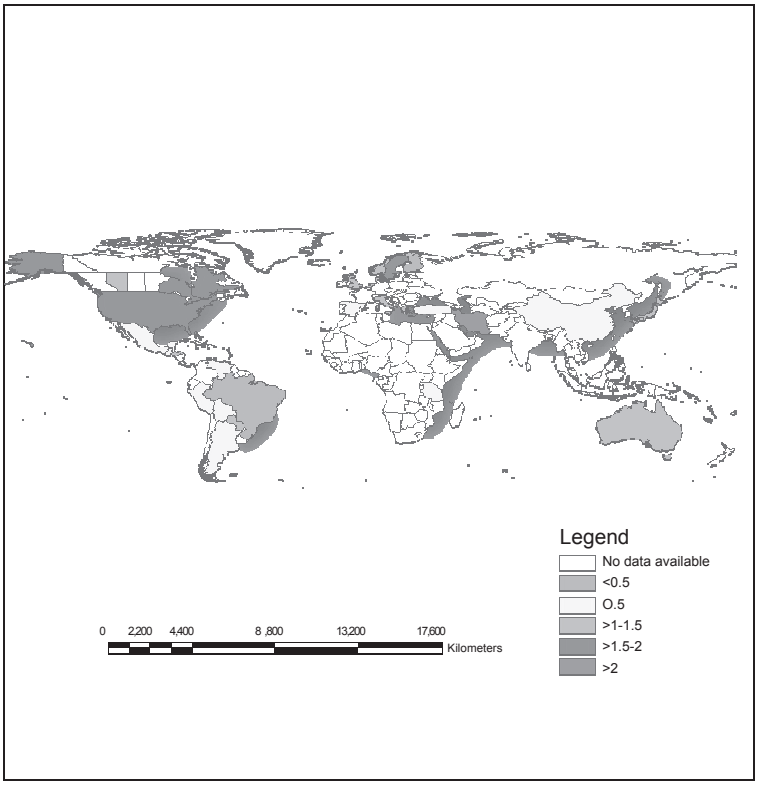

Figure 1. Guillain-Barré syndrome incidence among children (<15 years) in the world.

crude incidence of GBS for per division and per district in Bangladesh. In general, the crude incidence rates of GBS in children varied from 1.5 to 2.5 per 100,000 per year in Bangladesh. The incidence rate in the northern part of Bangladesh varied from 1.5 to 1.7 per 100,000 per year, whereas the rate in the southern part varied from 2.1 to 2.5 per 100,000 per year. A remarkable high incidence rate of more than 5.0 per 100,000 was observed in the Meherpur and Barisal district, in the southern part of Bangladesh. Thus**,** there appears to be a clear distinction between the three northern divisions and the three coastal divisions for which we currently have no explanation. A recent review reports that the overall incidence of GBS varies between 1.1/100,000/year and 1.8/100,000/year. The incidence of GBS increased with age after 50 years from 1.7/100,000/year to 3.3/100,000/year with lower rates reported in children (<15 years) of around 0.6/100,000/year (2). This review predominantly reports studies from Europe and North America. A limited number of rates was presented from other parts of the world, e.g. Asia and Africa. Thus, it is difficult to comment on possible geographical variation. A recent study carried out in Latin America and the Caribbean countries in children <15 years reports a GBS incidence of 0.82 cases per 100,000 children per year using the ongoing AFP surveillance

system (3). Therefore, the incidence rates in Bangladesh reported in this thesis appear to be at least 2-3 times higher compared to the incidence in the Americas and in Europe.

The use of the well-recognized National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) criteria or a comparable set of diagnostic criteria is critical as this allows comparisons to be made between studies. Therefore, our population-based study has some limitations. As our study was designed as a retrospective analysis of already collected clinical and demographic data focused on poliomyelitis, we cannot confirm if all cases fulfill the diagnostic criteria of the NINCDS. Further prospective surveillance studies are clearly indicated. Recently, a debate has been reopened on the association of H1N1 influenza virus infections and vaccination with the development of GBS. An assessment of the current or a modified AFP surveillance system for the purpose of GBS surveillance is, therefore, both timely and relevant.

Chapter 2.2 describes the clinical phenotype, course and outcome of GBS in Bangladesh. We conducted a prospective matched case-control study with a follow-up of six months including hospital-admitted patients in the Dhaka area of Bangladesh fulfilling the NINDS diagnostic criteria of GBS. Data were collected regarding age, sex, antecedent events, detailed neurological signs and symptoms, treatment, days to nadir, complications, duration of admission, GBS disability score and Medical Research Council (MRC) sum score at standard points (entry, and 2 weeks, 4 weeks, and 6 months after entry). Our study indicates that GBS in Bangladesh may predominantly affect young adult males, living in rural areas, and suffering from a severe, pure motor and axonal variant of GBS. The majority of patients (77%) did not receive specific treatment with IV immunoglobulin (IVIg). A poor outcome, defined as the inability to walk unaided at 6 months after disease onset, was found in 34 (43%) GBS patients. The demography and phenotype of GBS fluctuates significantly between different geographical areas. In Australia, Western Europe and North America, a preponderance of men is observed. The mean age of the patients in those areas is approximately 50 years. No seasonal variation has been reported for GBS incidence from developed countries (4-6). The most frequent subtype of GBS in those countries is AIDP, which accounts for at least 90% of GBS cases (7). However, in Asia, South and Central America, the axonal form of GBS constitutes 30% to 47% of cases (7) (Table 1). We reported that the majority (92%) of the GBS cases in Bangladesh have a pure motor variant with a predominantly axonal degeneration (67%). Also, the incidence of GBS, and of the AMAN subgroup in particular, appears to peak between January and March. Substantial variation in outcome exists and the prognosis of GBS is difficult to determine for individual GBS patients (1). A relatively large proportion of the patients in our cohort either died (14%) or remained severely disabled (29%) after a follow of 6 months.

Table 1: A comparative analysis of Guillain-Barré syndrome in developed and developing countries with Bangladesh

	Predominant age	Predominant season	Clinical presentation	Neurophysiology	Preceding infection	Anti-ganglioside antibodies	Prognosis	Ref
Developed countries	None	None	Variable	AIDP (90%)	*C. jejuni* (23-32%)	various gangliosides	variable	(7)
Developing countries	Young adult	Summer	Pure motor	Axonal (30-47%)	*C. jejuni* (19-66%)	GD1a or GM1	unclear	(7)
Bangladesh	Young adult (mean age 21 years)	January-March (Winter and spring)	Pure motor (>90%)	Axonal (67%)	*C. jejuni* (57%)	GM1 (50%)	poor	This work

We conclude that GBS patients in Bangladesh have a much poorer prognosis as compared to patients in developed countries (8). The poor outcome can be explained in part by the high frequency of preceding *Campylobacter* diarrhea and the severity of the disease in the acute phase, which are to defined poor prognostic factors (8, 9). Only 23% of GBS patients from our cohort received a specific treatment with IVIg. There was no appear to be the most frequently observed GBS variants in Bangladesh. A great majority of patients are children and young adults who do not receive any specific treatment. Our study has limitation; first, all patients in our cohort enrolled from three largest tertiary hospital in Dhaka area of Bangladesh which may admit more severe patients. Second, selection bias could possibly have been introduced as preferential selection of males in hospitalization is not an unknown phenomenon in developing countries.

C. jejuni and other antecedent infections

GBS is a typical post-infectious disease with a wide spectrum of clinical phenotypes, and differences in immunologic, electrophysiological and pathologic findings (10, 11). At present, infections that trigger the disease are identified in about two-third of all GBS patients. The most common triggers reported in case-control studies are preceding infections caused by *C. jejuni,* Cytomegalovirus (CMV), *Mycoplasma pneumoniae* and Epstein-Barr virus (EBV) (12). The etiology and diagnosis of preceding infections is complicated by the fact that in many GBS patients the antecedent infection is subclinical and may therefore not be noticed. *C. jejuni* has been identified as the most frequently diagnosed infection, preceding GBS in 14-66% of all cases (Chapter 1). However, there is still a paucity of information from the developing parts of the world. Prior to the study reported in this thesis, no systematic etiological study was ever done in Bangladesh.

In Chapter 2.2, we described the frequency of preceding *C. jejuni* infections in GBS patients and controls. Pretreatment serum samples, obtained within 2 weeks of neurological onset, were available in 97 (97%) of these patients for ELISA. Positive *C. jejuni* serology was found in 57% of the patients as compared to very low frequencies in the control group (8% of FC and 3 % of OND, $P<0.001$). Diarrhea in the four weeks preceding GBS was reported in 28 (51%) of the 55 *C. jejuni*-positive patients, as compared to 8 out of 42 (19%) *C. jejuni*-negative patients ($P<0.01$). The association between GBS in Bangladesh and recent *C. jejuni* infections is further supported by the culture of *C. jejuni* in 10 GBS patients. *C. jejuni* frequently induces a severe form of axonal GBS in young adults in Bangladesh. The geographical differences in demography and phenotype may relate to differences in exposure to specific infectious agents and/or to host-related or environmental factors. Studies from The Netherlands, UK, Mexico and Japan report that *C. jejuni* infections are the predominant cause of antecedent infections in GBS (9-14). Infections with *C. jejuni* are common in Chinese GBS patients. However, if stringent criteria for seropositivity are used, only 24% of these Chinese GBS cases were associated with *Campylobacter* (15). The frequency of *C. jejuni*-related GBS in many counties is not comparable due to the differences in diagnostic assays used. However, the diagnostic assay used in our study has been reported to be very sensitive and specific with GBS sera (16).

In addition, recent infections with CMV, EBV, and *M. pneumoniae*, were also demonstrated in GBS patients, but comparison with controls did not revealed statistical significance (Chapter 3). The seroprevalence of coxsackieviruses (36%) is significantly associated with GBS ($p<0.01$). In this study, 64% of coxsackieviruse-associated GBS patients have a positive serology for *C. jejuni* infection, as was found in other serological studies as well (12). A recent prospective multicentre study in children with GBS demonstrated that coxsackie viruses are associated as antecedent infection (17). Our findings on coxsackievirus-associated GBS must be confirmed in prospective large case-control studies using sensitive and specific serological assays and new molecular detection tools. Based on our prospective case control study, we conclude that antecedent *Campylobacter* infections play a prominent role in GBS in Bangladesh, but that in addition, several other agents may be involved may play an important role as well. Our data strongly indicate that infections with *C. jejuni* are the most important trigger for axonal variants of GBS in Bangladesh and are associated with significant morbidity and mortality. *C. jejuni* related GBS appears to be a dominant cause of AFP after the eradication of poliomyelitis in Bangladesh. This study generates some interesting questions concerning the high frequency of occurrence of the axonal variant of GBS in Bangladesh, associated with preceding *C. jejuni* infection: 1) Which anti-ganglioside antibodies are present in sera from GBS patients? 2) Which is the disease mechanism and are the *C. jejuni* lipooligosaccharides that cross-react with anti-ganglioside antibodies the only inducing neural damage as in other geographical locales?; 3) Are there genetic characteristics that distinguish GBS and MFS-associated *C. jejuni* strains from other *C. jejuni* strains? These issues are discussed in the following sections.

Anti-ganglioside antibodies in GBS

Previous studies showed that high titers of serum antibodies to gangliosides are associated with immune-mediated neuropathies, in particular with GBS. Antibodies to GM1, GD1a and GQ1b are associated with specific immunopathological subgroup in GBS. Interestingly, GM1 and GD1a are predominantly expressed in motor nerves and axons, and GQ1b has found to be enriched in cranial nerves (18). The study in Chapter (2.2 & 4) describes the presence of anti-gangliosides antibodies (to GM1, GD1a and GQ1b) which were more frequent as compared with controls ($P<0.001$). In GBS patients, antibodies to GM1 were more frequent (50%) than antibodies to GD1a (14%). *C. jejuni* infection was more often found in GBS patients with anti-ganglioside antibodies (78%) as compared to patients without anti-gangliosides antibodies (26%) ($P<0.001$). Anti-ganglioside antibodies of the IgG class are almost exclusively (49%) produced in patients with GBS. Our results are consistent with earlier findings that the presence of anti-GM1 antibodies is significantly associated with *C. jejuni* infection (18-20). Diarrhea in the four weeks preceding GBS was significantly associated with ganglioside-positive patients compared with ganglioside-negative patients ($P<0.01$). We did not find an association between anti-GM1/GD1a antibodies and overall disability at the peak of the illness. However, recent *C. jejuni* infection and anti-ganglioside antibodies are significantly associated with poor outcome of GBS patients after 6 months follow up.

Molecular mimicry and cross-reactive antibodies against *C. jejuni* LOS

Molecular mimicry is the mechanism by which infections trigger cross-reactive antibodies or T-cells that cause the symptoms of autoimmune disease (21). Many studies on the pathogenesis of GBS report findings that are consistent with the mimicry hypothesis, but none of these studies originate from a developing country such as Bangladesh. Several examples of molecular mimicry between microbial and "self" components are known, but in most cases no epidemiological relationship between autoimmune disease and microbial infection has been established. In 2004, Ang et al. proposed four criteria for auto immune-mediated disease and molecular mimicry: (1) The establishment of an epidemiological association between the infectious agent and the immune-mediated disease; (2) The identification of T-cells or antibodies directed against the patient's target antigens; (3) The identification of microbial mimics of the target antigen; and (4) Reproduction of the disease in an animal model. Many have concluded that GBS subsequent to *C. jejuni* enteritis fulfills all 4 criteria, although a reliable, reproducible animal model has not been described yet. Molecular mimicry may be responsible for the initiation of human autoimmune diseases (22). In general, autoimmune diseases occur in adults >50 years of age and the reported mean age of GBS patients in developed countries is in line with this observation. However, as the mean age in our prospective cohort was 21 years (Chapter 2.2), we wondered whether molecular mimicry was likely to be involved in this young age group.

The studies described in Chapter 4 provide serological evidence for the involvement of molecular mimicry in the pathogenesis of *C. jejuni*-related GBS in Bangladesh. In this regard, we aimed to determine the frequency of anti-LOS antibodies in GBS patients. In addition, we studied the cross-reactive activity of serum ganglioside antibodies with autologous *C. jeuni* LOS. Anti-LOS IgG antibody was significantly associated with GBS as compared with data from two control groups ($p<0.001$). Anti-LOS antibody presence is strongly associated with recent *C. jejuni* infection and anti-ganglioside antibodies from our cohort of GBS patients. In an earlier section, we described that *C. jejuni* is the causative agent of the predominant antecedent infection in GBS (Chapter 2.2). Some GBS patients in our study did not develop anti-ganglioside antibodies although anti-LOS antibodies were detected. This result is not new as others have reported on the absence of binding of anti-ganglioside antibodies to LOS of *C. jejuni* from MFS and GBS patients (23). These patients may have antibodies against ganglioside complexes (24). Preliminary data from our cohort suggests that patients with anti-LOS reactivity but without reactivity against single gangliosides have antibodies against complexes of different gangliosides (Islam et al., unpublished data, 25). This is in agreement with earlier studies reporting that ganglioside complexes are important target antigens in GBS as well as in MFS (26, 27). The current study supports the hypothesis that cross-reactive antibodies to single gangliosides or ganglioside complexes in these patients contribute to the development of GBS in Bangladesh. Our study results are in accordance with earlier studies that report that antecedent *C. jejuni* infections in GBS trigger the production of antibodies that cross-react with gangliosides (22, 23).

The role of molecular mimicry between LOS and gangliosides is further strengthened by the binding of mouse monoclonal anti-ganglioside antibodies with LOS of *C. jejuni* isolates from GBS and

enteritis controls (Chapter 4). Biochemical analysis of the LOS core structures identified the presence of sialic acid containing ganglioside-like moieties that show a large variation among different *C. jejuni* strains (Chapter 1). The GBS-associated *C. jejuni* strains express core oligosaccharides with a tetrasaccharide identical to GM1 (28), but also oligosaccharides similar to GD1a, GD3 and GT1a were identified (29). Our studies have clearly shown that LOS from GBS associated *C. jejuni* is recognized by anti-ganglioside antibodies from GBS patients. Serum from patients with GBS showed a dramatic reduction in anti-ganglioside reactivity after incubation with LOS from the autologous *C. jejuni* strains. Incubation with LOS that lacked ganglioside-mimics did not inhibit ganglioside-reactivity. In conclusion, these binding studies indicate that carbohydrate molecular mimicry exists between gangliosides and LOS from *C. jejuni* isolated from Bangladeshi GBS patients can result in the production of cross-reactive antibodies in GBS patients following *C. jejuni* infection.

The variety of ganglioside-like structures among *C. jejuni* strains may contribute to the heterogeneity of the anti-ganglioside antibody specificities in GBS. Our observations also indicate that a one-dimensional approach is inadequate in describing the relationship between antibody specificity and LOS carbohydrate composition. Several host but also pathogen-related factors may be involved in a continuing process of changing forms and shapes of molecules that potentially play a role as receptor or immune-modulating factor interacting between cells and pathogens.

Ganglioside mimics in GBS-associated *C. jejuni* strains

In the previous section, we described serological methods to identify ganglioside-like structures in the *C. jejuni* LOS. The set of *C. jejuni* isolates from Bangladeshi GBS patients available for the studies described in this thesis provided a unique opportunity to study both pathogen and host-related factors in GBS pathogenesis. Further molecular characterization of epitopes present in the carbohydrate part of *C. jejuni* LOS by mass spectrometry is described in Chapter 4. A structural analysis demonstrates that various ganglioside mimics are found in the LOS of neuropathy-associated strains in Bangladesh (Table 2). Interestingly, 57% of GBS-associated strains express a mixture of GM1a- and GD1a-like LOS. This finding suggests that a cluster or complex of the GM1a/GD1a ganglioside mimics, which is only expressed by strains with a class LOS A locus, may be the primary target antigens in a subgroup of GBS, rather than single ganglioside mimics. Our finding is consistent with the results described earlier (30, 31). However, it does not support the outcome of another study that reports that the expression of GD1a and not GM1 was associated with GBS (32). In Chapter 4, we described that *C. jejuni* strains isolated from GBS/MFS with ophthalmoplegia have a novel structure with two extension sites from the inner core and two branches mimicking gangliosides GA2 and GD3. The *Cst-II* variant in these strains is bi-functional (*Asn51*) which further supports the presence of di-NeuAc. The absence of a terminal Galβ-1,3-linked residue is consistent with a *cgtB* gene that has a frame-shift mutation. Others also report that the LOS core oligosaccharide from MFS-associated *C. jejuni* bears a GD3-like terminal trisaccharide rather than a GQ1b moiety (33). Our data strongly support the previous observation that the ganglioside–like structures in LOS may not always correspond exactly with the specificity of the serum anti-ganglioside antibodies (30).

Table 2: True molecular mimicry of *C. jejuni* LOS confirmed by mass spectrometry analysis

Strains	*C. jejuni* LOS class	LOS structure	Anti-ganglioside antibodies
BD-07	A	GM1a, GD1a	GD1a
BD-10	B	GA2/GD3	GD1a/GQ1b
BD-22	B	GA2/GD3	GM1
BD-27	A	GM1a, GD1a	GM1
BD-34	B	GA2/GD3	GM1
BD-39	A	GM1a, GD1a	GM1
BD-67	B	GA2/GD3	GM1/GD1a/GQ1b
BD-74	B	GA2/GD2	GD1a/GQ1b
BD-75	A	GM1a, GD1a	GM1/GD1a
BD-94	E	No ganglioside mimic	-

In conclusion, these antibody binding studies and biochemical studies give strong support for the hypothesis that carbohydrate molecular mimicry exists between gangliosides and LOS from *C. jejuni* isolated from Bangladeshi GBS patients which can be recognized by cross-reactive mouse monoclonal and patients serum antibodies. The cross-reactive antibody response associated with *C. jejuni* infections is probably the predominant cause of GBS in Bangladesh.

However, mass spectrometry showed that only 1 of 10 GBS-associated strain did not express ganglioside mimics. Absence of ganglioside mimics in a strain isolated from a GBS patient does not necessarily imply that ganglioside mimicry was not the causative mechanism for GBS in that patient. Co-infection with different *C. jejuni* strains may occur in GBS patients (30). It is possible that a ganglioside-mimicking *C. jejuni* strain induces GBS, but that a co-infecting strain without ganglioside mimics is isolated from the stool sample and wrongfully regarded as the strain that caused GBS. In addition, it is possible that the expression of ganglioside mimics down regulated in the course of infection or during subculture after isolation of the *C. jejuni* strain from the feces. This form of phase variability has been convincingly demonstrated for *C. jejuni* as a species.

Comparative genomic analysis of *C. jejuni* strains isolated from humans and poultry

Campylobacter strains with certain Penner heat-stable (HS) serotypes, including HS:19 and HS:41, are overrepresented among isolates from GBS cases as compared with enteritis cases in Japan, South Africa, China, and Mexico (34, 35). Several studies indicate that *C. jejuni* HS:19 and HS:41 have a clonal population structure and suggest that these serotypes might have unique virulence properties that are intricately linked to the development of GBS (31). However, data from the United Kingdom and The Netherlands suggest that such virulence properties may not be restricted to specific HS serotypes since various other serotypes are also associated with GBS in other parts of the world (36). In other studies, no clustering of the GBS or MFS-associated strains was found using several genotyping methods. To understand the molecular epidemiology of *C. jejuni*-related GBS in Bangladesh we analyzed *C. jejuni* strains isolated from GBS and relevant control strains. In addition, we describe the population structure analysis of *C. jejuni* strains isolated from GBS patients and enteritis patients, and poultry by various comparative genotyping methods.

In Chapter 5.1 we described that *C. jejuni* HS:23 serotype was highly prevalent (50%) among GBS-related *C. jejuni* strains from Bangladesh. *C. jejuni* HS:23 serotype was also common (28%) in uncomplicated enteritis patients in Bangladesh. In other reports, serotypes HS:19 or HS:41 are linked to the development of GBS (31, 37). Our serotyping results indicate that in addition to HS:19 and HS:41 strains, other serotypes also have the potential to induce anti-ganglioside antibodies and GBS. We found that *C. jejuni* HS:23 serotype and the new ST-3219 is highly prevalent among GBS-related *C. jejuni* strains from Bangladesh. This observation is consistent with previous reports that specific serotypes are overrepresented among GBS-related *C. jejuni* strains. These observations support the hypothesis that, although a great variety of *C. jejuni* serotypes can be isolated from GBS patients in some geographical areas (36), specific clonal serotypes and multi-locus types are prevalent in GBS patients in other places (37).

Chapter 5.2 describes the comparative genotyping analysis of *C. jejuni* strains isolated from GBS and enteritis patients in Bangladesh by MLST, AFLP, PFGE fingerprinting, and LOS typing. *C. jejuni* strains isolated from GBS patients in the developed world have been extensively characterized. We for the first time report the molecular characterization of GBS and enteritis related *C. jejuni* strains from Bangladesh. At present, many molecular typing methods are available to study the population structure of *Campylobacter* (34). PFGE detects genetic variation between strains using rare-cutting restriction endonuclease, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and it is a frequently used technique for outbreak investigations. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies (38). Despite the efforts to standardize protocols and interpretation criteria of PFGE data, comparison of inter-laboratory results usually remains difficult. Multilocus sequence typing (MLST) has been considered the state-of-the-art technique for bacterial population genetics (39) and is now recognized as the gold standard typing method for the phylogenetic study of the *Campylobacter* genus (40). AFLP is an alternative whole genome typing method, which has a high potential for strain identification (41-45).

We found 7 distinct clonal complexes with 22 different STs by MLST (Chapter 5.2). The predominant Bangladeshi lineage was the ST-403 complex. This ST-403 complex contained 50% of the GBS-related strains studied and 26% of the enteritis strains. Our result is consistent with earlier studies that the ST-403 complex has also been observed in humans, and has been reported to be the dominating genotype in a study in Curacao (46). According to the pubMLST database, ST-21 is the prevalent complex in the general population structure of *C. jejuni* (39), it is common in multiple hosts and has previously been described to be associated with infections in humans, and with livestock and environmental sources; as in chicken, cattle, contaminated milk and water (39, 47, 48). No representatives of ST-21 were present among GBS and enteritis related strains from Bangladesh. A recent study from Belgium demonstrates that strains from the ST-21 complex also shared a class C LOS locus (49). Both ST-21 and LOS C appear to be rare in Bangladesh. Our GBS-associated strains are assigned to different clonal complexes, which correlated with earlier data describing heterogeneity among neuropathogenic *C. jejuni* strains (36, 39). In our study, the ST-22 complex coincided with the HS:19 serotype. These types were only found among GBS-related *C. jejuni* ; ST-362 complex was the second most prevalent complex found in both GBS and enteritis strains. A significant number of new STs were identified in this study (ST-3442, ST-3741, ST-3743, ST-3748, ST-3968, ST-3969 and ST-3970), but these have not yet been assigned to any known complex. AFLP analysis demonstrated that not a single distinct subpopulation of *C. jejuni* strains was associated with GBS or enteritis. However, AFLP analysis revealed that HS:23 strains are clonal but substantial heterogeneity is found among non-HS:23 strains. Our population structure analyses demonstrate that a significant overlap of *C. jejuni* genotypes can be demonstrated by AFLP, PFGE and MLST. As in previous studies we could not identify GBS-specific genetic markers by comparing the genomes of *C. jejuni* strains from GBS vs non-GBS patients. (36, 39, 50, 51). A very recent study from Godschalk et al., did not report molecular markers specific to GBS on the basis of high-throughput AFLP (30). Based on our study, it can be concluded that *C. jejuni* serotype HS:23 strains are over represented among GBS related strains in Bangladesh and appear to be clonally related.

Biochemical analysis demonstrates that the lipooligosaccharides (LOS) of *C. jejuni* exhibit molecular mimicry with gangliosides in peripheral nerves (52). The LOS biosynthesis genes are clustered in the LOS locus. Variation in gene content is clearly reflected by the different classes of the LOS locus (53). In Chapter (5.1, 5.2), we determined the presence of five specific classes (A-E) of LOS. Interestingly, the class A locus was significantly associated with GBS without oculomotor symptoms whereas the class B locus associated with GBS with oculomotor symptoms. We showed that the class A and B LOS loci are associated with the expression of ganglioside mimics and with neuropathy. Others have reported a strong association between GBS-associated *C. jejuni* isolates and simultaneous presence of three LOS biosynthesis genes, *cst-II*, *cgtA* and *cgtB* (32). This observation is consistent with our findings, as these three genes are present in class A and B LOS loci, only. Comparison of the LOS loci of various *C. jejuni* strains has demonstrated that only the class A, B and C loci contain the genes that are necessary for the biosynthesis of ganglioside mimics (53, 54). Our observation that only *C. jejuni* with a class A and B LOS locus are associated with neuropathy is,

therefore, consistent with the ganglioside mimicry hypothesis. In our study 46% of the control *C. jejuni* isolated from enteritis patients also contained a class A or B LOS locus. This finding is in accordance with Dutch study as more than half of the enteritis-related *C. jejuni* strains contained a neuropathy-related LOS locus (55). Indeed, all GBS-related strains and 64% of the other clinical and environmental strains belonged to an LOS class (LOS class A, B, or C) that allowed the synthesis of a sialylated LOS (53). The LOS class may, therefore, be a necessary but not a sufficient determinant involved in the pathogenesis of GBS. Other, yet undetected host-related or pathogen-related factors may therefore interact in addition and drive the development of post-infection neuropathy (31).

In Chapter 4, we described that *C. jejuni* with the class A LOS locus are associated with GBS and GM1-like mimicry and that those strains with a class B locus are associated with MFS and GQ1b-like mimicry. There is only one difference in LOS cluster content between the class A and B. Class B contains a duplication of *cgtA*, encoding a β-1,4-GalNAc transferase. Because *cgtA* is not directly involved in the sialylation of the LOS outer core, it is less likely to be a determinant involved in the synthesis of cross-reactive structures and probably other factors are more likely to be involved. In our study, we did not find any class C LOS containing strain among the GBS-related *C. jejuni* strains (Chapter 5.1). Studies described in this thesis and by others show that most class A strains express a mixture of GM1a and GD1a mimics (31). Further research is needed to determine whether the GM1a/GD1a mixture is more effective than single ganglioside mimics in order to assist in the development of a crossreactive immune response that leads to GBS.

Chapter 5.3 describes the diversity and clonal relationships among *C. jejuni* from humans and poultry origin with three well-known typing methods, PFGE, MLST, and AFLP. We determined the population structure of 66 *C. jejuni* isolated from poultry, and also determined the similarities with *C. jejuni* isolated from enteritis patients by a comparative genotyping approach. MLST defined 16 sequence types (STs) and 5 clonal complexes (CCs) among poultry isolates, including 6 STs not previously documented in the PubMed database. We investigated the distribution of the *C. jejuni* isolated from humans and poultry origin by MLST clonal frame tree analysis to demonstrate the genetic relationship of certain sequence types. GBS isolates were found in ST-22, 403, and 362 complexes. The isolates from enteritis were most frequently represented with the ST-403 (28%), and ST-362 complexes (13%) (Chapter 5.2). The most frequent poultry ST-353 clonal complex was infrequently found among human isolates (4%). The ST-403 complex, the predominant ST in humans (GBS and enteritis) was not found among the isolates from poultry. However, ST-22, which was only found among the isolates from GBS patients. We also compared 5 predominant STs from human and poultry; among them 22% of all isolates overlapped genetically. In contrast to earlier studies, the ST-21 complex was the largest complex among poultry isolates of *C. jejuni* (39, 40, 56); however, this complex appears to be uncommon among *Camylobacter* poultry isolates from Bangladesh. The ST-353 complex, the most common complex in this present study, and the ST-574 and ST-354 complexes have all been found previously in poultry (57). The ST-353 complex is the most common complex among Senegalese poultry in accordance with our findings (58). Our comparative genotyping analysis suggests that the ST-403 isolates are uncommon or absent in poultry in Bangladeshi chicken. Our study and those of others suggest that the main source of ST-403 may be of non-poultry nature. A

recent study from England demonstrated that ST-403 has been found among *C. jejuni* isolated from dogs (59); this clonal complex has also been detected among porcine isolates and also occasionally in cattle (48, 60- 62). A large study carried out in New Zealand concluded that ST-403 strains were predominantly linked to cattle and not to poultry (63). A nationwide larger study will be necessary to confirm our preliminary observation and determine the source of the predominant STs in *Campylobacter*-related GBS in Bangladesh.

Highly heterogeneous fingerprints among *C. jejuni* strains in Bangladesh have been demonstrated by AFLP, and no typical pattern of isolates infecting either poultry or humans was identified. Others before us also reported on the high diversity of AFLP fingerprints among both human and chicken isolates (51). In this study, only 22% of human-related strains shared AFLP genotypes with poultry isolates. This finding is in accordance with our MLST data; and the most commonly identified AFLP type coincided with the ST-353 complex. Considering the AFLP and MLST data it could be concluded that other sources than poultry are accountable for human *Campylobacter*iosis in Bangladesh. Poultry might not be the sole source of *Campylobacter* infection in humans, and other sources might be considered as sources of human infection (64). However, our studies have some limitations: first, the number of isolates from poultry was too small to draw any straight forward conclusion for human *Campylobacter*iosis in Bangladesh; second, the precise origin of several strains was not known; third, as we considered only one bacterial colony per chicken sample, we cannot exclude the presence of more than one *Campylobacter* genotype/phenotype in poultry. Others also reported that it would be extremely difficult to disclose the sources of infection in humans as mixed infections have been demonstrated by analysis of fecal samples from flocks (65). A Dutch study reports that a single chicken may harbor a multiplicity of at least four different strains (45). Finally, substantial genetic diversity was found among *C. jejuni* strains isolated from poultry and humans in Bangladesh. Further studies with a larger numbers of isolates, including isolates from humans, poultry, other animals and environmental sources will provide more insight on the epidemiology of *Campylobacter* in Bangladesh.

Conclusions

The studies described in this thesis lead to the following conclusions:

- Despite the fact that polio has been eradicated in Bangladesh non-polio AFP is still prevalent in children <15 year of age. A significant proportion of these cases of non-polio AFP is caused by GBS. The AFP surveillance program can be used to obtain crude incidence data of GBS. We recommend a proper validation and further expansion of the surveillance system for this purpose (Chapter 2.1).

- Pure motor and axonal variants are the predominant forms of GBS in Bangladesh. Cranial nerve involvement is infrequent. *C. jejuni* is the locally predominant antecedent infectious agent in GBS. Anti-ganglioside antibodies are significantly

associated with *C. jejuni* infection. The axonal variant of GBS associated with *C. jejuni* infection, severe residual disability, and high mortality (Chapter 2.2).

- There is no significant association between cytomegalovirus, Epstein-Barr virus, and *Mycoplasma pneumoniae* and GBS in Bangladesh (Chapter 3).

- *O*ur results strongly support the hypothesis that anti-GM1 antibodies in GBS patients are induced by antecedent infections with GM1-mimicking *C. jejuni* strains. Molecular mimicry between gangliosides and *C. jejuni* LOS appears to be causally involved in the pathogenesis of *C. jejuni*-related GBS in Bangladesh. Antibodie*s* against gangliosides cross-reacted with corresponding *C. jejuni* LOS. The LOS structures of the *Campylobacter* strains isolated from GBS patients are similar to those in the corresponding ganglioside, suggesting that conformational epitopes may trigger the antibody response in these cases (Chapter 4).

- A new *C. jejuni* HS:23 serotype and a new sequence type (ST-3219) is highly prevalent among GBS-related *C. jejuni* strains from Bangladesh. LOS class A is associated with GBS and monosialylated ganglioside (GM1-like) mimics. LOS class B is associated with GBS/MFS and disialyated ganglioisde (GD3-like) mimics (Chapter 4, 5.1).

- Comparative genotyping analysis by MLST, AFLP and PFGE demonstrate that HS:23 strains from GBS or enteritis patients are clonal. We did not identify specific genetic determinants that differentiate GBS-associated *C. jejuni* strains from enteritis strains (Chapter 5.2).

- The population structure of *C. jejuni* isolated from poultry, and humans are highly heterogeneous. The most common ST-353 clonal complex in poultry is infrequent among human isolates. The predominant ST-403 complex in humans (GBS and enteritis) is not found among poultry.

Future perspective

GBS epidemiology: situation of Bangladesh in a global perspective

In order to acquire a global perspective, we compared our findings of the incidence of GBS in Bangladesh with similar data available in published reports from other countries both from developed and developing parts of the world (Fig.1). We compared the GBS incidence data <15 years of children. In order to make a fair comparison, we included only those data from published reports, which are comparable with our data. Our case definition therefore does not meet the strict NINDS criteria. Therefore, future studies should include a validation of the case definition used here. Minor modifications and additions in the AFP surveillance record form may increase the performance of the system to adequately detect GBS cases. Insight into the incidence of GBS in Bangladesh is important to a worldwide audience and to better document the geographical differences in incidence and clinical phenotype. In addition, changing patterns of antecedent infections triggering GBS may lead to local or global changes in the incidence of the disease. The emergence of e.g. H1N1 flu and H1N1 vaccination has been followed by increasing demands for a post-marketing surveillance of GBS, and to determine any changes in incidence following exposure to new environmental factors.

Extensive neurophysiological investigation in GBS patients in Bangladesh

In this study, we were able to perform EMG and nerve conduction study on 64 out of 100 patients. Electrophysiological studies showed that 67% of patients had an axonal variant of GBS, whereas 22% had AIDP. Eleven patients (14%) died and 23 (29%) remained severely disabled during 6 months follow up after disease onset. Despite its importance in GBS, the pathophysiology of weakness is only partly understood. At present, more advanced electrophysiological techniques are available (muscle scan, excitability testing, multichannel recordings) to improve insight in the pathophysiology of weakness in GBS, and to further classify GBS patients in electrodiagnostic and prognostic subgroups (66). New, non invasive electrophysiological tests performed at the onset of the disease may detect subgroups of patients with a bad prognosis who will likely benefit from more aggressive therapeutic interventions.

Antecedent infections and ganglioside antibodies

We identified the predominant cause of the antecedent infection in GBS patients and detected anti-ganglioside antibodies (GM1, GD1a and GQ1b) in more than half of the patients. Although *Campylobacter* infections in Bangladesh appear to play a prominent role in the pathogenesis GBS in Bangladesh, other agents may be involved as well. Cutting-edge microbial discovery laboratory platforms and modern and powerful high-throughput sequencing tools may detect other, yet unknown, pathogens that intervene in the pathogenesis of GBS. Antibodies against other glycoconjugates may be present in the *Campylobacter* negative GBS patients. Our preliminary data suggest that complex ganglioside antibodies are present in the serum samples from in our cohort of GBS patients. These antibodies seem to be even more prevalent than those against single gangliosde. The presence of

neuronal glycolipid targets in antigens of *C. jejuni* and GBS-related pathogens should be studied further.

Host-pathogen interaction

Although certain types of *Campylobacter* are implicated in GBS, it is host factors that drive the immune respons that will lead to GBS. Some *C. jejuni* strains isolated from diarrhea patients contain similar GM1 ganglioside-like epitopes (67), and yet these patients do not develop an anti-ganglioside immune respons nor GBS. We also described two clonally related *Campylobacter* strains isolated from two different GBS patients, expressing identical ganglioside-mimicking structures (GM1 and GD1a); however, one patient developed an anti-GD1a immune response while other patient developed an anti-GM1 response. The specific neuropathogenic *Campylobacter* types are therefore not sufficient to trigger GBS and other, yet unknown host factors are involved. Further research is necessary to elucidate the mechanism by which *C. jejuni* determine the fine-specificity of the anti-ganglioside antibodies.

Other *Campylobacter* factors

In this thesis, we did not detect any molecular markers specific for GBS-associated strains by our comparative genomic analysis; however, LOS class typing significantly differentiated GBS-related strains from enteritis-associated ones in Bangladesh. Many studies tried to identify *Campylobacter*-related GBS factors beside the LOS, but did not succeed (50). The availability of new molecular techniques may warrant some additional studies. Micro-arrays can be used to detect differences in mRNA expression levels. GBS factors that result in differential expression of mRNA may be detected with this approach. Comparison of the proteomes (protein expression) or glycomes (polysaccharide expression) may also be used to search for additional GBS factors including large number of *C. jejuni* strains. In our study, we found that one GBS patient did not have an anti-ganglioside antibody response and was infected with a *C. jejuni* strain without ganglioside mimic (Chapter 4). Therefore, mechanisms other than ganglioside mimicry may be involved in the development and progression of GBS.

Occurrence of *C. jejuni* in environment, and other sources in Bangladesh

We did a comparative genotyping analysis using humans and poultry isolates of *C. jejuni* in Bangladesh. The dissimilarity of the sequence types and genotypes of *C. jejuni* from humans and poultry suggests the presence of additional sources other than poultry in Bangladesh. Though poultry is considered to be the main vector of *C. jejuni*, other domestic farm animals such as cattle, sheep, and pigs, and pet animals such as cats and dogs, may act as a vector for human *Campylobacter*osis. Contaminated surface water may also be involved (68). Therefore, future studies should focus not only on poultry, but also on other animals and environmental sources including water. This will undoubtedly provide more insight on the epidemiology of *Campylobacter* in Bangladesh.

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Summary

Summary

Guillain-Barré syndrome (GBS) is probably the most frequent cause of acute flaccid paralysis (AFP) in Bangladesh. GBS is characterized by demyelination and axonal degeneration of peripheral nerves, leading to a typical acute, progressive, symmetric paralysis with areflexia of arms and legs. Some patients show involvement of cranial nerves or paresis of respiratory muscles for which they require ventilation. Other patients have the Miller Fisher syndrome (MFS), a variant of GBS is which the paresis is most evident in the muscles involved in eye movements. GBS and MFS are post-infectious immune mediated diseases and *Campylobacter jejuni* is considered to be the predominant cause of these antecedent infections. Molecular mimicry between *Campylobacter* lipooligosaccharides (LOS) and gangliosides in nervous tissue most likely induces a cross-reactive antibody response leading to nerve damage and the clinical symptoms of GBS or MFS.

The aim of the research described in this thesis was to define the clinical epidemiology, preceding infections and antibody response in GBS in Bangladesh. Most current knowledge of GBS is based on studies in patients from developed countries and very little is known about patients from developing countries. Considering the important role of preceding infections in the pathogenesis of GBS and the high exposure to infections in people from developing countries, we hypothesized that the disease frequency and characteristics of GBS in Bangladesh may differ from those in developed countries. In these studies we especially focused on *C. jejuni* since the high incidence of diarrhea in Bangladesh. *Campylobacter* strains from Bangladesh may also differ genetically or structurally from isolates from other geographical areas, influencing the pathogenesis and neurological manifestations. More specifically, in the studies described in this thesis was investigated in Bangladesh (i) the epidemiology of GBS in children, and the clinical presentation, course and outcome of GBS, (ii) the spectrum of antecedent infections and anti-ganglioside antibodies, (iii) the molecular mimicry of *C. jejuni* and gangliosides, and (iv) a comparative genomics of *C. jejuni* strains isolated from poultry and from patients with GBS and control enteritis.

First, the crude incidence rate of GBS in children (<15 years) was determined using the data from a WHO surveillance on AFP (**Chapter 2.1**). This surveillance is conducted to define the frequency of poliomyelitits, but can also be used to study the epidemiology of GBS which usually meets the clinical criteria for AFP. The incidence rate in the northern part of Bangladesh varied from 1.5 to 1.7 per 100,000 per year, whereas the rate in the southern part varied from 2.1 to 2.5 per 100,000 per year. A remarkable high incidence rate of more than 5.0 per 100,000 per year was found in the districts of Meherpur and Barisal, in the southern part of Bangladesh. In general, the crude incidence rates of GBS in children from Bangladesh varied from 1.5 to 2.5 per 100,000 per year.

In **Chapter 2.2** the clinical phenotype, course and outcome of GBS was described. We conducted a prospective matched case-control study in the Dhaka area of Bangladesh with a follow-up of six months including 100 hospital-admitted patients fulfilling the NINDS diagnostic criteria of GBS. In this cohort a relatively large proportion of patients were young males from rural areas in Bangladesh. The majority of patients suffered from a severe form of GBS

(92%), further characterized as a pure motor and axonal variant (67%). Most patients (77%) did not receive specific treatment with IV immunoglobulin (IVIg). Poor outcome, defined as the inability to walk unaided at 6 months after disease onset, was found in 34 (43%) patients with GBS.

In a case-control study with these GBS patients and 194 controls (patients with other neurological diseases and healthy family controls), we determined the types of preceding infections associated with GBS (**Chapter 2.2 and 3**). The predominant cause of the antecedent infection in GBS patients was *C. jejuni* (57%). Much lower frequencies of recent infections with *C. jejuni* were observed in the in the control groups (3% of patients with other neurological diseases and 8% of family controls, P<0.001). Diarrhea in the four weeks preceding GBS was reported in 28 (51%) of the 55 *C. jejuni*-positive patients, as compared to 8 (19%) of 42 *C. jejuni*-negative patients (P<0.01). In addition, recent infections with cytomegalovirus, Epstein-Barr virus and *Mycoplasma pneumoniae*, were also demonstrated in GBS patients, but in comparison with controls did not revealed statistical significance (**Chapter 3**). The seroprevalence of coxsackieviruses (36%) is significantly associated with GBS (P<0.01). In this study, 64% of coxsackievirus-associated GBS patients have a positive serology for *C. jejuni* infection. This putative association needs further confirmation.

In **Chapter 3.2** the research was described on the association between recent infection with *C. jejuni* and the presence of anti-ganglioside antibodies in patients with GBS. The presence of antibodies to the gangliosides GM1, GD1a and GQ1b in GBS patients was more frequent compared to the control groups (P<0.001). In GBS patients, antibodies to GM1 were predominant (50%), followed by antibodies to GD1a (14%). Preceding infections with *C. jejuni* were more often found in GBS patients with anti-ganglioside antibodies (78%) compared to patients without anti-gangliosides antibodies (26%) (P<0.001). Both the presence of recent *C. jejuni* infection and anti-ganglioside antibodies were significantly associated with poor outcome after 6 months of follow up.

Chapter 4 reports the serum antibody specificity to *C. jejuni* LOS in patients with GBS and the cross-reactivity of these antibodies to gangliosides. These studies were important to determine the involvement of molecular mimicry in the pathogenesis of *C. jejuni*-related GBS. Serum antibodies to *C. jejuni* LOS were significantly more frequently found in patients with GBS than in the two control groups (56% vs 3%, P<0.001). The presence of these anti-LOS antibodies in GBS patients was strongly associated with a positive serology for a recent *C. jejuni* infection and with the presence of anti-ganglioside antibodies. In addition, our studies showed that antibodies to gangliosides in serum from these patients cross-reacted with LOS from *C. jejuni* isolated from GBS patients. Moreover, mass spectrometry of the LOS from a selection of *C. jejuni* isolates from Bangladeshi GBS patients showed a similar carbohydrate structure as gangliosides. In conclusion, there is epidemiological, serological and structural evidence to support the hypothesis that infections with *C. jejuni* strains expressing ganglioside-mimicking LOS induce cross-reactive antibodies to gangliosides in patients with GBS. This molecular mimicry and cross-reactivity may play a role in the pathogenesis of half of the GBS patients.

Interestingly, these mass spectrometry studies identified a variety of ganglioside mimics in LOS from *C. jejuni* isolates **(Chapter 4)**. In 57% of these isolates the LOS was heterogeneous and

mimicked not one but several different gangliosides, such as the combination of GM1 and GD1a. Some patients also produced antibodies to combinations or complexes of GM1 and GD1a instead of antibodies to single gangliosides. Whether *C. jejuni* strains express mono- or disialylated LOS is determined by the polymorphism of the *cst-II* gene. This relation between polymorphisms in the bacterial *cst-II* gene and the expression of ganglioside-like LOS was confirmed in the Bangladeshi collection of *C. jejuni* isolates. We identified in *C. jejuni* strains isolated from GBS/MFS patients with ophthalmoplegia a novel LOS structure with two extension sites from the inner core and two branches mimicking GA2 and the ganglioside GD3. Our data also suggest that there is no absolute association between the presence of ganglioside mimics in *C. jejuni* LOS and cross-reactive serum antibodies to gangliosides, suggesting that other mechanisms than molecular mimicry may play a role in inducing GBS.

In **Chapter 5.1** we described that the HS:23 serotype is highly prevalent (50%) among GBS-related *C. jejuni* strains from Bangladesh. The comparative genotyping analysis of *C. jejuni* strains isolated from GBS and enteritis patients by MLST, AFLP, PFGE fingerprinting, and LOS typing is described in **Chapter 5.2**. Overlapping clonal groups were defined by MLST, AFLP and PFGE for strains from patients with GBS and gastroenteritis. *C. jejuni* HS:23 isolates from patients with GBS or enteritis were clonal and all strains belonged to ST-403 complex. We did not identify specific genetic determinants that differentiate *C. jejuni* strains associated with GBS versus strains from enteritis controls. In **Chapter 5.1 and 5.2**, we determined the presence of five specific genetic classes of LOS (A-E). The class A locus was significantly associated with GBS without oculomotor symptoms whereas the class B locus associated with GBS with oculomotor symptoms. We showed that the class A and B LOS loci are associated with the expression of ganglioside mimics and with neuropathy. We also described that *C. jejuni* with the class A LOS locus are associated with GBS and GM1-like mimicry and that those strains with a class B locus are associated with MFS and GQ1b-like mimicry. In **Chapter 5.3** we determined the genotypes of the *C. jejuni* isolated from poultry, and compared these with the genotypes of the *C. jejuni* isolated from patients. Comparative genomic studies showed that the population structure of *C. jejuni* isolated from poultry and humans are different and highly diverse. The most common ST-353 clonal complex in poultry was infrequent among human isolates. The predominant ST-403 complex in humans (GBS and enteritis) was not found among poultry.

Chapter 6 includes an extensive discussion of the results described in **Chapters 2 to 5** and suggestions for future research. The most important conclusions are that in Bangladesh a significant proportion of non-polio AFP in children is caused by GBS. Pure motor and axonal variants of GBS are also predominant in Bangladesh. *C. jejuni* is the most frequent antecedent infection in GBS and related with the induction of cross-reactive anti-ganglioside antibodies. Our data confirm that molecular mimicry between gangliosides and *C. jejuni* LOS appears to be causally involved in the pathogenesis of *C. jejuni*-related GBS in Bangladesh.

Samenvatting

Samenvatting

Het Guillain-Barré syndrome (GBS) is waarschijnlijk de meest voorkomende oorzaak van acute hypotone verlamming (of 'acute flaccid paralyisis', AFP) in Bangladesh. GBS wordt gekenmerkt door demyelinisatie en axonale degeneratie van perifere zenuwen welke leidt tot een progressieve, symmetrische verlamming en areflexie van armen en benen. Bij een deel van de patiënten ontstaat tevens uitval van hersenzenuwen of verlamming van de ademhalingsspieren waarvoor de patiënten moeten worden beademend. Andere patiënten krijgen een Miller Fisher syndroom (MFS), een variant van GBS waarbij er vooral sprake is van een verlamming van de spieren die betrokken zijn bij oogbewegingen. GBS en MFS zijn post-infectieuze immuun-gemedieerde ziekten en *Campylobacter jejuni* wordt beschouwd als de meest voorkomende oorzaak van deze vooafgaande infecties. Moleculaire mimicry tussen *C. jejuni* lipo-oligosacchariden (LOS) en gangliosiden in zenuwweefsel veroorzaakt mogelijk een kruis-reactieve antistofrespons welke resulteert in zenuwbeschadiging en de klinische symptomen van GBS en MFS.

Het doel van het onderzoek beschreven in dit proefschrift was om de klinische epidemiologie, voorafgaande infecties en antistofrespons van GBS in Bangladesh te bepalen. De meeste huidige kennis over GBS is gebaseerd op studies bij patiënten uit ontwikkelde landen en heel weinig is bekend over patiënten van ontwikkelingslanden. Gegeven de belangrijke rol van voorafgaande infecties in de pathogenese van GBS en de hoge mate van bloodstelling van mensen in ontwikkelingslanden aan infecties, hebben wij gehypothetiseerd dat de frequentie en kenmerken van GBS in Bangladesh mogelijk verschilden van die in ontwikkelde landen. In deze studies hebben we ons vooral gericht op *C. jejuni* vanwege de hoge incidentie van diarree in Bangladesh. *C. jejuni* stammen in Bangladesh verschillen mogelijk genetisch of biochemisch van isolaten uit andere geografische gebieden en zij beinvloeden daardoor mogelijk de pathogenese en neurologische manifestaties. Meer specifiek, in de studies beschreven in dit proefschrift werd onderzocht in Bangladesh: (i) de epidemiologie van GBS bij kinderen en de klinische presentatie, het ziektebeloop en de prognose van GBS, (ii) het spectrum van voorafgaande infecties en anti-ganglioside antistoffen, (iii) de moleculaire mimicry van *C. jejuni* en gangliosiden, en (iv) vergelijkende genetische analyse van *C. jejuni* stammen geisoleerd van gevogelte en van patiënten met GBS en controle entritis.

Eerst werd de incidentie van GBS bepaald in kinderen (<15 jaar) waarbij gebruik werd gemaakt van data van een WHO surveillence studie gericht op AFP (**Hoofdstuk 2.1**). Deze surveillence studie wordt uitgevoerd om de incidentie van poliomyelitis te bepalen, maar kon ook gebruikt worden om de epidemiologie van GBS te onderzoeken aangezien GBS meestal ook voldoet aan de klinische kriteria voor AFP. The incidentie in het noordelijk deel van Bangladesh varieerde van 1,5 tot 1,7 per 100.000 pe jaar, terwijl de incidentie in het zuidelijk deel van Bangladesh varieerde van 2.1 tot 2.5 per 100.000 per jaar. Een opmerkelijk hoge incidentie van meer dan 5,0 per 100.000 per jaar werd gevonden voor de districten Meherpur en Barisal in het zuiden van Bangladesh. In het algemeen varieerde de incidentie van GBS bij kinderen in Bangladesh tussen 1.5. en 2.5 per 100.000 per jaar.

In **Hoofdstuk 2.2** werd het klinische fenotype, het ziekte beloop en de prognose van GBS beschreven. Wij hebben een prospectieve gematchte case-controlstudie uitgevoerd in het gebied rond Dhaka in Bangladesh met een follow-up van 6 maanden bij 100 in het zieknhuis opgenomen patiënten die volden aan de diagnostische kriteria van de NINDS voor GBS. In dit cohort kwamen jonge mannen afkomstig van de niet-verstedelijkte gebieden van Bangladesh relatef vaak voor. De meerderheid van de patiënten in Bangladesh leden aan een ernstige vorm van GBS (92%), nader gekarakteriseerd als een puur motore en axonale variant (67%). De meeste patiënten kregen geen specifieke behandeling met IV immunoglobulinen (IVIg). Een slecht herstel, gedefinieerd als onvermogen om zonder hulp te kunnen lopen na 6 maanden na het begin van de ziekte, werd gevonden in 34 (43%) van de patiënten met GBS.

In een case-control studie met deze GBS patiënten en 194 controle personen (patiënten met andere neurologische ziekten en gezonde familiecontroles), werden door ons bepaald welke type voorafgaande infecties ware geassocieerd met GBS (**Hoofdstuk 2.2 en 3**). De meestvoorkomende oorzaak van de voorafgeaande infecties bij patiënten met GBS was *C. jejuni* (57%). Veel lagere frequenties van een recente infectie met *C. jejuni* werden gevonden bij de controlegroepen (3% van de patiënten met andere neurologische ziekten en 8% van de gezonde familiecontroles, P<0.001). Diarree in de 4 weken voorafgaand aan GBS werden gemeld door 28 (51%) van de 55 *C. jejuni*-positieve patiënten, vergeleken met 8 (19%) van de 42 *C. jejuni*-neagatieve patiënten (P<0.01). Daarnaast werden ook recente infecties cytomegalovirus, Epstein-Barr virus en *Mycoplasma pneumoniae* aangetoond in patiënten met GBS, maar vergeleken met de controles bleken deze niet significant vaker voor te komen (Hoofdstuk 3). De seroprevalentie van coxsackievirus (36%) bleek significant geassocieerd te zijn met GBS (P<0.01). In deze studie had 64% van de GBS patiënten met een positieve coxsackie-serologie tevens een positieve serologie voor *C. jejuni*. Deze mogelijke associatie moet worden bevestigd.

In **hoofdstuk 3.2** werd het onderzoek beschreven naar de associatie tussen recente infecties met *C. jejuni* en de aanwezigheid van anti-ganglioside antistoffen bij patiënten met GBS. De aanwezigheid van antistoffen tegen de ganglisodien GM1, GD1a en GQ1b bij patiënten met GBS bleek meer frequent dan in de controlegroepen (P<0.001). In GBS patiënten bleken antistoffen tegen GM1 (50%) het vaakste voor te komen, gevolgd door antistoffen tegen GD1a (14%). Voorafgaande infecties met *C. jejuni* warden vaker gevonden bij GBS patiënten met anti-ganglioside antistoffen (78%) dan bij patiënten zonder anti-gangliside antistoffen (26%) (P<0.001). Zowel de aanwezigheid van recente *C. jejuni* infecties als anti-ganglisoide antistoffen bleken significant geassocieerd te zijn met een slecht klinisch herstel na 6 maanden van follow-up.

Hoofdstuk 4 beschrijft de specificiteit van antistoffen in het serum van patiënten met GBS voor *C. jejuni* LOS en de kruisreactiviteit van deze antistoffen met gangliosiden. Deze studies waren belangrijk om te bepalen of moleculaire mimicry een rol speelt in de pathogenese van *C. jejuni*-gerelateerde GBS. Serum antistoffen tegen *C. jejuni* LOS werden significant vaker gevonden bij patiënten met GBS dan bij de twee controlegroepen (56% vs 3%, P<0.001). De aanwezigheid van deze anti-LOS antistoffen bij de GBS patiënten bleek sterk geassocieerd te zijn met een positieve serologie voor een recente *C. jejuni* infectie en met de aanwezigheid van anti-ganglioside antistoffen.

Daarnaast.werd in onze studies aangetoond dat antistoffen tegen gangliosiden in het serum van patiënten kruisreageerden met het LOS van *C. jejuni* isolaten van patiënten met GBS. Verder werd met behulp van massa spectrometrie aangetoond dat het LOS van een selectie van *C. jejuni* isolaten van Bangladese GBS patiënten een vergelijkbare suikerstructuur heeft als gangliosiden. Er zijn derhalve epidemiologische, serologische en structurele aanwijzingen ter ondersteunig van van de hypothese dat in Bangladesh infecties met *C. jejuni* stammen die op ganglioside gelijkend LOS tot expressie brengen bij GBS patiënten kruisreactieve antistoffen tegen gangliosiden kunnen veroorzaken. Deze moleculaire mimicry en kruisreactiviteit speelt mogelijk een rol in de pathogenese bij ongeveer de helft van de patiënten met GBS in Bangladesh.

Interessant genoeg bleek uit deze massa spectometrie-studies dat de *C. jejuni* isolaten uit Bangladesh een varieteit aan verschillende ganglioside mimics tot expressie kunnen brengen **(Hoofdstuk 4).** Bij 57% van deze isolaten was het LOS heterogeen en vertoonde deze gelijkenis met niet één maar met meerdere verschillende gangliosiden, zoals de combinatie van GM1 en GD1a. Sommige patiënten produceerden antistoffen tegen combinaties of complexen van GM1 en GD1a in plaats van antistoffen tegen enkelvoudige gangliosiden. Of een *C. jejuni* mono- of disialyl-gangliosiden tot expressie brengt wordt bepaald door het polymorfisme van het *cst-II* gen. Deze relatie tussen de polymorfismen van het bacteriële *cst-II* gene en de expressie van op ganglioside-gelijkend LOS werd bevestigd in de Bangladese collectie van *C. jejuni*-isolaten. Wij identificeerden in *C. jejuni* isolaten van patiënten met GBS/MFS en ophthalmoplegia een nieuwe LOS-structuur met in de 'inner core' van het LOS twee plaatsten voor extensies en met twee mimics voor GA2 en het ganglioside GD3. Onze resultaten suggereren verder dat er geen absolute associatie bestaat tussen de aanwezigheid van ganglioside mimics in *C. jejuni* LOS en kruis-reactive antistoffen tegen gangliosiden in het serum. Deze bevindingen suggereren mogelijk dat er ook andere mechanismen dan moleculaire mimicry een rol spelen bij het ontstaan van GBS.

In **Hoofdstuk 5.1** beschreven we dat het HS:23 serotype vaak voorkomt (50%) bij *C. jejuni*-isolaten van patiënten met GBS uit Bangladesh. De vergelijkende genotype analyse van *C.jejuni*-isolaten van GBS patiënten en eneteritis patiënten met behulp van MLST, AFLP en PFGE en met LOS biosynthese klassetypering werden beschreven in **Hoofdstuk 5.2**. Overlappende klonale groepen werden bepaald met behulp van MLST, AFLP en PFGE van patiënten met GBS en gastroenteritis. *C. jejuni* HS:23 isolaten van GBS patiënten en enteritis patiënten bleken klonaal te zijn en alle stammen bleken te behoorden tot het ST-403-complex. We vonden geen specifieke genetische determinanten die differentieerden tussen *C. jejuni* van GBS patiënten en controlepersonen met enteritis. In **Hoofdstuk 5.1 en 5.2** bepaalden we de aanwezigheid van vijf specifieke genetische klassen van LOS (A-E). Het klasse A locus was significant geassocieerd met GBS met oculomotore symptomen. We toonden aan dat klasse A en B van LOS loci waren geassocieerd met de expressie van ganglioside mimics en met het voorkomen van neuropathie. We beschreven verder dat *C. jejuni* met het klasse A LoS locus waren geassocieerd met GBS en met op GM1-gelijkend LOS en dat isolaten met het klasse B locus waren geassocieerd met het MFS en op GQ1b-gelijkend LOS. In **Hoofdstuk 5.1** bepaalden we het genotype van *C. jejuni* bij de isolaten uit gevogelte en vergeleken deze met het genotype van *C. jejuni* bij de isolaten van patiënten. In een

vergelijkend genomische analyse bleek de populatiestructuur van de *C. jejuni* geisoleerd uit gevogelte en mensen verschillend en bovendien heel divers. Het bij isolaten van gevogelte meest voorkomend clonale complex ST-353 bleek zeldzaam te zijn bij isolaten van mensen. Het meest voorkomende ST-403 complex bij isolaten van mensen (GBS en controle enteritis) werd niet gevonden bij isolaten van gevogelte.

Hoofdstuk 6 bevat een uitgebreide discussie over de resultaten beschreven in de hoofdstukken 2 tot en met 5. De meest belangrijke conclusies zijn dat in Bangladesh een groot deel van de non-polio AFP bij kinderen wordt veroorzaakt door GBS. Puur motore en axonal vormen van GBS zijn in Bangladesh het meest voorkomend. *C. jejuni* is de meest frequente voorafgaande infectie bij GBS en gerelateerd met de vorming van kruis-reactieve anti-ganglioside antistoffen. Onze resulaten bevestigen dat moleculaire mimicry tussen *C. jejuni* LOS en gangiosiden een belangrijke oorzaak lijkt te zijn in de pathogenese van *C. jejuni*-gerelateerde GBS in Bangladesh.

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Curriculum vitae

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Zhahirul Islam was born on November 2nd, 1977, in Netrakona, Bangladesh. In 1992, he completed his secondary education from Lakshmigonj High School, Netrakona. After his secondary education, he completed higher secondary education from Notre Dame College, Dhaka. Then, he enrolled at the Department of Biochemistry and Molecular Biology, University of Dhaka, and completed his BSc. (honors) in 2001 and MSc. in 2002. Mr. Z. Islam conducted his MSc thesis research (Dr. Kaisar Ali Talukder) on "Phenotypic and genotypic characterization of provisional serotype *Shigella flexneri* 1c and clonal relationships with 1a and 1b strains isolated in Bangladesh" at the Enteric and Food Microbiology Laboratory, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). After completing his MSc, he joined the same laboratory at ICDDR,B as a senior research technician and worked on the molecular epidemiology of *Shigella* spp. and *Campylobacter* spp. among diarrheal patients in Bangladesh. In 2004, he got special training on Real-Time PCR at the Armed forces Research Institute of Medical Sciences in Bangkok, Thailand (Dr. J. Maiden). In 2004, he initiated his PhD project on *Campylobacter* and GBS in Bangladesh with Prof. Hubert Ph Endtz and Dr. Bart C Jacobs from Erasmus MC, Rotterdam, The Netherlands. In 2005, he got pre-doctoral training under the supervision of Prof. Hubert Ph Endtz at the Department of Medical Microbiology and Infectious Diseases in Erasmus MC, Rotterdam. He started his PhD in 2006 at Department of Medical Microbiology and Infectious Diseases in Erasmus MC, The Netherlands in collaboration with Dhaka Medical College Hospital, Dhaka and ICDDR,B, Bangladesh.

List of publications

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1. **Islam Z,** Jacobs BC, van Belkum A, Mohammad QD, Islam MB, Herbrink P, Diorditsa S, Luby SP, Talukder KA, Endtz HP. Axonal variant of Guillain-Barré syndrome associated with Campylobacter infection in Bangladesh. Neurology. 2010;74:581-587.
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5. **Islam Z**, Jacobs BC, Mohammad QD, Diorditsa S, Endtz HP. Acute flaccid paralysis surveys to determine incidence rates for GBS. *Submitted*.
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