Guillain-Barré syndrome in Bangladesh: genetic polymorphism and pathogenesis

Ph.D. Thesis

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Statutory Declaration

The thesis titled as "**Guillain-Barré syndrome in Bangladesh: genetic polymorphism and pathogenesis"** for the requirement of the degree of Doctor of Philosophy (Ph.D.) in the Faculty of Biological Science, University of Dhaka. This study has been carried out in Laboratory of Gut-Brain Signaling, Laboratory Sciences and Services Division, icddr,b, Dhaka, Bangladesh in collaboration with the Department of Biochemistry and Molecular Biology, University of Dhaka. Any published or unpublished writings discussed have been clearly referenced in the text. To the best of our knowledge no part of the work presented here has not been submitted for any other degree or qualification or to pass an examination.

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Dedicated

To

My Beloved Parents

Mr. Sekander Hayat & Ms. Amena Meher

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Abstract

Guillain-Barré syndrome (GBS) is a rapidly progressive, immune-mediated, paralytic disorder of the peripheral nervous system, which has led to significant morbidity and disability in the post-poliomyelitis era. Annually, there are 1 to 2 cases of GBS per 100,000 people worldwide. The pathological spectrum of GBS comprises acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), and acute motor sensory axonal neuropathy (AMSAN). Numerous microbial infections, including *Campylobacter jejuni* (*C. jejuni*), have been linked with the risk of developing of GBS. Molecular mimicry between lipooligosaccharides of *C. jejuni* and host nerve gangliosides is postulated to be an important mechanism by which an aberrant immune response triggers neuronal damage. However, the low occurrence of *C. jejuni*-induced enteritis GBS (1 in 1000-5000 cases), the family history of GBS and rare phenomena of recurrent GBS clearly indicate that in addition to the molecular mimicry theory, genetic host factors are probably involved in the pathogenesis of GBS. We aimed to identify the contribution of several immune response-related genetic host factors in the pathogenesis of GBS in a well-documented Bangladeshi cohort comprising 303 patients with GBS and 303 healthy individuals. The gene alterations studied included polymorphisms in human leukocyte antigen (*HLA)-DQB1* and single nucleotide polymorphisms (SNPs) in nucleotide oligomerization domain (*NOD)*, immunoglobulin G Fc-gamma receptors (*FcγRs*), and the promoters of interleukin-10 (*IL-10*) and matrix metalloproteinase-9 (*MMP9*).

The *HLA-DQB1* gene complex is highly polymorphic and possesses dense linkage disequilibrium (LD). Variation in the gene *HLA-DQB1* and in haplotype patterns may play crucial roles by altering the ability of the immune system to recognizing self and foreign antigens implicated in the pathogenesis of GBS. The current study indicates that *HLA-DQB1* polymorphisms are not associated with susceptibility to GBS. Haplotype 9 (*DQB1**0303 - *0601) is less common among patients with GBS than in healthy control individuals (P = 0.006, OR = 0.49, 95% CI = 0.30-0.82; Pc = 0.06). Patients with the *C. jejuni*-triggered axonal variant of GBS possess a higher frequency of haplotype 5 (*DQB1**0501-*0602; P = 0.024, OR = 4.06, 95% CI = 1.25-13.18; Pc = 0.24), and the *DQB1**0201 alleles were predominant in the demyelinating subtype of GBS before correction of P-value (P = 0.027,

OR = 2.68, 95% CI = 1.17-6.17; *P*c = 0.35). Thus, our findings indicate that *HLA-DQB1* polymorphisms are not risk factors for the development of GBS. Moreover, clinical features and serological subgroups of GBS are not influenced by these genetic markers.

NOD receptors play an important role in the first line of innate immunity defense by sensing microorganisms. This study of NOD polymorphisms in 303 patients with GBS and 303 healthy control individuals implies there is no significant association between NOD polymorphisms (*NOD1*-Glu266Lys and *NOD2*-[Arg702Trp; Gly908Ar]) and GBS susceptibility or severity. Moreover, polymorphisms in *NOD2* are rare in both patients with GBS and in healthy individuals from Bangladesh.

FcγR is a key immune system regulator that bridges cellular and humoral immunity by modulating diverse effector functions, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators. Our investigation on FcγR polymorphisms in patients with GBS and healthy individuals indicates an association of the FcγRIIIa-V158F genotype with the severe form of the disease ($P = 0.005$, OR = 2.24, 95% CI = 1.28-3.91; Pc = 0.015). Patients with a recent *C. jejuni* infection possess a higher frequency of the homozygous genotypes FcγRIIIa-V/V158 ($P \leq 0.001$, OR = 0.36, 95% CI = 0.23-0.56; Pc ≤ 0.003) and FcγRIIIb-NA2/2 ($P = 0.004$, OR = 1.70, 95% CI = 1.18-2.44; Pc = 0.012) compared to patients with *C. jejuni* negative serology. However, no association was evident between GBS susceptibility and FcγR genotypes or haplotype patterns. There was a higher frequency of haplotype 1 (FcγRIIa-H131R - FcγRIIIa-V158F - FcγRIIIb-NA1/2) and the FcγRIIIb-NA2/2 genotype in patients positive for anti-GM1 antibodies than in patients who are negative for these antibodies ($P = 0.031$, OR = 9.61, 95% CI = 1.24-74.77, Pc = 0.279; $P = 0.027$, OR = 1.62, 95% CI = 1.06-2.5, Pc = 0.081; respectively).

This study of IL-10 promoter polymorphisms in patients with GBS indicates that the homozygous -819 TT genotype is more prevalent in patients with the axonal variant of GBS than in patients with the demyelinating subtypes of GBS ($P = 0.042$, OR = 8.67, 95% CI = 1.03-72.97; Pc = 0.123) or healthy individuals ($P = 0.005$, OR = 4.2, 95% CI = 1.55-11.40; Pc = 0.015). The -1082G/A, -819C/T and -592 C/A polymorphisms in *IL-10* were not significantly associated with disease susceptibility. Moreover, the haplotype combinations

GCC/GTA, GCC/ATA and GCC/GCA are common in severe forms of GBS (P = 0.008, OR = 3.22, 95% CI = 1.4-7.43; Pc = 0.024).

MMP-9 is an inflammatory mediator that is activated by pro-inflammatory cytokines and participates in macrophage recruitment. Our research on the association of the *MMP9* (-1562 C/T) promoter polymorphism with the susceptibility and severity of GBS reveal the involvement of the variant allele and CT genotype in the severe form of GBS (P = 0.012, OR = 2.0, 95% CI = 1.14-3.38; Pc = 0.024 and P = 0.01, OR = 2.28, 95% CI = 1.22-4.22; Pc = 0.03, respectively). However, the *MMP9* (-1562 C/T) promoter polymorphism was not associated with susceptibility to GBS.

In summary, we conclude that genetic polymorphisms in *HLA-DQB1*, *NOD*, immunoglobulin G *FcγR*, and the *IL-10* and *MMP9* promoter regions are not risk factors for the development of GBS. However, the contribution of these polymorphisms to the clinical features and serological subgroups of GBS, including antecedent infections, presence of auto-antibodies, severe or mild muscle weakness, and outcome of the disease, cannot be ignored and will enrich our knowledge about host-pathogen interactions in the pathogenesis of GBS. A large cohort of patients with GBS from multi-ethnic regions is required to confirm our findings on the contribution of genetic host factors to the pathogenesis of GBS.

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Abbreviations

ACD	Albuminocytological dissociation
Abs	Antibodies
ADCC	Antibody-dependent cellular cytotoxicity
AFP	Acute Flaccid Paralysis
AIDP	Acute Inflammatory Demyelinating Polyradiculoneuropathy
AMAN	Acute Motor Axonal Neuropathy
AMSAN	Acute Motor and Sensory Axonal Neuropathy
APC	Antigen Presenting Cell
ASO-PCR	Allele specific oligonucleotide-polymerase chain reaction
CI	Confidence Interval
CMV	Cytomegalovirus
C. jejuni	*Campylobacter jejuni*
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic acid
EMG	Electromyography
et al.	et alia' meaning 'and others'
etc.	Etcetera
GBS	Guillain-Barré syndrome
GM1	Monosialotetrahexosylganglioside
HGH	Human Growth Hormone
HLA	Human Leukocyte Antigen
Fab	Fragment, antigen-binding
FcγR	Fc gamma receptor
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IGOS	International GBS Outcome Study
IQR	Interquartile range
IVIg	Intravenous Immunoglobulin
i.e.	That is
LMIC	Low and middle-income countries
LOS	Lipooligosaccharide
MFS	Miller-Fisher syndrome
MHC	Major Histocompatibility complex
MMP	Matrix Metalloproteinase
MRC	Medical Research Council
MS	Multiple Sclerosis
NA	Neutrophil antigen
NCBI	National Center for Biotechnology Information
NINDS	National Institute of Neurological and Communicative Disorders and Stroke
NOD	Nucleotide Binding Oligomerization Domain
NLRs	Nucleotide- binding oligomerization domain like receptors
OR	Odds Ratio

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PCR	Polymerase Chain Reaction
PNS	Peripheral nervous system
PRMs	Pathogen recognition molecules
P-value	Estimated probability
Pc	*P* corrected
RFLP	Restriction Fragment Length Polymorphism
Rpm	Revolutions per minute
Rs	Reference SNP
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SVPE	Small volume plasma exchange
TBE	Tris-Borate-EDTA
TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
Ta	Annealing temperature
Tm	Melting temperature
Ml	Microliter

Chapter 1

General Introduction

1. General introduction

1.1 Overview

Guillain-Barré syndrome (GBS) is a life-threatening, post-infectious, immune-mediated neurological disease that exhibits characteristics of flaccid paralysis in the post-poliomyelitis world, affecting 1 to 2 individuals per 100,000 people per year.[1,2] The pathological spectrum of GBS includes acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN) and acute motor sensory axonal neuropathy (AMSAN).[3,4] Demyelination and axonal damage provoked by autoimmunity after infection are the apparent causes of GBS, but the exact mechanism is yet to be elucidated.[5,6] Approximately two-thirds of patients with GBS report antecedent infections, with either respiratory or gastrointestinal symptoms, days to weeks before the onset of neurological signs and symptoms.[4,7,8] *Campylobacter jejuni* (*C. jejuni*), a common diarrhea-causing bacterial pathogen, is the single most identifiable agent linked with GBS[4,6,9,10] *C. jejuni* infections are associated with a severe, pure motor, axonal variant of GBS that usually has a poor outcome.[4,6,11,12] Molecular mimicry between the outer core structures present on *C. jejuni* and the peripheral nerve gangliosides of the host is thought to induce autoimmune reactions.[13–16] The time between onset of infection and the first neurological manifestations of GBS is reported to be 1-3 weeks in most cases.[17] Although certain types of *Campylobacter* are implicated in GBS, only a small percentage (0.1%) of patients with *C. jejuni*-induced enteritis develop GBS, which indicates that host factors such as genetic susceptibility could be involved in triggering the pathological process.[8,10,18–20] Some hosts respond to the lipooligosaccharide (LOS) structures of *C. jejuni* that mimic nerve gangliosides (e.g. GM1, GD1a, GQ1b and others) by producing anti-ganglioside antibodies linked to neuronal damage,[4,21,22] thus triggering different subtypes of GBS.[23–26] Many studies on the clinical and epidemiological features, pathogenesis and disease management of GBS have been conducted in the developed world[2,3,9,11,27–31]: however, research did not commence on GBS in Bangladesh until a decade later.[4,14,32–35]

Following the worldwide eradication of poliomyelitis, GBS is currently the most dangerous and potentially devastating non-polio acute flaccid paralysis (AFP) in Bangladesh.[32] Throughout the country, the crude incidence rate of GBS in children under 15 years of age is 1.5-2.5 cases per 100,000,[32] which is 2.5 to 4 times greater than the rest of the world. GBS is accompanied by long-term rehabilitation, severe residual disabilities and a high

mortality rate in Bangladesh.[14,33,34] Acute motor axonal neuropathy (AMAN) resulting from *C. jejuni* infection is the predominant subtype of GBS in Bangladesh.[4] The most common autoantibodies against *C. jejuni* LOS are GM1, GD1a and GQ1b.[4] Molecular mimicry between gangliosides and *C. jejuni* LOS is the pathogenic mechanism in most cases of *C. jejuni*-related GBS in Bangladesh.[14] However, in some cases, the immune response against gangliosides not only results from molecular mimicry involving *C. jejuni* LOS, but may also be linked to the genetics of host susceptibility[14] as only a subset of patients with diarrhea or *C. jejuni*-induced enteritis (1 in 1000-5000 cases) develop GBS.[8,10,18–20] Moreover, the occurrence of GBS within families[36–38] and the recurrence of GBS[39] also indicate the involvement of genetic factors in disease development. Thus, in addition to pathogen-derived factors, genetic susceptibility plays an important role in the pathogenesis of GBS.

Single nucleotide polymorphisms (SNPs) or the study of polymorphisms in relation to disease susceptibility, severity or prognosis has been widely used to assess the contribution of genetic factors to the pathogenesis of GBS.[40] SNPs or polymorphisms are widely distributed throughout the genome and are, by definition, present in at least 1% or more of the general population.[41,42] Various immune response genetic host factors are likely to be involved in each step from exposure to infection to development of neuropathy (***Figure 1.1***). Polymorphisms or SNPs in these host factors may affect host defense, recognition of microorganisms, cross-reactive immune system activation, complement activation, macrophage recruitment, development of neuropathy, and recovery from disease.[35,43–45] Within protein-coding genes, SNPs can be located in: [1] the promoter region, which is involved in transcriptional regulation of the gene expression; [2] the coding region, which is translated to a protein; [3] the intron, which is not translated to a protein but is involved in splicing; and [4] the untranslated region (UTR), which affects the stability of RNA. SNPs can therefore lead to differences in protein expression levels, alter the function of a protein or result in the absence of a protein.[40,46] Analyzing the associations between polymorphisms and pathogenesis is a key step in developing effective treatment options for clinical disorders. Previously, the associations of polymorphisms in tumor necrosis factor-alpha (*TNF-α*),[47] the *CD1A* and *CD1E* genes,[48] the *FAS* promoter [49] and Toll-like receptor-4 (*TLR-4*)[35] with the pathogenesis of GBS have been studied in Bangladeshi patients with GBS. However, the association of polymorphisms in human leukocyte antigen *(HLA)-DQB1*, nucleotide-binding

oligomerization domain (*NOD*), immunoglobulin G Fc-gamma receptor (*FcγR*), interleukin-10 (*IL-10*) promoter and the matrix metalloproteinase-9 promoter (*MMP9-1562C/T*) with GBS pathogenesis remains to be elucidated for Bangladeshi patients with GBS.

1.1.1. HLA-DQB1

The HLA gene complex is highly polymorphic. Both the *DQA1* and *DQB1* genes are polymorphic; however, *DQB1* is more polymorphic than *DQA1* and is the major determinant of the DQ antigen.[50,51] Variation in the *HLA-DQB1* and *-DRB1* alleles is implicated in the pathology of autoimmune diseases including GBS. The findings from previous studies regarding the relationship between *HLA-DQB1* polymorphisms and the possible link to disease development were inconsistent. *HLA-DQB1**060x and *HLA-DRB1**0701 were significantly associated with the development of GBS in an Indian population,[45] whereas a Dutch study reported no significant association between *HLA-DQB1* polymorphisms and GBS.[30]A significant association of the *DQB1*03* allele was identified in an English population of *C. jejuni*-positive patients with GBS,[27] whereas other studies did not find such an association.[52] This lack of consensus regarding the association of *HLA-DQB1* polymorphisms with the pathogenesis of GBS, coupled with the importance of the *HLA-DQB1* immunological mechanism in pathogen recognition, prompted our interest in investigating the highly polymorphic *HLA-DQB1* alleles in a well-documented cohort of patients to further characterize the pathological basis of immune-mediated tissue damage in GBS.

1.1.2. NOD

NOD1 and NOD2 are cytosolic receptor proteins of the innate immune system and form part of the first line of defense in the host.[53] Pathogen-associated molecular patterns (PAMPs) are evolutionarily conserved structures on microorganisms that are recognized by NOD receptors.[54] Genetic variations in innate immunity genes have been reported to be associated with a range of inflammatory disorders, including both TH2-driven atopic diseases and TH1-dominated autoimmune diseases.[55] Polymorphisms in NOD1 and NOD2 genes may shift the

balance between pro- and anti-inflammatory cytokines, which modulates the risk of infection and causes chronic inflammation or various autoimmune diseases.[56–58] Several studies have reported that variations in the NOD1 (Glu266Lys, rs6958571) and NOD2 (Arg702Trp, rs2066844 and Gly908Arg, rs2066845) genes are associated with atopic dermatitis,[59] asthma,[60] Crohn's disease (CD),[56] inflammatory bowel disease,[61] and sarcoidosis.[62] Based on the significance of NOD1 and NOD2 polymorphisms in genetic susceptibility to various diseases, we aimed to determine the association of these polymorphisms (NOD1: Glu266Lys, rs6958571; NOD2: Arg702Trp, rs2066844 and Gly908Arg, rs2066845) with the susceptibility and severity of GBS.

1.1.3. Immunoglobulin G FcγR

Immunoglobulin G FcγRs are important immune-response modulating molecules that link cellular and humoral immunity through interactions with IgG.[63] Autoreactive antibodies produced against nerve gangliosides in patients with GBS act via FcγRs to trigger effector functions such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and release of inflammatory mediators.[64,65] Three relevant biallelic functional polymorphisms in the immunoglobulin G Fc receptors—FcγRIIA: H131/R131 (rs1801274), FcγRIIIA: V158/F158 (rs396991) and FcγRIIIB: NA1/NA2—play important roles in the affinity of the IgG-FcγR interaction, therefore, these polymorphisms may be associated with the susceptibility, severity and pathogenesis of GBS. Several studies have reported associations between FcγR polymorphisms and GBS susceptibility in British, Dutch and Norwegian populations;[63] however, inconclusive findings were reported when the AIDP subtype was the predominant form of GBS in the population. As the *C. jejuni*-associated axonal subtype is the predominant GBS subtype in our Bangladeshi population,[4] it is of utmost importance to investigate the role of FcγR polymorphisms in the pathogenesis of GBS in these patients. Moreover, treatment of GBS with intravenous immunoglobulins (IVIg) works by blocking the FcγR.[66,67] Taken together, these points emphasize the importance of researching FcγR polymorphisms as genetic host factors involved in GBS.

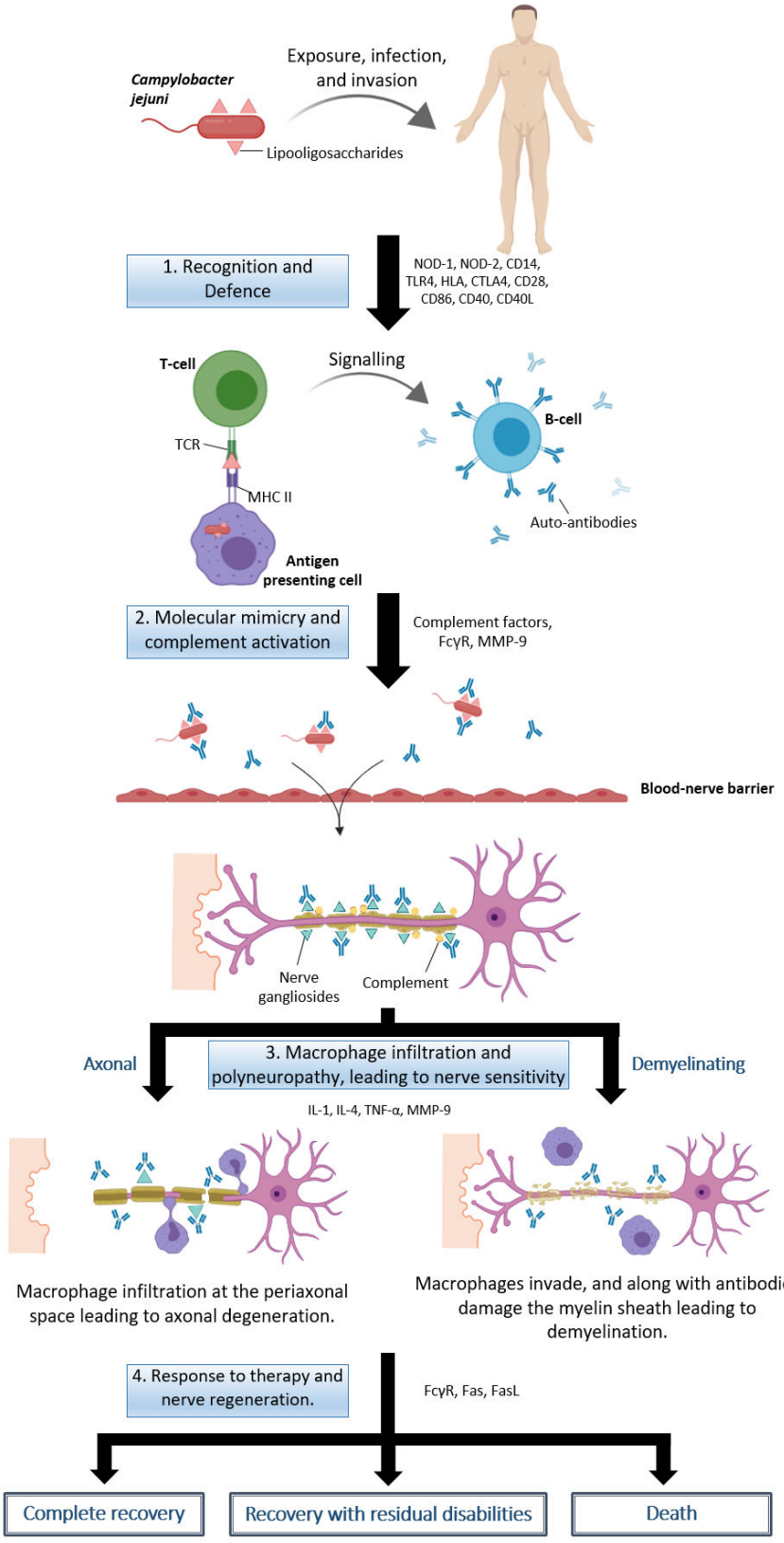

Figure 1.1 Pathogenesis of Guillain-Barré syndrome (GBS)

1.1.4. IL-10 promoter polymorphisms

IL-10 is a regulatory cytokine that plays a pivotal role in the pathogenesis of a number of diseases, particularly inflammatory and autoimmune diseases such as GBS.[68] IL-10 was first described by Fiorentino et al. as a product of T helper-type 2 (Th2) cells that inhibited cytokine production from Th1 cells.[69] Among the various polymorphic sites in the promoter region of the IL-10 gene, the loci -1082 G/A (rs 1800896), -819 C/T (rs 1800871) and -592 C/A (rs 1800872) are most common and predominantly control IL-10 expression. Variability in the IL-10 gene may dysregulate the immune response and leads to autoimmunity.[70] Previous studies on the link between IL-10 gene polymorphisms and the risk of developing GBS were inconclusive.[44,68,71] Myhr et al. reported that the genotypes -592 CC and -819 CC were associated with GBS susceptibility, but there was no association with *C. jejuni* infection or disease severity.[68] A significant correlation between high expression of IL-10-secreting blood mononuclear cells (MNCs) and anti-ganglioside antibody production with axonal damage was described by Press et al.[71,72] In contrast, Geleijns et al. found no such association between *IL-10* gene promoter polymorphisms and susceptibility to GBS, *C. jejini* infection, anti-ganglioside antibody production or the severity of GBS.[44] Considering the importance of both the pro- and anti-inflammatory effects of IL-10 in disease pathogenesis, we aimed to investigate the distribution of functional polymorphisms in the IL-10 gene in patients with GBS (compared with healthy control individuals) and analyze the association of these polymorphisms with the susceptibility and severity of GBS in a Bangladeshi population.

1.1.5. MMP-9

MMP-9 is an enzyme of the gelatinase subfamily of matrix metalloproteinases (MMPs). MMPs participate in macrophage recruitment and infiltration of the blood-nerve barrier.[44] Increased serum levels of MMP-9 have been found in several autoimmune diseases including systemic lupus erythematosus (SLE), systemic sclerosis, rheumatoid arthritis, multiple sclerosis[73] and GBS.[74] Due to the action of MMP-9 in the proteolytic degradation of tissues and in immune cell recruitment, this enzyme is thought to play a notable part in the pathology of GBS. An SNP exists at position -1562 in *MMP9* and results in a cytosine being replaced by thymine.[75] The polymorphic allele (T allele) has stronger promoter activity, leading to

increased expression of the enzyme.[75] These data suggest that this polymorphism in the *MMP9* promoter may influence the disease progression of GBS and perhaps susceptibility. Therefore, an in-depth study of the MMP-9 gene in patients with GBS is crucial to determine such associations and hence elucidate suitable treatments for GBS.

1.2. Rationale

This thesis focuses on Bangladeshi patients with GBS and aims to determine whether the *HLA-DQB1* alleles, SNPs in NOD, FcγR polymorphisms, *IL-10* promoter polymorphisms and the *MMP9* (-1562 C/T) promoter polymorphism are major causative factors in the susceptibility or severity of GBS. Identification of host factors that contribute to the high endemicity of GBS in Bangladesh is imperative. This study will determine the role of genetic host susceptibility factors in the pathogenesis of GBS in Bangladesh. Understanding the pathogenesis of GBS through genetic approaches has already been explored in cohorts from the Western world with GBS. However, as the severity and frequencies of GBS subtypes are different in South-Asian countries compared with the Western world, it is likely that genetic susceptibility might differ among these populations. Therefore, it is important to study genetic polymorphisms in Bangladeshi patients with GBS to obtain more information about the pathogenesis of GBS in this population.

In Bangladesh, most patients with GBS are so poor that they can neither afford costly conventional treatments nor bear the expense of a long hospital stay. This lack of medical intervention means a significant proportion of patients become crippled and ultimately are a burden for the nation. Traditional explanations for the pathophysiology of weakness experienced in GBS are incomplete; these explanations are also unable to predict the responses of individual patients with GBS to expensive treatment. A better understanding of the pathophysiology of weakness in GBS would enable the development of a more specific therapy. Molecular mimicry is the most popular hypothesis promulgated regarding nerve damage and weakness in GBS; however, other potential issues in the pathogenesis of GBS are not solely nullified by this hypothesis.

The outcomes of this study could potentially help to identify a genetic marker for GBS, which in turn would enable the recognition of disease-prone individuals. Moreover, this

knowledge could aid in the development of convenient treatment options and new therapeutics that could improve the quicker recovery of the vast population of patients affected by GBS worldwide.

1.3. Outline of the thesis

GBS is a rare disease in the Western world. However, GBS is the most common form of flaccid paralysis and has varied clinical presentations in Bangladesh. A study of 100 patients with GBS in Bangladesh found that 57% had serological evidence of a recent *C. jejuni* infection, 67% had an axonal variant of GBS, and there was a high mortality rate (14%).[4] Host genetic predisposition connected to geographical location may be responsible for the particular subtype of GBS. To obtain a clearer perception of the multifaceted nature of GBS, it is imperative to conduct studies in different ethnic populations with GBS. This thesis illustrates the contribution of host genetic factors in the susceptibility, severity and pathogenesis of GBS in Bangladesh. Throughout the thesis, we present current approaches in molecular genetics and examples related to the polymorphisms to illustrate the exciting associations between SNPs and disease development in GBS.

Chapter 1 provides a general introduction on GBS in Bangladesh and the links to the polymorphisms of interest. We describe selected gene polymorphisms and their possible association with the pathogenesis of GBS.

Chapter 2 reviews literature from around the world concerning GBS. We describe the theoretical background, clinical presentation and pathogenesis of GBS to illustrate various phenomena. The hypothesis and objectives of this study are also stated in this chapter.

Chapter 3 endeavors to integrate empirical and experimental population genetics with theory. In particular, we present various methods for analyzing parameters of population genetics, as well as other statistical software useful for the analysis of polymorphisms in Bangladesh. Examples and theory relevant to studies of SNPs or polymorphisms associated with the development of GBS are also described.

Chapters 4-8 describe several genetic association studies of gene polymorphisms in patients with GBS compared with healthy control individuals in Bangladesh. An analysis of whether these host genetic factors are associated with the susceptibility to disease or clinical features

or serological subgroups is presented. We considered axonal and demyelinating cases of GBS, positive or negative serology for *C. jejuni* infection, the presence of anti-ganglioside antibodies, severe or mild form of GBS (at entry), and good or poor outcome (after 6 months follow-up) as subgroups for the GBS population. We present data on *HLA-DQB1* polymorphisms and haplotype patterns in GBS (***Chapter 4***), *NOD* polymorphisms in GBS pathogenesis (***Chapter 5***), the contribution of the immunoglobulin G FcγRIIIa-V158F polymorphism to the severity of GBS (***Chapter 6***), IL-10 promoter polymorphisms in patients with GBS in Bangladesh (***Chapter 7***), and the association between the *MMP9* (-1562C/T) polymorphism and the severity of GBS (***Chapter 8***).

Chapter 9 discusses the main findings and importance of the results outlined in *Chapters 4-8*. Recent literature and classic references are used to consider the experimental and theoretical points of view, and to discuss the impact of host genetic polymorphisms in the development of GBS and specific subgroups in patients from Bangladesh.

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Chapter 2

Literature Review

2. Literature review

2.1. Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) is a rapid onset, paralytic disorder of the peripheral nervous system associated with an aberrant immune response. Studies on the electrophysiological and pathological features have classified the disease into two major subtypes: (i) the demyelinating subtype (acute inflammatory demyelinating polyradiculoneuropathy [AIDP]), and (ii) the axonal subtype (acute motor axonal neuropathy [AMAN] or acute motor and sensory axonal neuropathy [AMSAN]).[1–4] In addition, there are other well-defined variants of GBS such as Miller-Fisher syndrome (MFS), which presents as ophthalmoplegia (weakness or paralysis of the muscles responsible for eye movement), ataxia (loss of full control of body movements), and areflexia (having no or less reflexes).[5–7] In North America and Europe, the most common form of GBS is AIDP (56-87% of cases),[8,9] while the more severe, axonal subtype is predominant in South America, Central America, and Asia.[10–12]

GBS is characterized by relatively symmetrical muscle weakness of the limbs, developing over a period of several days or a few weeks, and reaching clinical nadir within 2-4 weeks. An evident marker of the disease is albuminocytological dissociation (ACD), which is a combination of high protein levels in the cerebrospinal fluid with a normal white blood cell count. Although the majority of patients with GBS will recover, approximately 3-14% of patients die, 20-30% of cases require mechanical ventilation, and some patients experience severe residual deficits.[3,13–15]

Currently the most beneficial treatments for GBS are plasma exchange (PE) therapy and intravenous IgG (IVIg) administration. However, both treatments are expensive and do not always ensure a full recovery.[16] Considerable research on GBS is therefore required to make advancements in convenient and inexpensive treatment plans.

2.2. Historical background

The clinical entity of GBS was first described by Jean-Baptiste Octave Landry in 1859 in relation to 10 patients with mysterious ascending paralysis.[17] In 1916, three French physicians (Georges Guillain, Jean Alexandre Barré, and Andre Strohl) noted two French soldiers experiencing motor weakness, areflexia, and diminished deep tendon reflexes, along with the typical findings in the cerebrospinal fluid (ACD). This disorder was eventually named 'Guillain-Barré syndrome'. Historically, GBS was thought to be one disease, but several variants have since been recognized.

2.3. Epidemiology

GBS occurs throughout the Western hemisphere without geographical clustering or seasonal variations. Population-based studies in 2016 indicated that the crude mean annual incidence rate of GBS varied from 0.6 to 1.9 cases per 100,000 populations. The reported incidence of GBS in Western countries in 2011 ranged from 0.89 to 1.89 cases (median, 1.11) per 100,000 people per year, although an increase of 20% is seen with every 10-year rise in age after the first decade of life, and the ratio of males to females with the syndrome is 1.78 (95% CI, 1.36 to 2.33).[18] The current crude mean annual incidence rate is reported as varying from 1 to 2 per 100,000 population.[19] GBS can occur at any age, but adults are more frequently affected than children, and males are more susceptible than females (ratio 3:2).[3]

2.4. Course of the disease

The majority of patients with GBS develop the syndrome following a bacterial or viral infection.[12,20–23] In response to the infection, the body produces anti-ganglioside antibodies, depending on the antecedent infection and the GBS subtype ***(Figure 2.1).*** Patients start exhibiting disease symptoms at around 2 weeks, and reach nadir (maximum progressive weakness) by approximately 4 weeks. In some cases, a plateau phase persists before the recovery phase begins, which may last for weeks, months, or even years.

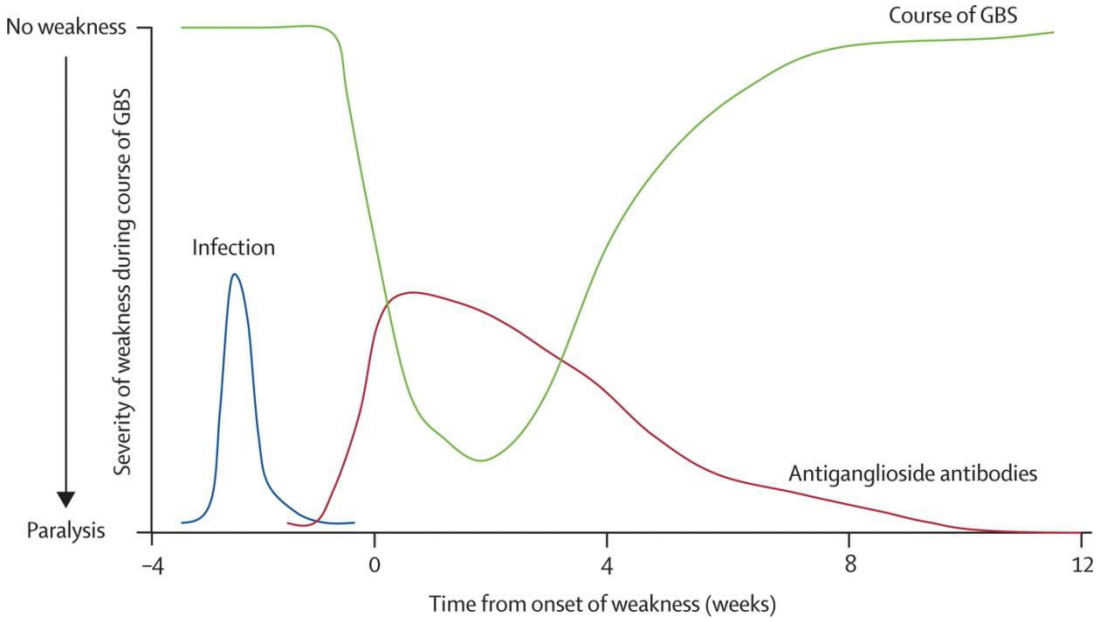

Figure 2.1 Course of Guillain-Barré syndrome (GBS).[24]

2.5. Disease symptoms

Typically a gastrointestinal or respiratory infection occurs 2-4 weeks prior to the presentation of GBS,[16] which features marked muscle weakness in the lower limbs, and is symmetric. Muscle weakness usually ascends the body affecting the upper limbs, truncal and respiratory muscles. Sometimes even the cranial nerves are involved, resulting in facial, oculomotor, or bulbar Weakness.[3] Patients also complain of paresthesia, which is a burning or prickling sensation usually felt in the hands, arms, legs, or feet, but can occur in other parts of the body. Autonomic dysfunction is common including blood pressure or heart rate instability, pupillary dysfunction, and bowel or bladder dysfunction.[19,25] The sensation, which happens without warning, is usually painless and described as tingling or numbness, skin crawling, or itching. However, weakness and pain that can be mostly muscular, radicular or neuropathic are felt and reported frequently.[19]

2.6. Diagnosis of GBS

Diagnosis of GBS is mainly based on clinical signs and symptoms since there are no reference tests or specific diagnostic markers that allow positive confirmation of a diagnosis of GBS. Diagnostic criteria for GBS were derived in 1978 at the request of the National

Institute of Neurological and Communicative Disorders and Stroke (NINDS).[26] The basis for issuing diagnostic criteria was related to the swine flu vaccine incident of 1976-1977.[27,28] At a conference on GBS in 1981, clarification of these diagnostic criteria (***Table 2.1***) was offered.[26] The criteria were reconfirmed in 1990 and still remain the gold standard for clinical diagnosis of GBS.[26]

Table 2.1: Diagnostic criteria for typical Guillain-Barré syndrome described by Asbury and Cornblath (NINDS criteria)[26]

Features required for diagnosis	• Progressive weakness in both arms and legs which may begin as weakness only in the legs • Areflexia or hyporeflexia
Features strongly supportive of the diagnosis	• Progression of symptoms over days to 4 weeks • Relative symmetry of symptoms • Mild sensory symptoms or signs • Cranial nerve involvement • Autonomic dysfunction • Absence of fever at onset • Pain • High protein concentration in CSF • ≤10 leukocytes/mm^3 in CSF • Typical electrodiagnostic features, e.g. conduction block
Features that should raise doubt about the diagnosis	• Severe pulmonary dysfunction • Sharp sensory level • Bladder or bowel dysfunction at onset • Fever at onset • Marked persistent asymmetry of weakness • Persistent bladder or bowel dysfunction • Presence of polymorphonuclear cells in CSF

NINDS, National Institute of Neurological Diseases and Stroke; CSF, cerebrospinal fluid.

2.7. Subtypes of GBS

Historically, GBS was thought to be one distinct disease, however in-depth analysis has revealed several variants, which are now classified on the basis of electrophysiological data and the sites of damage in the body.

AIDP is the most highly occurring form of GBS worldwide (70%) and is found to be the dominating subtype in North America and Europe.[8] Demyelination is mediated by macrophages, and nerve cells are infiltrated by lymphocytes, making AIDP a T cell-mediated disorder. Nerve cells being attacked by complement-mediated antibody adds to the problem.[29] Re-myelination alleviates symptoms.

Patients with the AMAN subtype exhibit rapidly ascending symmetrical weakness and resultant respiratory failure. The majorities of patients (75%) report a preceding infection with *C. jejuni*, and consequently show positive serology for *Campylobacter*. Patients with AMAN also typically have high titers of antibodies to gangliosides (GM1, GD1a, and GD1b). Contrary to AIDP, lymphocytic infiltration is not observed in AMAN.[30] In fact, demyelination does not occur, and only the axons are affected as a result of complement-mediated antibodies attacking the nodes of Ranvier, exhibiting an alternative mechanism.[31]

AMSAN variants of GBS show all the traits of AMAN, and the sensory nerves are also affected. Like in AMAN, progression is rapid and symmetric, but both sensory and motor dysfunction occur.[32] Only 5% of all cases of GBS are classified as MFS, which presents as a trio of symptoms—ataxia, areflexia, and ophthalmoplegia.[33]About one-third of patients with GBS do not meet any of these criteria and are defined as ‘equivocal’ or ‘inexcitable’.[19]

2.8. Pathogenesis

GBS develops through the action of various components of the immune system, culminating in extensive nerve damage. There is no single common physiological process for the disease in general; however, the involvement of anti-ganglioside antibodies, complement activation, and association with certain preceding events are always present in every form of the disease.

2.8.1. Antecedent infections in GBS

Preceding symptoms of diarrhea or upper respiratory tract infections have been found in two-thirds of cases of GBS.[16,20] *C. jejuni* is the leading (25-55%) infectious agent in triggering the development of GBS in adult patients.[12,20,34] Other infectious agents, including *Cytomegalovirus* (6-15%),[20,21,35] *Mycoplasma pneumonia* (3-21%),[20,35,36] *Haemophilus influenza* (1-9%) [20,37,38] Epstein-Barr virus (1-10%) ,[20,35] and Hepatitis E virus (5%),[23,39] have been associated with the development of GBS. Herpes simplex virus (1%),[20] Varicella-zoster virus (1-1.3%),[20,40] Influenza virus (2-3%),[20,41] and *Salmonella enterica* (1%)[42,43] have also been reported as infectious agents in GBS.

2.8.2. Anti-ganglioside antibodies

Gangliosides are a large family of glycosphingolipids, predominantly distributed on the cell surface membrane and anchored in the external leaflet of the lipid bilayer by a ceramide moiety.[44] Cross-reactive auto-antibodies are elevated in the sera of patients with GBS during the acute phase [45,46] and associated with the clinical spectrum.[47–50] This high proportion of anti-ganglioside antibodies is thought to contribute to neuronal pathology by inducing complement-mediated axonal injury and demyelination.[51] Gangliosides GM1, GM1b, GD1a, GalNac-GD1a, GD2, GD3, LM1, GQ1b, GT1a, and GM2 have been identified as targets for auto-antibody production in GBS.[44,52] Antibodies to GM1, GM1b, GD1a, and GalNac-GD1a are particularly common in AMAN [53] and, with the exception of GalNacGD1a, in AMSAN.[1]The MFS subtype is especially associated with antibodies to GQ1b.[1,54]

2.8.3. Molecular mimicry

The hypothesis of molecular mimicry is based on epidemiological, clinical and experimental evidence of the association of infectious agents with autoimmune diseases.[44] The term “molecular mimicry” was coined by Damian in 1964 to define the sharing of antigens between microbes and hosts.[55] There are four proposed criteria for molecular mimicry: (i) establishment of an epidemiological association between the infectious agent and the immune-mediated disease; (ii) identification of T cells or antibodies directed against the patient’s target antigens; (iii) identification of microbial mimics of the target antigen; and (iv)

reproduction of the disease in an animal model. The fulfillment of all four criteria clearly indicate that GBS is a true case of molecular mimicry ***(Figure 2.2)***.[44]

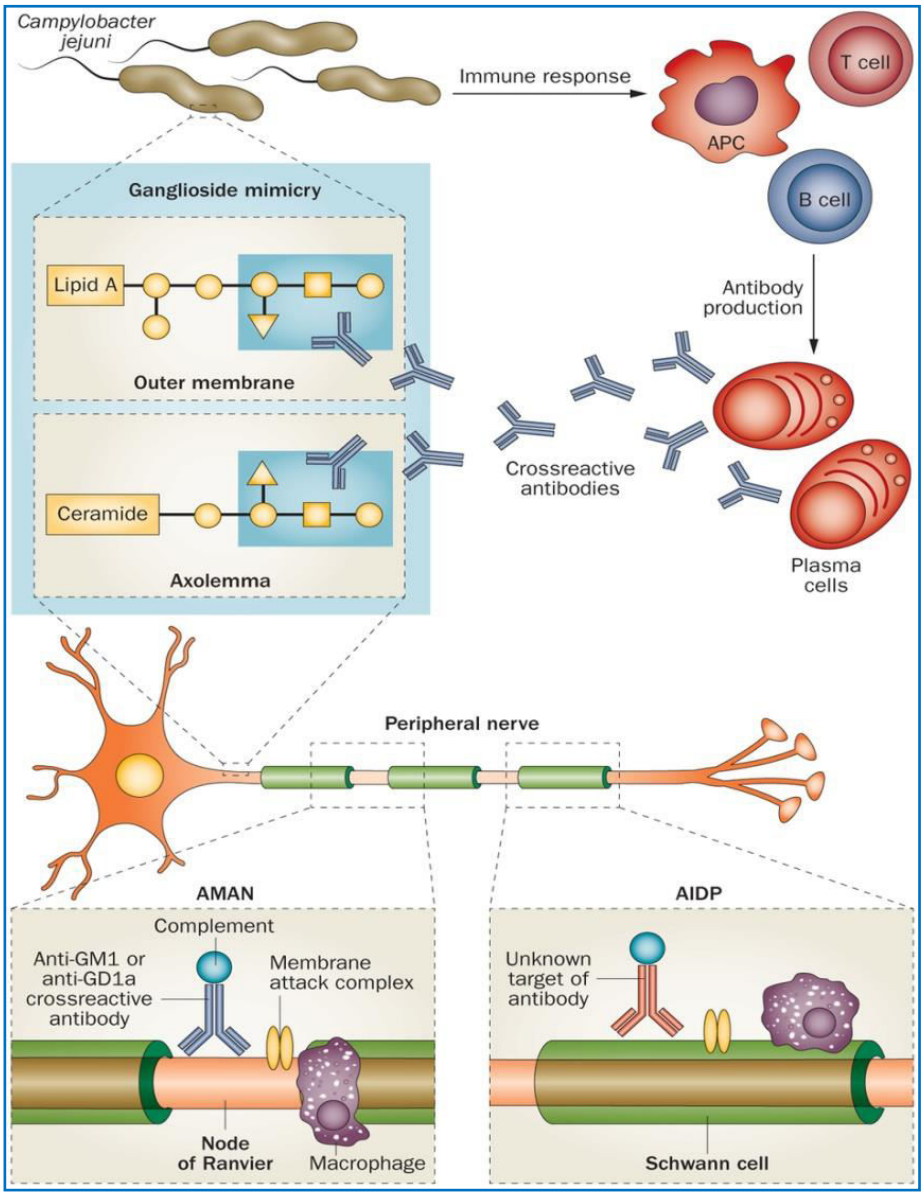

Figure 2.2 Immunopathogenesis of Guillain-Barré syndrome (GBS): molecular mimicry and anti-ganglioside antibodies [25]

In the case of *C. jejuni*-triggered GBS (***Figure 2.3***), antigens present on the capsule of *C. jejuni* closely resemble the ganglioside structure present on the surface of host nerves. Antibodies produced by the body in response to a *C. jejuni* infection therefore have the ability to cross-react with the host's myelin, causing demyelination and giving rise to GBS. Hence, GBS is considered to be auto-immune in nature. The type of antibody generated determines the variant of GBS that the patient develops (either axonal, demyelinating, or MFS). However, host susceptibility appears to play a greater role than molecular mimicry, as only a small percentage of patients (1 in 1000-5000 patients) suffering from a *C. jejuni*-induced enteric infection go on to develop GBS. [56-59]

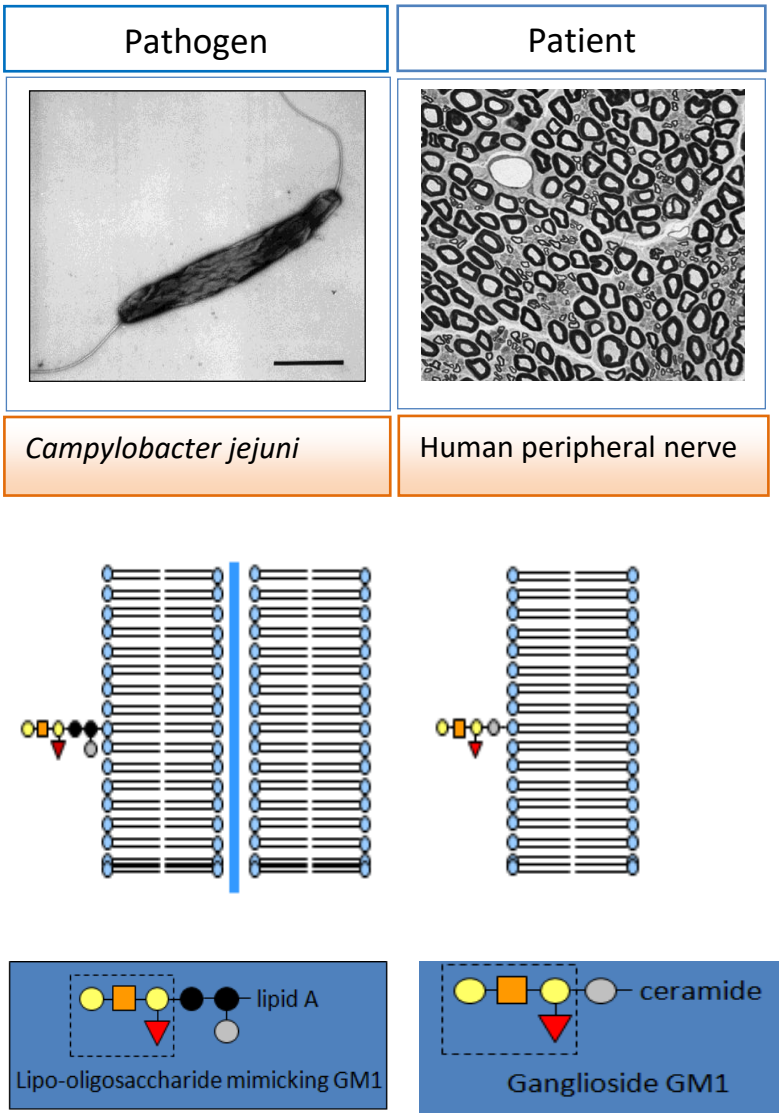

Figure 2.3 Molecular mimicry between lipo-oligosaccharides of *C. jejuni* and human peripheral nerve GM1 ganglioside.[60]

2.8.4. Host factors

As previously stated, less than 1 in 1000-5000 patients with a *C. jejuni* infection develop GBS.[56–59] In addition, the extent of nerve damage and severity of disease appear to be greater in some patients than others. In fact, the clinical presentation of GBS can differ within a subgroup of patients having the same antecedent infection, reaffirming the role of host factors in GBS. Numerous reports have cited the occurrence of GBS in multiple members of a family.[61,62] Three families had affected siblings, and four families had an affected parent and offspring.[62] Such familial association is usually observed in cases of genetically inherited diseases, suggesting an active role for at least one genetic component in GBS. Moreover, recurrence of GBS (RGBS)[63–65] also indicates the importance of genetic host factors in the susceptibility and severity of GBS.

2. 8. 5. Genetic polymorphisms

Genetic polymorphisms are changes in gene sequences among individuals, groups, or populations.[66] These gene changes may significantly hinder protein production and normal physiological processes, and thereby contribute to disease development. Some of these genetic variants are a result of single nucleotide polymorphisms (SNPs), whereas others are multi-site variants. SNPs may result in amino acid substitutions, leading to altered protein function or splicing, may change the structure of enhancer sequences during splicing,[67] or may affect mRNA stability.[68,69] SNPs can alter transcription factor binding motifs, changing the efficacy of enhancer or repressor elements,[70] and can also alter the structure of translation initiation codons that may lead to downregulation of the wild-type transcript.[71]

Investigating the associations of genetic polymorphisms with pathogenesis is a key step in developing new treatment strategies for clinical disorders. Several polymorphisms have been studied to determine probable roles in the etiology of GBS, including the genes encoding mannose-binding lectin,[72] tumor necrosis factor alpha (TNF-α),[73–75] Fc-gamma receptor (FcγRs),[76] Toll-like receptor-4 (TLR-4),[77–79] matrix metalloproteinase 9 (MMP9) ,[74] CD1A and CD1E gene polymorphisms,[80,81] and *FAS* promoter polymorphisms.[82,83] However, confirmation of the polymorphisms in large and unselected groups of patients, along with their functional effects, needs to be established to identify these polymorphisms as disease-

causing factors. Furthermore, for previous studies to be conclusive, they need to be conducted on GBS-affected populations throughout diverse geographical locations, since different populations are affected in different ways. Polymorphisms in *TNF-α*, *CD1A*, *CD1E*, *TLR-4*, and the FAS-FASL region have previously been reported in Bangladeshi patients with GBS.[75,79,81,83]

This thesis aims to yield an improved understanding of the contributions of a range of polymorphisms on the pathogenesis of patients with GBS in Bangladesh. The gene changes to be studied include human leukocyte antigen-*DQB1 (HLA-DQB1)* polymorphisms, nucleotide-binding oligomerization domain (*NOD*) polymorphisms, SNPs in immunoglobulin G Fc-gamma receptors (*FcγRs*), Interleukin-10 (*IL-10*) promoter polymorphisms, and a matrix-metalloproteinase-9 (*MMP9*) promoter polymorphism (-1562 C/T).

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2.9. Hypothesis

Genetic polymorphisms of immune-response host factors are likely to be involved in the susceptibility and severity of GBS as well as in the pathogenesis of the disease in Bangladesh**.**

2.10. Objective

2. 11. General Objective

The general objective of the study was to investigate the polymorphisms of candidate markers of human leukocyte antigen (HLA)-*DQB1*, nucleotide oligomerization domain (*NOD*), immunoglobulin G FcγRs, interleukin-10 (*IL-10*), matrix metallopeptidase-9 (*MMP9*) and to evaluate their relationship with the pathogenesis of GBS in Bangladesh.

2. 12. Specific objectives

- To determine human leukocyte antigen (*HLA*) Class II *DQB1* polymorphisms and haplotype (HLA-*DQB1* *0201, *030x, *0401, *050x and *060x) in patients with GBS and healthy controls.
- To analyze nucleotide oligomerization domain (*NOD*) polymorphisms: *NOD1* (Glu266Lys) and *NOD2* (Arg702Trp & Gly908Arg) gene polymorphisms in study subjects.
- To investigate Immunoglobulin G Fc-gamma receptor polymorphisms *FcγRIIa* (*FcγRIIa-R131* and *FcγRIIa-H131*), *FcγRIIIa* (*FcγRIIIa-V158* and *FcγRIIIa-F158*), *FcγRIIIb* (*FcγRIIIb-NA1* and *FcγRIIIb –NA2*), respectively and haplotype in patients with GBS and healthy individual of Bangladesh.
- To describe the functional polymorphisms of interleukin-10 (*IL-10*), -1082 G/A, -819 C/T and -592 C/A in patients with respect to healthy population of Bangladesh.
- To determine matrix metallopeptidase-9 (*MMP9*) (-1562C/T) polymorphism in patients and controls.
- To evaluate the association of candidate polymorphic genotypes and alleles with clinical and serological features (presence of anti-GM1 antibody and *C. jejuni* serology).
- To describe the candidate polymorphic genotypes and alleles in association with susceptibility and severity of GBS patients in Bangladesh.
- To investigate the outcome and prognosis of the disease after six months of follow-up in relation with candidate gene polymorphisms.

Chapter 3

Study population and methods

3. Study population and methods

3.1. Place of study

This study was carried out at Laboratory of Gut-Brain Signaling, Laboratory Sciences and Services Division (LSSD), icddr, b, Dhaka, Bangladesh in close collaboration with the Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka, Bangladesh.

3.2. Demography of study population

In this study, three hundred and three (n = 303) Bangladeshi patients with GBS (208 males, 95 females; with a median age of 29-years-old [interquartile range, 17-42]), treated at Dhaka Medical College and Hospital (DMCH) were included according to the inclusion-exclusion criteria.[1] Patients with GBS were diagnosed using the clinical features and electrophysiological criteria described by Asbury and Cornblath[1] and enrolled between 2010 and 2016. Most of the patients were young adult (male, 69%) and lived predominantly in rural areas of Bangladesh (72%). Data were collected regarding age, sex, residence antecedent events, detailed neurological signs and symptoms, treatment, complication and duration of admission.

Table 3.1: Demography of GBS patients (n = 303)

Characteristics		**Percentage of patients**
Sex	Male	69%
	Female	31%
Age **29 (17-42)**	Child	29%
	Adult	71%
Area of living	Rural	72%
	Urban	28%

Clinical, electrophysiological and serological data of patients with GBS were obtained. Blood specimen was collected by venipuncture before medication and the disease outcome was evaluated by assessing the clinical data at specific time-points (at entry, 2 weeks, 4 weeks and after 6 months).

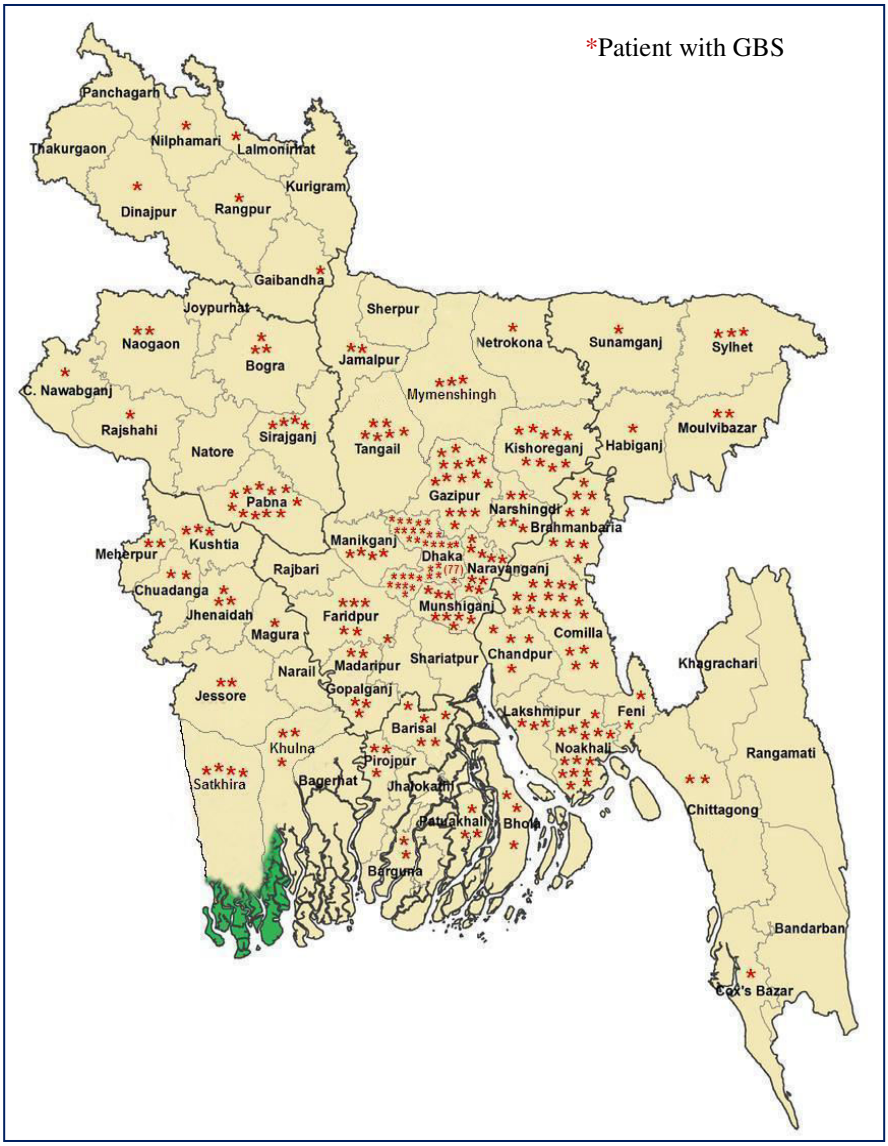


Figure 3.1 Geographical distribution of Guillain-Barré syndrome (GBS) patients enrolled in this present study in Bangladesh.

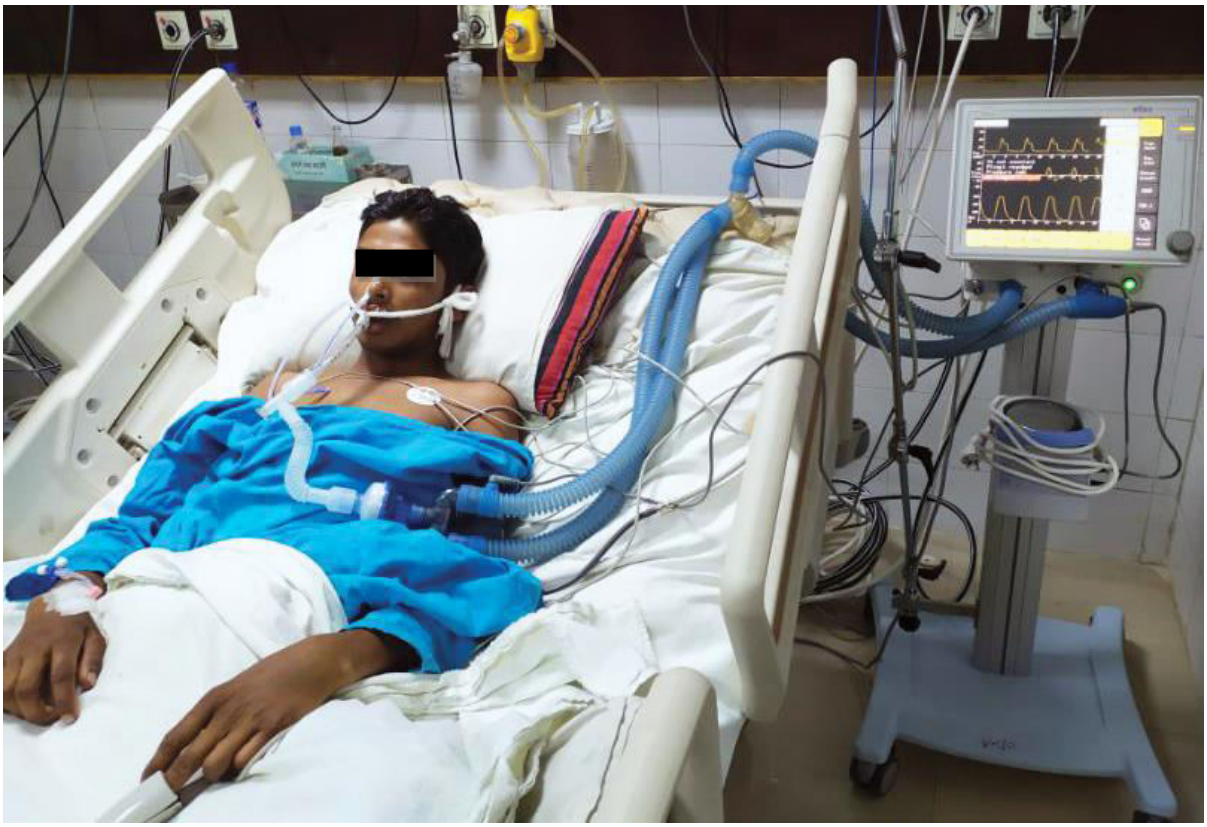


Figure 3.2 Artificially ventilated severe patient with Guillain-Barré syndrome (GBS).

In this cohort, 75% (227/303) patients had an antecedent illness with diarrhea 57% (129/227), respiratory infection 20% (45/227), fever 11% (25/227), and others 12% (28/227). Among 303 patients with GBS, 61% patients had recent *C. jejuni* infection and 38% patients were anti-GM1 ganglioside antibody, 15% were anti-GD1a ganglioside antibody and 9% were anti-GQ1b ganglioside antibody serology positive. Patients with GBS were classified on the basis of electrophysiological studies.[2] Electrophysiological studies of 82% (247/303) of GBS patients revealed axonal variant of GBS 59% (146/303, including acute motor axonal neuropathy [AMAN] and acute motor and sensory axonal neuropathy [AMSAN]); demyelinating type 27% (68/247, acute inflammatory polyradiculoneuropathy [AIDP]) and unclassified GBS cases with inexcitable nerves or equivocal, were 13% (33/247) respectively. Prior to data collection, clinical examination and specimen collection, a written informed consent was taken from all the patients with GBS (or authorized person).

3. 3. Healthy controls

Three hundred and three (n = 303) Bangladeshi healthy individuals (204 males and 99 females) were recruited as healthy controls with median age of 34 years old (interquartile range, 28-46).[2] Healthy individuals were genetically unrelated and matched with patients, all were free from neurological diseases, recent infection, chronic diseases, major surgery and other medical illnesses. Written informed consent was obtained from all the healthy individuals before data collection and specimen collection. Healthy controls were recruited during sample enrolment period.

3.4. C. jejuni and anti-ganglioside antibody serology

Serology against *C. jejuni* and antibodies against GM1, GD1a and GQ1b gangliosides were measured by enzyme linked immunosorbent assay (ELISA).[3–5]

3.4.1. C. jejuni serology

3.4.1.1. Measurement of IgG serology

The microtitre wells were coated with unlabelled *Campylobacte*r antigen in 0.1M NH_4HCO_3 buffer and stored at -20^{0}C.Tris bactopeptone buffer (0.01 MTris-HCl, pH 8.0, neutralised bacteriological peptone [Oxoid, Basingstoke, UK] 5% v / v, Tween-20 0.25% v / v) was used for dilution of sera (1:100). Tris-bactopeptone buffer contained an *Escherichia coli* J5 acid glycine extract (5% v/v) to minimize a specific reactivity. The plates were then incubated at 37^0 C for 1 hour. After incubation plates were washed with PBS solution (pH 7.4). Hundred microliter enzyme conjugate (campy-PO-IgG diluted 1:1900 in Phosphate-buffered saline-1% *Bovine serum albumin* [PBS-1%BSA]) was added to each micro-plate wells. After incubation at 37^{0}C for 1 hour, the plates were washed with PBS solution (pH 7.4). Hundred micro-liter substrate solution (3, 3′, 5, 5′-tetramethylbenzidine) was added in each well and incubated for 30 minutes at room temperature in dark. Hundred micro-liter stop solution (3M H_2SO_4) was added to the well to stop the reaction. The absorbance was taken at 450 nm using a microtiter plate reader after 5 minutes of adding the stop solution

3.4.1.2. Measurement of IgM or IgA serology

Micro-titer wells of ELISA plates were coated with diluted anti-human IgM or IgA antibodies) (Dako, Glostrup, Denmark) and stored at -20˚C. Tris bactopeptone buffer (0.01 M Tris-HCl, pH 8.0, neutralised bacteriological peptone [Oxoid, Basingstoke, UK] 5% v / v, Tween-20 0.25% v / v) was used for dilution of sera (1:100). Tris-bactopeptone buffer contained an *Escherichia coli* J5 acid glycine extract (5% v / v) to minimize a specific reactivity. The plates were then incubated at 37^0 C for 1 hour. After incubation plates were washed with PBS solution (pH 7.4). Hundred micro-liter enzyme conjugate (campy-PO-IgM or campy-PO-IgA, 1:1900 diluted in PBS-1%BSA) was added to each wells and incubated at 37^0 C for 1 hour. After washing, 100 µl substrate solution (3, 3′, 5, 5′-tetramethylbenzidine) was added to the wells and incubated for 30 minutes at room temperature in dark. Hundred microliter stop solution (3M H_2SO_4) was added to the plate to stop the reaction. The absorbance was taken at 450 nm using a microtiter plate reader after 5 minutes of adding the stop solution.

3.4.2. Anti-ganglioside antibody detection (IgG and IgM)

Micro-titer ELISA plates were coated by adding 100 micro liter (µl) absolute ethanol without GM1 solution and absolute ethanol with GM1 solution in respected 96 wells (marked) and incubated overnight at room temperature (±20 ^{0}C). After incubation (when wells were completely dry) 200 µl PBS-1%BSA solution was added and incubated 2 hours at room temperature (±20 ^{0}C) then another 2 hours in a refrigerator (±4 ^{0}C), respectively. After blocking the plate, 100 µl diluted serum and control samples (1:100 in PBS-1%BSA) were added and incubated overnight at ±4 ^{0}C. After overnight incubation plates were washed perfectly (at least 6 times) with PBS (pH 7.8) to remove the unbound product. Plates were ready for another 90 minutes incubation at ±20 ^{0}C after adding 100 µl diluted peroxidase-conjugate (1:2500 diluted in PBS-1%BSA) in each well; 1 plate IgG and the other IgM. After incubation plates were washed and 100 µl substrate solutions were added then incubated 10 minutes at dark. Absorbance was measured at 490 nm within 15 minutes after adding 100 µl stop solution (2N HCL) to each well to stop the reaction. The end result is obtained by subtracting the average extinction of the GM1-negative wells from the GM1-positive wells.

3. 5. Muscle strength assessment

Severity of the disease was measured based on Medical Research Council (MRC)-sum score (ranging from 0-60)[6,7] at nadir (maximum muscle weakness). The MRC-sum score was defined as the summation of MRC score of six muscles in the upper and lower limbs on both sides, ranging from 60 (normal strength) to 0 (quadriplegic).[6] The rapidity of progression was indicated by the number of days from the onset of weakness to nadir, defined as the lowest MRC sum score with an severity of GBS, was defined using the MRC sum score for six muscles in the upper and lower limbs on both sides.[8,9]

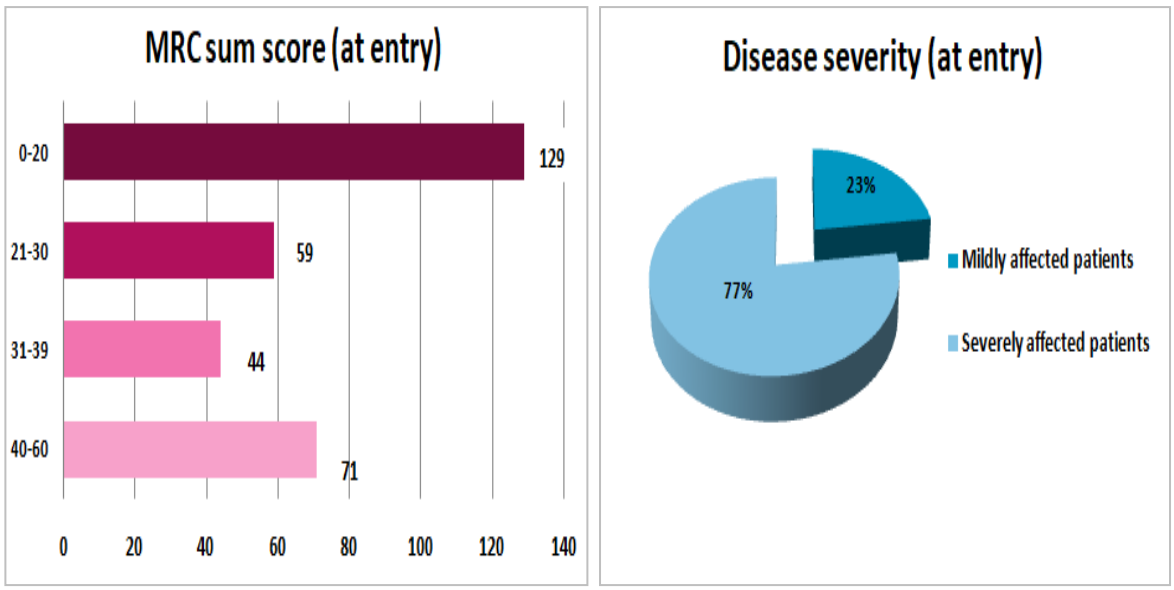


Figure 3.3 Muscle strength assessments. GBS disease severity based on (MRC)-sumscore (ranging from 0-60) indicated maximum patients with GBS were severely affected (77%).

Patients with GBS at nadir with MRC-sumscore <40 were defined as severely affected patients and with MRC-sumscore ≥ 40-60 were defined as mildly affected patients.[8,10] In this cohort 77% (232/303) patients with GBS were severely affected and 23% (71/303) were mildly affected.

3. 6. Outcome and prognosis of the disease assessment

In this cohort, outcome of the disease was assessed based on GBS disability score (GBS-DS) described by Hughes et al. ranging from 0 (Healthy) to 6 (Death)[8,11] after six months of follow-up. According to the GBS-DS a 'good outcome' was defined as the ability of patients to ambulate without assistance (GBS-DS of 0, 1, and 2) and a 'poor outcome', as the inability of patients to ambulate independently or death (GBS-DS of 3, 4, 5 and 6).[8,11] After six months, sixty-nine percent patients with GBS had good outcome with severe residual disability (29%) and thirty-one percent had poor outcome during course of the disease.

3.7. Genomic DNA isolation

Whole blood was collected from all 606 participants into lithium heparin anti-coagulant coated blood collection tubes for genomic DNA isolation. The QIAamp® DNA Blood Midi Kit (100; Qiagen, Hilden, Germany) was used to isolate genomic DNA according to the manufacturer's instructions. The eluted DNA samples were dissolved in 1×TE-buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at -80°C. DNA samples were diluted in Milli-Q water to a final concentration of 10ng/µL and stored at -20°C until genotyping.

3.8. Detection and genotyping of candidate genes

Sequence-specific polymerase chain reaction (PCR-SSP),[12,13] allele-specific polymerase chain reaction (AS-PCR)[14,15] and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[8,16] were used to detect the alleles and genotypes. For PCR-SSP and PCR-AS specific internal positive controls were used.[17,18] PCR was performed using a MJ Research PTC-200 Thermal Cycler with specific reaction condition and primer pairs.[8,12,14–16] Primers were designed or collected from published papers (***Tables 3.2a, 3.2b, 3.2c and 3.2d***).[8,12,14–18] For polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) a number of restriction endonuclease were used to digest the PCR products according to the manufacturer's instructions (***Table 3.2a, 3.2b, 3.2c and 3.2d***).

Table 3.2a: List of sequence specific primer sequences used for detection of HLA-DQB1gene polymorphisms

Allele	Method	Primer sequence (5′→3′)	PCR product size (bp)	Length
HLA-DQB1				
DQB1*0201	SSP-PCR	Forward- GTGCGTCTTGTGAGCAGAAG Reverse- GCAAGGTCGTGCGGAGCT	205	20 mer 18 mer
DQB1*0201/0302	SSP-PCR	Forward- GACGGAGCGCGTGCGTCT Reverse- CTGTTCCAGTACTCGGCGG	129	18 mer 19 mer
DQB1*0301/4	SSP-PCR	Forward- GACGGAGCGCGTGCGTTA Reverse- AGTACTCGGCGTCAGGCG	122	18 mer 18 mer
DQB1*0303	SSP-PCR	Forward- GACGGAGCGCGTGCGTTA Reverse- CTGTTCCAGTACTCGGCGT	129	18 mer 19 mer
DQB1*0401	SSP-PCR	Forward- CACCAACGGGACCGAGCT Reverse- GGTAGTTGTGTCTGCATACG	200	18 mer 20 mer
DQB1*0501	SSP-PCR	Forward- CGGAGCGCGTGCGGGG Reverse- GCTGTTCCAGTACTCGGCAA	128	16 mer 20 mer
DQB1*0502	SSP-PCR	Forward- TGCGGGGTGTGACCAGAC Reverse – TGTTCCAGTACTCGGCGCT	117	18 mer 19 mer
DQB1*0503	SSP-PCR	Forward- TGCGGGGTGTGACCAGAC Reverse – GCGGCGTCACCGCCCGA	87	18 mer 17 mer
DQB1*0601	SSP-PCR	Forward- GCC ATG TGC TACTTCACCAAT Reverse-CAC CGT GTC CAA CTC CGCT	198	21 mer 19 mer
DQB1 *0602	SSP-PCR	Forward-CGTGCG TCT TGT GAC CAGAT Reverse-GCT GTT CCA GTA CTC GGC AT	121	20 mer 20 mer
DQB1*0603/608	SSP-PCR	Forward-GGA GCG CGT GCG TCT TGTA Reverse- GCT GTT CCA GTA CTC GGC AT	127	19 mer 20 mer
DQB1*0604	SSP-PCR	Forward-CGT GTA CCA GTT TAA GGG CA Reverse-GCA GGA TCC CGC GGT ACC	254	20 mer 18 mer
DQB1*0605	SSP-PCR	Forward-CGT GTA CCA GTT TAA GGG CC Reverse-GCA GGA TCC CGC GGT ACC	254	20 mer 18 mer
Internal control				
DRB1		Forward-TGCCAAGTGGAGCACCCAA Reverse-GCATCTTGCTCTGTGCAGAT	796	19 mer 20 mer

SSP-PCR, sequence specific polymerase chain reaction; bp, base pair; *HLA-DQB1,* Human leukocyte antigen-*DQB1*.

Table 3.2b: Primer sequences and enzymes used to detect NOD1 (Glu266Lys, rs6958571 and NOD2 (Arg702Trp, rs2066844 and Gly908Arg, rs2066845) polymorphisms

SNP	Method	Primer sequence (5′→3′)	PCR product size (bp)	Length	Restriction Endo-nuclease
NOD1 (Glu266Lys)	*PCR-RFLP*	Forward-AAGTGACAGGCTGTGTCTGC Reverse-CTTCCCACTGAGCAGGTTG	232	20 mer 19 mer	Bsob1 (5'-CYC**G**RG-3')
NOD2 *(Arg702 Trp)*	*PCR-RFLP*	Forward- CTG GCA GGG CTG TTG TCC Reverse- TGGCGGGATGGAGTGGAA	141 141,72, 69 72, 69	18 mer 18 mer	Msp1 (5'-CCGG-3')
NOD2 *(Gly908 Arg)*	*PCR-RFLP*	Forward- ACATATCAGGTACTCACT GA Reverse-GATCACCCAAGGCTTCAG	113 113, 60,53 60, 53	20 mer 18 mer	Hha I (5'-G**C**GC-3')

SNP, single nucleotide polymorphism; *NOD*, Nucleotide oligomerization domain; PCR, polymerase chain reaction, bp, base pair.

Table 3.2c: List of allele specific primer sequences used for detection of Immunoglobulin G (IgG) FcγR polymorphisms

Immunoglobulin G FcγRs	Method	Primer sequence (5′→3′)	PCR product size (bp)	Length
SNP				
FcγRIIa- H131	PCR-AS	Forward-ATCCCAGAAATTCTCCCA Reverse-CAATTTTGCTGCTATGGGC	253	18 mer 19 mer
FcγRIIa- R131	PCR-AS	Forward-ATCCCAGAAATTCTCCCG Reverse-CAATTTTGCTGCTATGGGC	253	18 mer 19 mer
FcRγIIIa-F158	PCR-AS	Forward- TCACATATTTACAGAATGGCAATGG Reverse-TCT CTG AAGACA CAT TTC TAC TCC CTA C (G specific)	138	25 mer 31 mer
FcRγIIIa-V158	PCR-AS	Forward- TCACATATTTACAGAATGGCAATGG Reverse-TCT CTG AAG ACA CAT TTC TACTCC CTA A (T specific)	138	25 mer 31 mer
FcγRIIIb-NA1	PCR-AS	Forward-CTCAATGGTACAGGGTGCTC Reverse-GGCCTGGCTTGAGATGAGGT	118	20 mer 20 mer
FcγRIIIb-NA2	PCR-AS	Forward-CTCAATGGTACAGCGTGCTT Reverse-CACCTGTACTCTCCACTGTCGTT	171	20 mer 23 mer
Internal control				
HGH (FcγR)		Forward-GCCTTCCCAACCATTCCCTTA Reverse-CTCACGGATTTCTGTTGTGTTTC	428	21 mer 23 mer

FcγR, Fc gamma receptor*;* SNP, single nucleotide polymorphism; PCR-AS, allele specific polymerase chain reaction; bp, base pair; HGH, human growth hormone; NA, nuetrophil antigen.

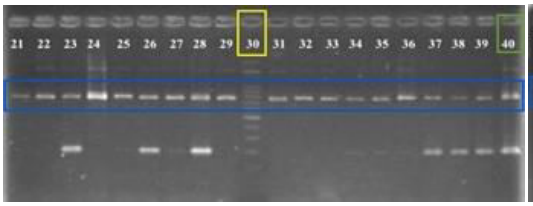
Table 3.2d: Primer sequences and enzymes used to detect promoter polymorphisms of Interleukin-10 (IL-10) -1082 G/A (rs1800896), -819 C/T (rs1800871) and -592 C/A (rs1800872) and MMP9-1562 C/T

SNP	Method	Primer sequence (5′→3′)	PCR product size (bp)	Length	Restriction Endo-nuclease
IL-10 (-1082 G/A)	*PCR-RFLP*	Forward CTCGCTGCAACCCAACTGGC Reverse TCTTACCTATCCCTACTTCC	139 139, 106 106	20 mer 20 mer	*MnlI*
IL-10 (-819 C/T)	*PCR-AS*	Forward-C specific CCCTTGTACAGGTGATGTAAC Forward-T specific CCCTTGTACAGGTGATGTAAT Reverse CCTAGGTCACAGTGACGTGG	483	21 mer 21 mer 20 mer	NA
IL-10 (-592 C/A)	*PCR-RFLP*	Forward CCTAGGTCACAGTGACGTGG Reverse GGTGAGCACTACCTGACTAGC	412 412, 236, 176 236, 176	20 mer 21 mer	*RsaI*
MMP9 (-1562 C/T)	*PCR-RFLP*	Forward-AAA TGG CAG AGC CGG GAT Reverse-ACCAGCAGCCTCCCTCACT	608 608, 266, 342 266, 342	18 mer 19 mer	*Sph1*

PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; bp, base pair; NA, not applicable.

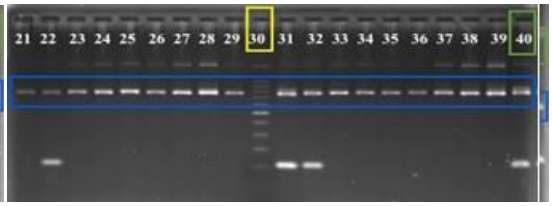
After digestions at specific sites, the products were electrophoresed on 2-3% agarose gels stained with 0.05 μg/mL ethidium bromide and the product bands were visualized using a Molecular Imager® Gel Doc™ XR+system (Bio-Rad Laboratories Inc, USA) (***Figure 3.4***). Sanger-sequencing was performed for detection of polymorphisms of some samples using by Genetic Analyzer ABI 3500 automated DNA sequencer using the same primer sequences used for PCR amplification of interest.

A.



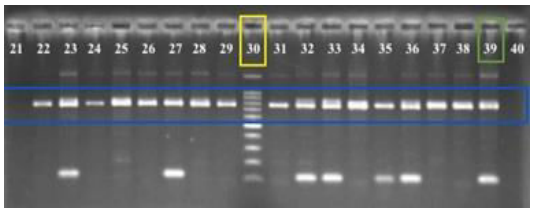
HLA-DQB1*0201 (PCR product size; 205 bp)

B.



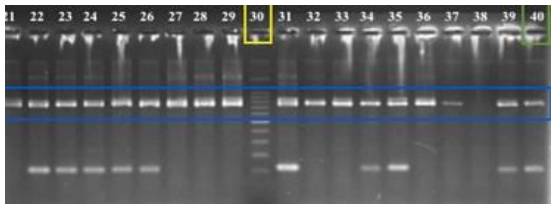
HLA-DQB1*0301/4 (PCR product size; 122 bp)

C.



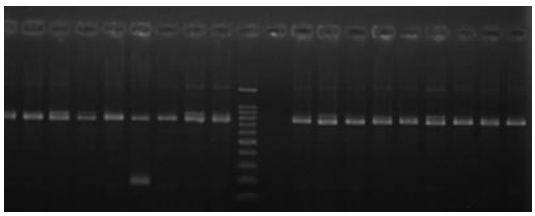
HLA-DQB1*0302 (PCR product size; 129 bp)

D.



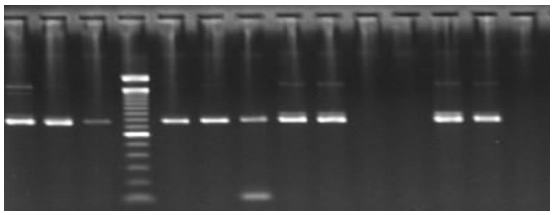
HLA-DQB1*0303 (PCR product size; 129 bp)

E.



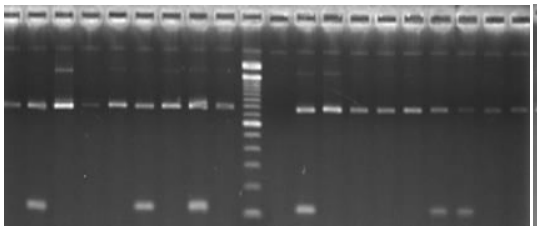
HLA-DQB1*0401 (PCR product size; 200 bp)

F.



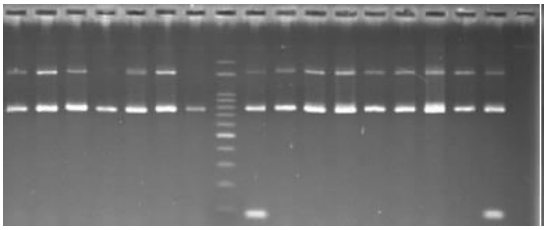
HLA-DQB1*0501 (PCR product size; 128 bp)

G.



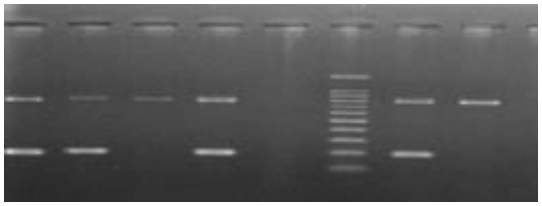
HLA-DQB1*0502 (PCR product size; 117 bp)

H.



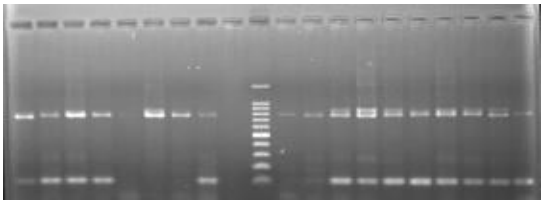
HLA-DQB1*0503 (PCR product size; 87 bp)

I.



HLA-DQB1*0601 (PCR product size; 198 bp)

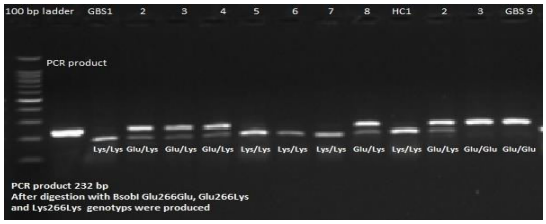
J.



HLA-DQB1*0602 (PCR product size; 121 bp)

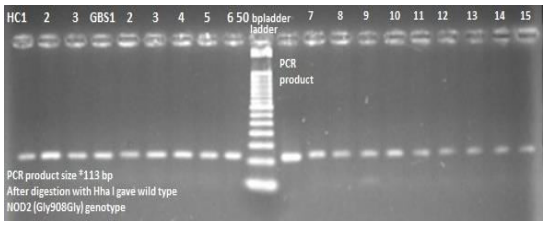
Continued

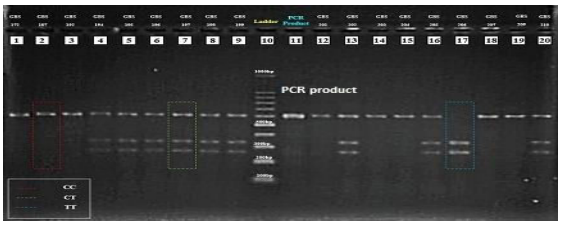
K. ***L.***



NOD1-Glu266Lys (PCR product size; 232,170 bp) ***NOD2-Arg702Trp (Wild type only; 72, 69 bp)***

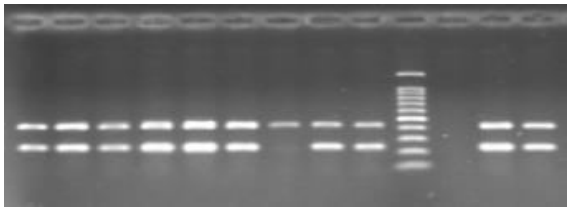
M. ***N.***

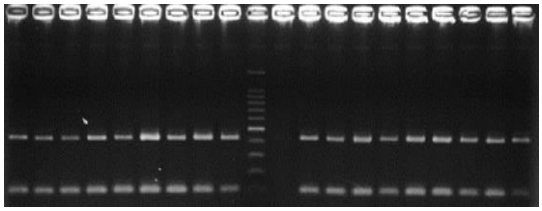




NOD2-Gly908Arg (Wild type only; 113 bp) ***MMP-9 (-1562C/T) (PCR product size; 608, 342, 266)***

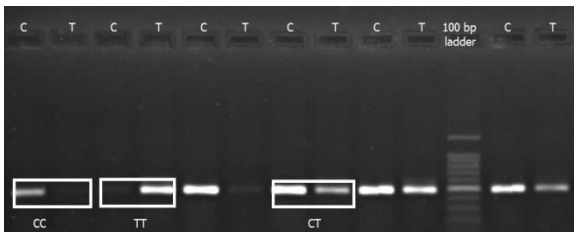
O. ***P.***

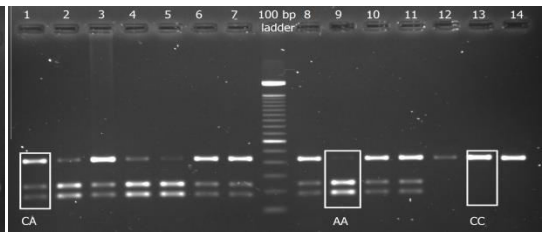




Fc gammaRII-H/H131 (PCR product size; 253bp) ***Fc gammaRIIIb-NA1 (PCR product size; 118bp)***

Q. ***R.***





IL-10, -819 (PCR product size; 483bp) ***IL-10, -592 (PCR product size; 412, 236, 176bp)***

Figure 3.4 Detection of polymorphisms (A-R). Detection of *HLA-DQB1* polymorphisms (A-J) with internal positive control (796 bp); *NOD* polymorphisms (K-M); MMP9 (N); Fc gamma receptor polymorphisms (O-P; not images of all polymorphisms were presented) with internal positive control (428 bp); IL-10 polymorphisms (Q-R; not images of all polymorphisms were presented).

3. 9. Statistical analysis

Hardy-Weinberg equilibrium was assessed using Pearson's chi-square test for healthy control group. Statistical calculations were done with the chi-square test or Fisher's exact test with Yates' continuity correction and Logistic regression analysis. Odds ratios (ORs) and 95% confidence intervals (CLs) were calculated and used to investigate the associations between polymorphism of candidate genes and the risk for GBS development. The level of significance was defined as a *P*-value less than 0.05. The Bonferroni method was applied to correct *P*-values for multiple comparisons. Statistical analyses were performed using Microsoft® Excel 2007, GraphPad Prism (version 5.01, GraphPad Software, Inc. La Jolla, CA, USA), SPSS (version 16.0, Company, Chicago, IL, USA), D´statistics and genotype package in R statistics. For sequencing analysis Chromas 2.6.6 and MEGA6 software were used.

4.0. Ethical considerations

The studies of this thesis were reviewed and approved by the Institutional Review Broad (IRB) and ethical committees at icddr,b and Dhaka Medical College and Hospital Dhaka, Bangladesh.

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Chapter 4

HLA-DQB1 polymorphisms and haplotype in GBS

Human leukocyte antigen-*DQB1* polymorphisms and haplotype patterns in Guillain-Barré syndrome

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Abstract

***Objective*:** The etiology of Guillain-Barré syndrome (GBS) remains enigmatic, although genetic and environmental factors are speculated to be associated with this autoimmune condition. We investigated whether polymorphisms and the haplotype structures of the human leukocyte antigen (HLA)-DQB1 gene relates to the autoimmune response to infection and affect the development of GBS.

***Methods*:** *HLA-DQB1* polymorphic alleles (*0201, *030x, *0401, *050x, *060x) were determined for 151 Bangladeshi patients with GBS and 151 ethnically matched healthy controls using sequence-specific polymerase chain reaction. Pairwise linkage disequilibrium and haplotype patterns were analyzed based on D′statistics and the genotype package in R statistics, respectively. Association studies were conducted using Fisher's exact test and logistic regression analysis, and the Bonferroni method was applied to correct for multiple comparisons.

***Results*:** No associations were observed between *HLA-DQB1* alleles and susceptibility to disease in the comparison between GBS patients and healthy subjects. Haplotype 9 (*DQB1**0303-*0601) tended to be less frequent among patients with GBS than healthy controls (P = 0.006, OR = 0.49, 95% CI = 0.30-0.82; Pc = 0.06). Haplotype 5 (*DQB1**0501-*0602) and the *DQB1**0201 alleles were more frequent in the *Campylobacter jejuni*-triggered axonal variant of GBS (P = 0.024, OR = 4.06, 95% CI = 1.25-13.18; Pc = 0.24) and demyelinating subtype (P = 0.027, OR = 2.68, 95% CI = 1.17-6.17; Pc = 0.35), though these associations were not significant after Bonferroni correction.

Interpretation: This study indicates *HLA-DQB1* polymorphisms are not associated with susceptibility to GBS. In addition, these genetic markers did not influence the clinical features or serological subgroup in patients with *C. jejuni*-triggered axonal variant of GBS.

Introduction

Guillain-Barré syndrome (GBS) is a post-infectious immune-mediated neuropathy that includes the symptoms of flaccid paralysis. Molecular mimicry between the outer core structures of *Campylobacter jejuni* and host nerve gangliosides is one apparent cause of GBS, and instigates a tissue-damaging autoimmune response that determines disease presentation.[1–5] However, the exact mechanisms that lead to induction of nerve fiber demyelination and axonal damage after antecedent *C. jejuni* infection remain to be elucidated. Several subtypes of GBS have been associated with specific *Campylobacter* strains, though a single strain can lead to different subtypes of GBS and only a small percentage (1 in 1000-5000 cases) of patients with *C. jejuni* enteritis develops GBS.[6,7] Thus, molecular mimicry is not the only pathogenic mechanism underlying *C. jejuni*-triggered GBS.[4] Host genetic factors may play a role by modifying regulatory elements that influence GBS susceptibility and disease pathogenesis. In particular, genetic polymorphisms and the resulting haplotype variations may play an important role in the pathogenesis of GBS.

The human leukocyte antigen (HLA) gene complex is extensively polymorphic. The *HLA-DQB1* gene, the major stimulus of the DQ antigen, is the most polymorphic HLA variant[8–10] and also exhibits the most dense linkage disequilibrium (LD).[11] *HLA-DQB1* allele variations and haplotype patterns may affect the recognition of self and non-self antigens and have been implicated in the pathology of a number of autoimmune diseases.[12] As one of the most polymorphic regions in the HLA gene complex, *HLA-DQB1* has been a focus of inquiry to investigate the genetic and pathophysiological basis of GBS and the associated immune-mediated tissue damage.[13]

Several case-control studies have investigated whether there is an association between HLA-class I or II antigens and GBS susceptibility and subgroups.[14–18] Most of these studies did not find any association or observed weak associations with regard to disease susceptibility to GBS. For example, the *DQB1**060x alleles were significantly associated with increased risk of developing GBS in the Indian population, but no association was found in the Dutch population.[14,15] One study reported an increased frequency of *DQB1**03 alleles among *C. jejuni*-infected patients with GBS compared to *C. jejuni*-negative patients, though other studies did not find any association with recent *C. jejuni* infection.[16,17] In our view, these

differences could be the consequence of limited sample sizes, as well as geographical variations and differences in GBS subtype.

In this study, we used one of the largest cohorts of GBS patients from low/middle-income countries (LMIC) to evaluate the association of *HLA-DQB1* polymorphisms with GBS disease susceptibility and the clinical features and serological subgroups of GBS. HLA allele distributions vary between patients with different subtypes of GBS.[18] Therefore, considering the varied regional distribution of HLA alleles and high endemicity and severity of GBS in Bangladesh, we also investigated the association between *HLA-DQB1* polymorphic alleles and haplotype patterns with GBS among patients and healthy controls in Bangladesh.

Materials and Methods

Study population

A total of 151 patients with GBS (102 males and 49 females; median age, 29 years [interquartile range, 17-42 years]) diagnosed with GBS at Dhaka Medical College and Hospital (DMCH) using the National Institute of Neurological Disorders and Stroke (NINDS) criteria were enrolled in this study.[19] Patients with GBS were matched with 151 genetically unrelated healthy individuals (77 males and 74 females; median age, 35 years [interquartile range 28-40 years]) without any history of neurological disorders, serious comorbidities (infection, stroke, myocardial infarction, major surgery, etc.) or chronic medical illnesses, with no specific predilection for race, religion or socioeconomic status during control selection. Written informed consent was obtained from all participants before data collection, clinical examination and specimen collection. This study was approved by the Institutional Review Board (IRB) and ethical committees of the icddr, b, and Dhaka Medical College and Hospital, Dhaka, Bangladesh.

Peripheral blood and clinical data were collected at entry before treatment for all enrolled patients. The majority of patients with GBS (130/151, 86%) had a history of a preceding illness, either diarrhea (71/130, 55%) or respiratory infection (24/130, 18%) or another preceding illness (35/130, 27%). Electrophysiological studies were performed for 104/151 (69%) patients with GBS; subtype was classified as the axonal type (59/151 [57%]: 55,

AMAN and 4, AMSAN); the demyelinating type (27/151, [26%]; AIDP), or unclassified GBS with inexcitable nerves or equivocal findings (18/104 [17%]).[20–22] The severity of disease was assessed at study entry using the Medical Research Council (MRC) sum score at nadir (maximum muscle weakness).[23] Patients with a MRC sum score at nadir of <40 were considered severely affected and between 40 and 60, mildly affected.[24] Disease outcome was measured using the GBS disability score after six months' follow-up.[25,26] Antibodies against the lipooligosaccharide (LOS) of *C. jejuni* and antibodies against GM1, GD1a and GQ1b were measured serologically using enzyme linked immunosorbent assays (ELISAs).[27,28]

Genomic DNA isolation

Whole blood was collected from all 302 participants into lithium heparin anticoagulant-coated blood collection tubes for genomic DNA isolation. The QIAamp® DNA Blood Midi Kit (100; Qiagen, Hilden, Germany) was used to isolate genomic DNA according to the manufacturer's instructions. The eluted DNA samples were dissolved in 1× TE-buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at -80°C. DNA samples were diluted in Milli-Q water to a final concentration of 10 ng/µL and stored at -20 °C until genotyping.

HLA typing

Sequence-specific PCR (PCR-SSP) was performed for *HLA-DQB1* typing using previously published primer sequences and reaction conditions.[29] A primer pair was added to each PCR reaction as an internal positive control to amplify the third intron of the *DRB1*genes.[30]

Statistical analysis

The associations between the *HLA-DQB1* alleles and susceptibility to GBS and the clinical or serological features of GBS were assessed using Fisher's exact test with Yates' continuity correction and logistic regression analysis. Allele frequencies were reported as *P*-values, odds ratios (ORs) and 95% confidence intervals (CIs). *P*-values less than 0.05 were considered statistically significant. *HLA-DQB1*allelic frequency was estimated by simple counting and the data were processed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), Graph-Pad Prism (version 5.01, GraphPad software, Inc., La Jolla, CA, USA) and SPSS (16.0 version, Chicago, IL, USA). Pair wise linkage disequilibrium (LD) was analyzed based on D′statistics for each of the 13 *HLA-DQB1* loci assessed. Haplotype structures and frequencies were estimated from genotypic data and their associations with GBS susceptibility and the clinical and serological subgroups were assessed using logistic regression analysis. Individual alleles with an allele frequency > 10% and haplotype frequency > 4% within the population were included in the association studies. The Bonferroni method was conducted to correct for multiple comparisons, whereby the *P* value was multiplied with the number of comparisons and denoted as *P*c (*P*c, *P* corrected).

Results

Influence of HLA-DQB1 polymorphisms and haplotype patterns on GBS susceptibility

The influence of 13 *HLA-DQB1*polymorphic loci on susceptibility to GBS was assessed by comparing patients and healthy controls. No alleles were significantly associated with GBS disease susceptibility (***Table 4.1***). However, a trend towards a lower frequency of the *DQB1**0601 allele was observed in patients with GBS, but this was not significant when corrections for multiple comparisons were made (*P* = 0.045, OR = 0.60, 95% CI = 0.38-0.96; *P*c = 0.58; ***Table 4.1***).

Table 4.1: Frequency distribution of *HLA-DQB1* polymorphisms in patients with GBS and healthy controls

Allele	GBS	HC	*P* value	Odds ratio (95%CI)
	***n* = 151 (%)**	***n* = 151 (%)**		
DQB1*0201	56 (37)	48 (32)	0.397	1.26 (0.78-2.03)
DQB1*0301/4	35 (23)	37 (25)	0.893	0.92 (0.55-1.58)
DQB1*0302	63 (42)	70 (46)	0.487	0.83 (0.53-1.30)
DQB1*0303	64 (42)	78 (52)	0.134	0.69 (0.44-.1.08)
DQB1*0401	39 (26)	27 (18)	0.125	1.60 (0.92-2.78)
DQB1*0501	31 (21)	36 (24)	0.580	0.83 (0.48-1.42)
DQB1*0502	12 (8)	21 (14)	0.139	0.53 (0.25-1.12)
DQB1*0503	20 (13)	17 (11)	0.726	1.20 (0.60-2.40)
DQB1*0601	51(34)	69 (46)	0.045[a]	0.60 (0.38-0.96)
DQB1*0602	87 (58)	81 (54)	0.562	1.17 (0.75-1.85)
DQB1*0603/8	7 (5)	6 (4)	1.00	1.17(0.39-3.58)
DQB1*0604	3 (2)	4 (3)	1.00	0.74 (0.16-3.39)
DQB1*0605	4 (3)	5 (3)	1.0	0.79 (0.21-3.02)
DQB1*03	114 (75)	122 (81)	0.330	0.73 (0.42-1.27)
DQB1*05	64 (42)	72 (48)	0.418	0.80 (0.51-1.27)
DQB1*06	111 (74)	117 (77)	0.5	0.80 (0.48-1.36)

GBS, Guillain-Barré syndrome; HC, healthy controls; 95% CI, 95% confidence interval; a, *P*c = 0.58 (*P*c, *P* corrected).

In haplotype analysis, a total of 136 different profiles were observed among the 2^{13} possible combinatorial patterns for the 13 *HLA-DQB1* polymorphic loci. Eighty-eight and 90 profiles were observed among the patients with GBS and healthy controls, respectively (***Figure 4.1***).

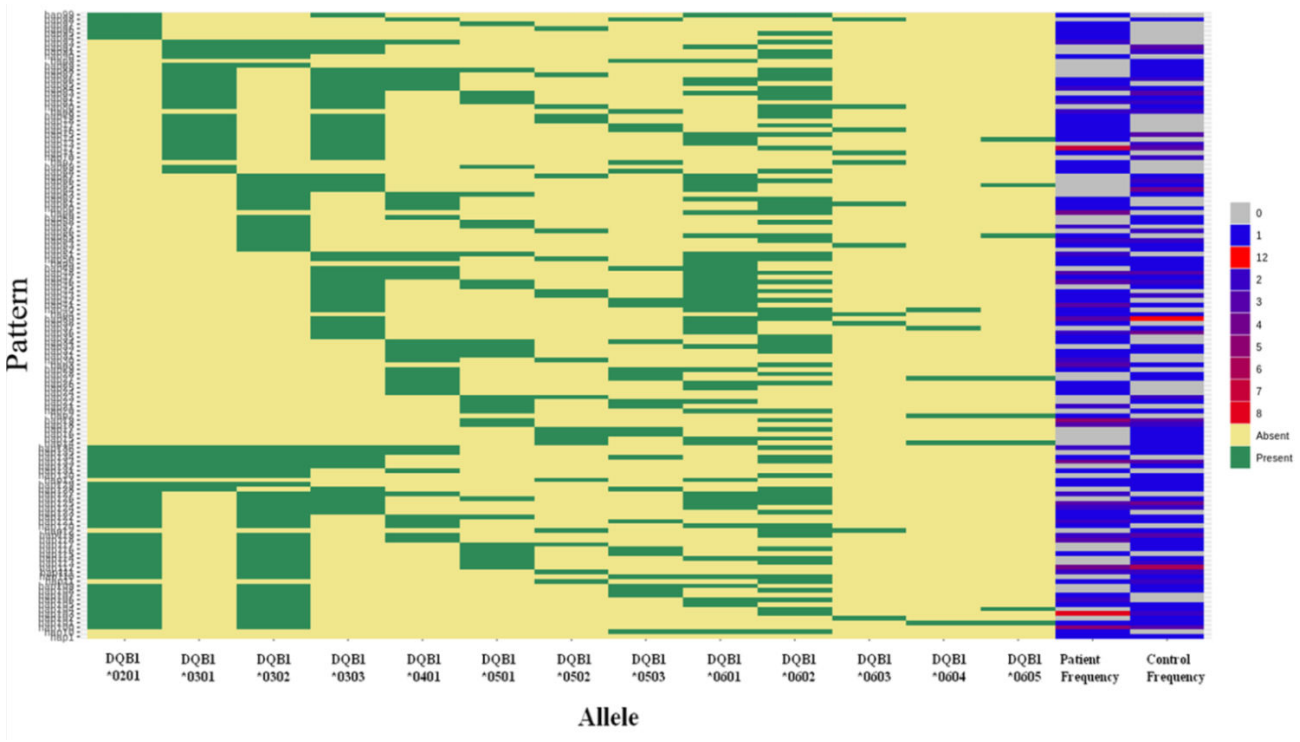

Figure 4.1 Allelic profiles of *HLA-DQB1* in patients with GBS and healthy controls. The 136 patterns for the13 *HLA-DQB1* alleles are presented on the right. Green indicates the presence and yellow indicates the absence of specific alleles for the13 *HLA-DQB1* loci. The frequencies of the patterns among patients with GBS and healthy controls are presented as color gradients with the frequencies shown on the left.

Forty-two profiles were common to both groups, with 46 profiles unique to patients and 44 unique to healthy controls (***Figure 4.1***). Of the 136 haplotype patterns, 10 haplotypes (Haplotype 1-10) were predominant (frequency > 4%); these 10 haplotypes represented 64% of total predicted haplotype variation. Haplotype 9 tended to be associated with GBS (*DQB1**0303-*0601, *P* = 0.006, OR = 0.49, 95% CI = 0.30-0.82; *Pc* = 0.06; ***Table 4.2***); no other haplotypes were significantly associated with GBS.

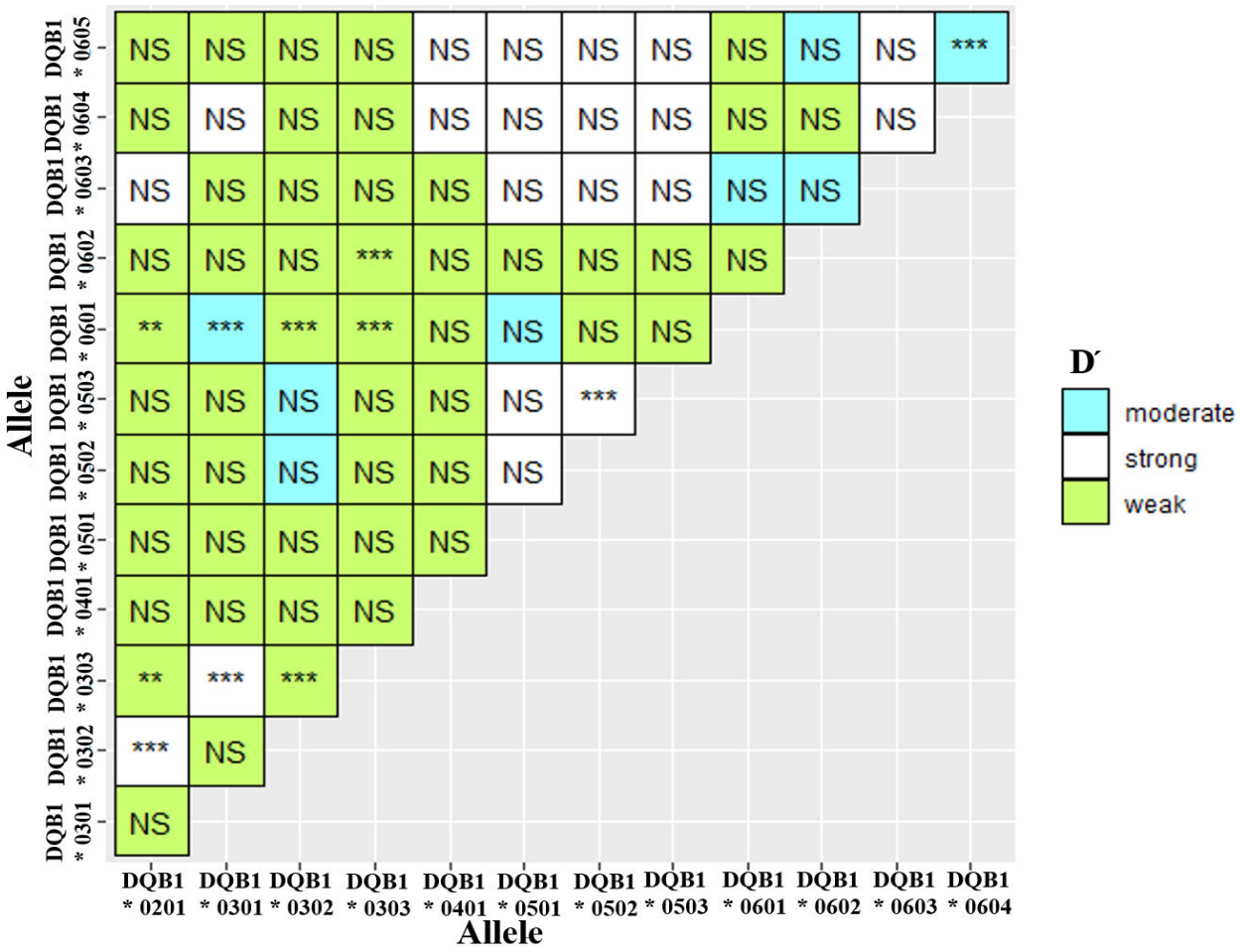

Figure 4.2 Pair wise linkage disequilibrium (LD) among the 13 HLA-DQB1 loci based on D´ statistics. D´ >0.75 indicated strong LD with white shade, D´ 0.5-0.74 indicated moderate LD with cyan shade and D´ <0.49 indicated weak LD with green shade. *P* value overwrite above the respective LD where ***<0.005, **<0.05, *<0.01, Not significant>0.1.

Table 4.2: Logistic regression-derived odd ratios for the associations of predominant haplotype (1-10) with GBS and GM1 auto-antibodies

Haplotype No.	*HLA-DQB1* alleles	GBS vs. healthy controls		Anti-GM1-Ab (positive vs. negative)	
		***P* value**	**OR (95%CI)**	***P* value**	**OR (95%CI)**
1	*0303 -*0601 -*0602	0.140	0.64 (0.36- 1.16)	0.184	0.58 (0.26- 1.30)
2	*0301 -*0303 -*0602	1.00	1.0 (0.53- 1.87)	0.581	1.23 (0.59- 2.59)
3	*0201 -*0302 -*0602	0.529	1.22 (0.66- 2.26)	0.247	0.60 (0.26- 1.42)
4	*0201 -*0302 -*0501	0.105	0.44 (0.16- 1.19)	0.265	0.43 (0.10- 1.90)
5	*0501 -*0602	0.265	0.65 (0.31- 1.38)	0.881	1.07 (0.44 -2.60)
6	*0201 -*0302	0.538	1.16 (0.72- 1.89)	0.498	0.81 (0.44 -1.49)
7	*0201 -*0302 -*0303 -*0601 -*0602	1.00	1.0 (0.28- 3.52)	0.984	2.32-07 (0.00 -Inf)
8	*0201 -*0301 -*0302 -*0303 -*0602	0.363	1.79 (0.51- 6.23)	0.596	1.44 (0.37 -5.60)
9	*0303 -*0601	0.006[a]	0.49 (0.30-0.82)	0.029[b]	0.47 (0.24-0.93)
10	*0303 -*0401 -*0601-*0602	0.430	1.53 (0.53- 4.41)	0.467	0.57 (0.12 -2.59)

OR, Odds ratio; 95% CI, 95% confidence interval; Anti-GM1-Ab, anti-GM1 antibody sero-positive or sero-negative; a, *P*c = 0.06 (*P*c, *P* corrected); b, *P*c = 0.29 (*P*c, *P* corrected).

Pairwise linkage disequilibrium analysis based on D′statistics indicated significant LD between patients and healthy controls for the *0201-*0302, *0301-*0303, *0301-*0601, *0502-*0503 and *0604-*0605 *HLA-DQB1* alleles after correction (***Figure 4.2***).

Association of HLA-DQB1 polymorphisms with the clinical features and serological subtypes of GBS

Next, we performed subgroup analysis based on the subtype of GBS and *C. jejuni* sero-positivity (***Table 4.3 & 4.4***). The *DQB1**0201 alleles were significantly more frequent among patients with the demyelinating subtype compared to healthy controls, but this trend was not significant when corrected for multiple comparisons (P = 0.027, OR = 2.68, 95% CI = 1.17-6.17; Pc = 0.35; ***Table 4.3***). The *DQB1**0601 alleles were significantly less frequent among patients with the axonal subtype of GBS compared to healthy controls, but significance was lost after correcting for multiple comparisons (P = 0.029, OR = 0.48, 95% CI = 0.25-0.92; Pc = 0.37; ***Table 4.3***). Haplotype 5 (*0501-*0602) was significantly more prevalent in *C. jejuni* sero-positive patients with the axonal variant compared to *C. jejuni* sero-positive or sero-negative patients with demyelinating subtype or unclassified GBS; but, this trend was not significant after Bonferroni correction (P = 0.024, OR = 4.06, 95% CI = 1.25-13.18; Pc = 0.24; ***Table 4.5***). The *DQB1**0401 alleles were less frequent in *C. jejuni* sero-positive patients with the axonal subtype than *C. jejuni* sero-positive or sero-negative patients with other subtypes of GBS, but significance was lost after correcting for multiple comparisons (P = 0.045, OR = 0.39, 95% CI = 0.16-0.97; Pc = 0.58; ***Table 4.5***).

Table 4.3: Distribution of *HLA-DQB1* polymorphic alleles in patients with the axonal and demyelinating subtypes of GBS and healthy controls

Allele	Axonal subtype *n* = 59 (%)	Demyelinating subtype *n* = 27 (%)	Healthy controls *n* = 151 (%)	Axonal vs. HC		Demyelinating vs. HC	
				P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
DQB1*0201	21 (36)	15 (55)	48 (32)	0.626	1.19 (0.63-2.23)	0.027[a]	2.68 (1.17-6.17)
DQB1*0301/4	14 (24)	6 (22)	37 (24)	1.00	0.96 (0.47-1.94)	1.00	0.88 (0.33-2.34)
DQB1*0302	23 (40)	15 (55)	70 (46)	0.357	0.74 (0.40-1.37)	0.409	1.45 (0.63-3.30)
DQB1*0303	25 (42)	11 (41)	78 (52)	0.282	0.69 (0.38-1.26)	0.403	0.64 (0.28-1.48)
DQB1*0401	11 (19)	8 (30)	27 (18)	1.00	1.05 (0.48-2.29)	0.190	1.93 (0.77-4.88)
DQB1*0501	17 (29)	4 (15)	36 (23)	0.482	1.29 (0.66-2.54)	0.334	0.56 (0.18-1.71)
DQB1*0502	3 (5)	1 (4)	21 (14)	0.091	0.33 (0.09-1.16)	0.206	0.24 (0.03-1.85)
DQB1*0503	6 (10)	4 (15)	17 (11)	1.00	0.89 (0.33-2.39)	0.745	1.37 (0.42-4.44)
DQB1*0601	17 (29)	12 (44)	69 (46)	0.029[b]	0.48 (0.25-0.92)	1.00	0.95 (0.42-2.17)
DQB1*0602	37 (63)	17 (63)	81 (54)	0.279	1.45 (0.78-2.70)	0.407	1.47 (0.63-3.42)
DQB1*0603/8	2 (4)	0 (0)	6 (4)	1.00	0.85 (0.17-4.33)	*nc*	-
DQB1*0604	0 (0)	1 (4)	4 (3)	*nc*	-	1.00	1.41 (0.15- 13.15)
DQB1*0605	0 (0)	2 (7)	5 (3)	*nc*	-	0.597	2.34 (0.43-12.7)

HC, healthy controls; 95% CI, 95% confidence interval; *nc,* not calculated; a, *P*c = 0.35 (*P*c, *P* corrected); b, *P*c = 0.37 (*P*c, *P* corrected).

Table 4.4: Distribution of *HLA-DQB1* polymorphic alleles in healthy controls and *C. jejuni* sero-positive and *C. jejuni* sero-negative patients with GBS

Allele	Healthy controls	*Cj*-positive patients	*C. jejuni* sero-positive *n* = 95/151		Axonal type			Demyelinating type		
					C. jejuni					
	n = 151 (%)	*n* = 95 (%)	HC vs. *Cj* (+)		*n* = 59 (57%)	sero+ *n* = 47 (80%)	sero– *n* = 12 (20%)	*n* = 27 (26%)	sero+ *n* = 12 (44%)	sero– *n* = 15 (56%)
			P value	Odds ratio (95% CI)						
DQB1*0201	48 (32)	35 (37)	0.489	1.25 (0.73-2.15)	21 (36)	17	4	15 (55)	6	9
DQB1*0301/4	37 (24)	18 (19)	0.348	0.73 (0.38-1.36)	14 (24)	9	5	6 (22)	2	4
DQB1*0302	70 (46)	38 (40)	0.357	0.77 (0.46-1.30)	23 (40)	16	7	15 (55)	6	9
DQB1*0303	78 (52)	37 (39)	0.066	0.60 (0.35-1.00)	25 (42)	20	5	11 (41)	4	7
DQB1*0401	27 (18)	22 (23)	0.329	1.38 (0.74-2.61)	11 (19)	7	4	8 (30)	3	5
DQB1*0501	36 (23)	22 (23)	1.00	0.96 (0.53-1.76)	17 (29)	14	3	4 (15)	1	3
DQB1*0502	21 (14)	5 (5)	0.034[a]	0.34 (0.13-0.95)	3 (5)	3	0	1 (4)	0	1
DQB1*0503	17 (11)	14 (15)	0.436	1.36 (0.64-2.91)	6 (10)	5	1	4 (15)	3	1
DQB1*0601	69 (46)	30 (32)	0.033[b]	0.55 (0.33-0.94)	17 (29)	15	2	12 (44)	5	7
DQB1*0602	81 (54)	58 (61)	0.291	1.35 (0.80-2.28)	37 (63)	29	8	17 (63)	8	9
DQB1*0603/8	6 (4)	4 (4)	1.00	1.06 (0.29-3.87)	2 (4)	2	0	0 (0)	0	0
DQB1*0604	4 (3)	2 (2)	1.00	0.79 (0.14-4.40)	0 (0)	0	0	1 (4)	1	0
DQB1*0605	5 (3)	2 (2)	0.710	0.63 (0.12-3.30)	0 (0)	0	0	2 (7)	1	1

Cj, Campylobacter jejuni; sero +, *C. jejuni* sero-positive; sero -, *C. jejuni* sero-negative; HC, healthy control; 95% CI, 95% confidence interval; a, *P*c = 0.44 (*P*c, *P* corrected); b, *P*c = 0.42 (*P*c, *P* corrected).

Table 4.5: Association studies of axonal subtype patients with anti-ganglioside antibodies, HLA-DQB1 alleles, haplotype and recent infection with *C. jejuni*

Association variables	**Axonal subtype** ***n* = 59 (%)**	**Demyelinating type** ***n* = 27 (%)**	***P* value**	**Odds ratio (95% CI)**
Anti-GM1-Ab	35 (59)	04 (15)	<0.001[a]	8.38 (2.57-27.34)
Anti-GD1a-Ab	09 (15)	03 (11)	0.608	1.44 (0.36-5.81)
Anti GQ1b-Ab	03 (5)	02 (7)	0.671	0.67 (0.11 -4.26)
	Axonal type with ***Cj* (+)** ***n* = 47 (%)**	**Either demyelinating type or unclassified with *Cj* (-) or *Cj* (+)** ***n* = 104 (%)**		
Anti-GM1-Ab	29 (62)	28 (27)	<0.001[a]	4.37 (2.11 -9.08)
HLA DQB1*0401	07 (15)	32 (31)	0.045[b]	0.39 (0.16 -0.97)
Hap 5 (*0501-*0602)	08 (17)	05 (4.8)	0.024[c]	4.06 (1.25-13.18)

Cj (+), *C. jejuni sero-positive*; *Cj* (-), *C. jejuni sero-negative;* 95% CI, 95% confidence interval; a, statistically significant; b, *P*c = 0.58 (*P*c, *P* corrected); c, *P*c = 0.24 (*P*c, *P* corrected).

Association of HLA-DQB1 polymorphisms and haplotype variations with autoantibodies in patients with GBS

The distribution of *HLA-DQB1* polymorphisms among anti-ganglioside antibody (Ab) sero-positive patients with GBS is presented in ***Table 4.6***.

Table 4.6: Distribution of HLA-DQB1 alleles in anti-ganglioside antibody sero-positive patients with GBS and in healthy controls

Alleles	**Healthy controls** ***n* = 151 (%)**	**Anti-ganglioside antibody sero-positive GBS** ***n* = 73 (48%)**		
		Anti-GM1-Ab	**Anti-GD1a-Ab**	**Anti-GQ1b-Ab**
		***n* = 58 (38%)**	***n* = 23 (15%)**	***n* = 14 (9%)**
DQB1*0201	48 (32)	22 (38)	7 (30)	5 (36)
DQB1*0301/4	37 (24)	13 (22)	4 (17)	2 (14)
DQB1*0302	70 (46)	23 (40)	6 (26)	5 (36)
DQB1*0303	78 (52)	22 (38)	10 (43)	4 (28)
DQB1*0401	27 (18)	10 (17)	5 (22)	1 (7)
DQB1*0501	36 (23)	12 (21)	4 (17)	0 (0)
DQB1*0502	21 (14)	5 (9)	2 (9)	2 (14)
DQB1*0503	17 (11)	11 (19)	6 (26)	2 (14)
DQB1*0601	69 (46)	13 (22)	11 (48)	6 (43)
DQB1*0602	81 (54)	37 (64)	16 (69)	11 (78)
DQB1*0603/8	6 (4)	0 (0)	0 (0)	0 (0)
DQB1*0604	4 (3)	1 (2)	0 (0)	1 (7)
DQB1*0605	5 (3)	1 (2)	0 (0)	0 (0)

GM1, GD1a, GQ1b, ganglioside.

Overall, 48% (73/151) of patients with GBS were anti-ganglioside antibody sero-positive: 38% (58/151) were anti-GM1 antibody sero-positive, 15% (23/151) were anti-GD1a antibody sero-positive and 9% (14/151) were anti-GQ1b antibody sero-positive (***Table 4.6***). Among the anti-GM1 antibody positive patients, the frequency of the *DQB1**0601 allele was significantly lower in sero-positive patients compared to sero-negative patients, but this was not significant when the *P*-values were corrected for the number of alleles (*P* = 0.022, OR = 0.42, 95% CI = 0.20-0.88; *P*c = 0.28; ***Table 4.7***). Moreover, haplotype 9 (DQB1*0303-*0601) was less common among anti-GM1 antibody sero-positive patients than sero-negative patients, but this trend was not significant after correction (*P* = 0.029, OR = 0.47, 95% CI = 0.24-0.93; *P*c = 0.29; ***Table 4.2***).

Table 4.7: Distribution of *HLA-DQB1060x polymorphisms within anti-GM1 antibody sero-positive and sero-negative patients with GBS**

Allele	Presence of anti-GM1 antibody			
	Positive *n* = 58 (%)	Negative *n* = 93 (%)	*P*-value	Odds ratio (95% CI)
DQB1*0601	13 (22)	38 (41)	0.022 [a]	0.42 (0.20-0.88)
DQB1*0602	37 (64)	51 (55)	0.311	1.45 (0.74-2.85)
DQB1*0603/8	0 (0)	7 (8)	*nc*	-
DQB1*0604	1 (2)	1 (1)	1.00	1.61 (0.10-26.32)
DQB1*0605	1 (2)	3 (3)	0.6	0.53 (0.05-5.18)

nc, not calculated; 95% CI, 95% confidence interval; a, *P*c = 0.28 (*P*c, *P* corrected).

Association of HLA-DQB1 polymorphisms with severity and disease outcome in GBS

The patients with GBS were classified as severely affected (74%) or mildly affected (26%) based on MRC sum score. The *DQB1**0303 alleles were significantly more frequent among severely affected patients than mildly affected patients with GBS, but this significance was lost after correcting for multiple comparisons (*P* = 0.025, OR, 2.49; 95% CI, 1.13-5.48; *P*c = 0.32; ***Table 4.8***). However, no significant associations were observed between GBS disease severity and the ten most common haplotype patterns. Furthermore, no significant associations were evident between the candidate alleles or haplotype patterns and disease outcome at six months' follow-up.

Table 4.8: Distribution of *HLA-DQB1* allele frequency among patients with different severities of GBS

Allele	Mildly affected	Severely affected	*P* value	Odds ratio (95% CI)
	n = 40 (%)	*n* = 111 (%)		
DQB1*0201	13 (33)	42 (38)	0.572	0.79 (0.36-1.69)
DQB1*0301/4	9 (23)	26 (23)	1.00	0.94 (0.40-2.24)
DQB1*0302	18 (45)	45 (41)	0.709	1.2 (0.57-2.48)
DQB1*0303	11 (28)	54 (49)	0.025[a]	2.49 (1.13-5.48)
DQB1*0401	10 (25)	29 (26)	1.00	0.94 (0.41-2.16)
DQB1*0501	11 (28)	20 (18)	0.253	1.72 (0.74-4.02)
DQB1*0502	2 (5)	11 (10)	0.515	0.47 (0.10-2.25)
DQB1*0503	3 (8)	17 (15)	0.281	0.44 (0.12-1.62)
DQB1*0601	10 (25)	40 (36)	0.243	0.59 (0.26-1.34)
DQB1*0602	24 (60)	64 (58)	0.853	1.10 (0.52-2.30)
DQB1*0603/8	2 (5)	5 (5)	1.00	1.12 (0.21-5.99)
DQB1*0604	2 (5)	1 (1)	0.171	5.78 (0.51-65.67)
DQB1*0605	1 (3)	3 (3)	1.00	0.92 (0.09-9.13)

Mildly affected at nadir, MRC-sum score ≥ 40; severely affected at nadir, MRC-sum score < 40; 95% CI, 95% confidence interval; a, *P*c = 0.32 (*P*c, *P* corrected).

Discussion

This study investigated the association between *DQB1* alleles and haplotype patterns and GBS susceptibility in Bangladesh. Associations between HLA complex genes and human autoimmune diseases have been described; however, studies of HLA typing among populations with different genetic backgrounds have reported inconclusive associations with GBS.[14,15,17,31–33] In this study, we observed no association between *DQB1* alleles or haplotype patterns and disease susceptibility to GBS; the *DQB1* alleles and haplotype patterns had no influence on the clinical and serological subgroups of GBS in Bangladesh after the *P*-values were corrected.

GBS is a heterogeneous disorder with respect to severity, prognosis and clinical features.[24] In this study the *DQB1**0303 alleles were significantly associated with the severe form of GBS before correcting for multiple comparisons, implying *HLA-DQB1* polymorphisms may possibly influence disease severity and the extent of the inflammatory response at the peripheral nerves. Though a Dutch study reported no association between *HLA-DQB1* alleles and disease severity, the *HLA-DRB1**01 allele was associated with the need for mechanical ventilation in patients with GBS.[14]

The associations of individual *HLA-DQB1* polymorphic alleles with GBS have been studied; however, haplotype studies were not performed.[14–16] In this study, we found individual *DQB1* alleles or haplotype were not associated with the development of GBS. However, haplotype 9 (*HLA-DQB1**0601**-***0303) was less frequent among patients with GBS in Bangladesh compared to healthy controls and LD analysis also indicated their association among *DQB1* *0601and*0303 alleles. Moreover, no significant LD was observed between the alleles of the 10 most common haplotype. This implies that the presence of both alleles (*HLA-DQB1**0601**-***0303) may exert a reciprocal effect towards the development of GBS in the Bangladeshi population.

The *DQB1**03 allele is significantly associated with *C. jejuni* infection.[16] However, our study revealed a relatively lower frequency of the *DQB1**0303 and *0601 alleles and a slightly higher frequency of the *0502 alleles in *C. jejuni* sero-positive patients compared to healthy controls. This discrepancy may be due to local evolutionary pressure among infectious agents in different ethnic populations. A previous study also indicated the

contribution of *HLA-DQB1**030x alleles to regional variation in GBS.[31] Further analysis revealed haplotype 5 (*0501-*0602) was more frequent in the *C. jejuni*-associated axonal variant of GBS compared to other subtypes of GBS. This observation may be one factor explaining the higher prevalence of the axonal subtype of GBS in Bangladesh compared to other regions of the world. Furthermore, this also may explain how human ancestry and race modify *C. jejuni* strains interact with an individual's immune system to trigger different subtypes of GBS.[20] In our Bangladeshi population, a higher frequency of the *DQB1**0201 alleles were observed in the demyelinating variant of GBS. However, it is important to confirm and compare our results with studies of other ethnic populations from different regions of the world where the demyelinating variant of GBS predominates.

C. jejuni-triggered GBS is frequently associated with anti-GM1 antibodies, and GM1 acts as a target pathogenic antigen that triggers the axonal variant of GBS.[28,34] HLA class II genes are recognized by CD4+ Th cells and are known to influence antibody responses by activating B cells.[35] A previous study observed no association between HLA alleles and the presence of anti-GM1antibodies.[14] However, the *HLA-DRB1**0803 and *HLA-DQA1**0301 alleles were more frequent in Japanese[36] and Chinese[32] anti-GM1 antibody-positive patients with GBS, respectively, while no significant association was observed between the *HLA-DRB1* and *HLA-DQB1* alleles and anti-GM1antibody positivity in Dutch patients with GBS.[14] We did not observe a significant association between *HLA-DQB1* alleles and anti-GM1 antibody positivity in Bangladeshi GBS patients.

HLA-DQB1 alleles have diverse effects on susceptibility to autoimmune diseases. A stronger association between the *DQB1**06 alleles and disease susceptibility and a lower frequency of the *DQB1**03 alleles were observed in multiple sclerosis.[37] Similar studies on *HLA-DQB1* polymorphisms showed a higher risk of type I diabetes among individuals with the *DQB1**0201/*0302 alleles, whereas the *DQB1**0301, *DQB1**0601,**DQB1**0602, *DQB1**0603 and *DQB1**05 alleles protect against the development of type I diabetes.[38] Furthermore, the *DQB1**04 alleles confer susceptibility to rheumatoid arthritis whereas the *DQB1**06 alleles protect against the development of rheumatoid arthritis.[39]

This study has several limitations. Even though we used one of the largest GBS cohorts from developing countries, the sample size was relatively small for investigation of a large

number of haplotypes in GBS patients. Here, we only explored the association of *HLA-DQB1* alleles with disease susceptibility and subgroups, without considering other HLA alleles that are also important in GBS pathogenesis.

In conclusion, *HLA-DQB1* gene polymorphisms and haplotype were not associated with susceptibility to GBS in the Bangladeshi population. However, the importance of *HLA-DQB1* polymorphisms in the pathogenesis of GBS still remains unclear. Extensive analysis of a larger cohort of patients (e.g. from the IGOS study)[25]from various ethnic backgrounds is required to confirm our findings on *HLA-DQB1* alleles and haplotype and the development and progression of GBS.

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Conflict of interest

The authors do not have any conflicts of interest to report.

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Chapter 5

NOD polymorphism and GBS in Bangladesh

NOD polymorphism confers no risk for susceptibility and severity of Guillain-Barré syndrome

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Graphical abstract

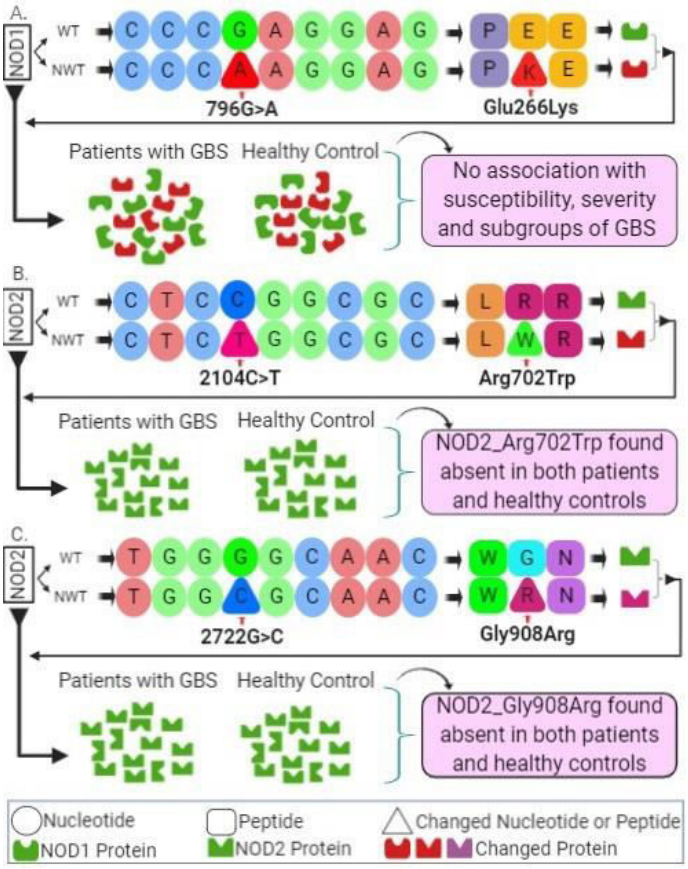

Figure 5.1 Graphical abstract

Abstract

Objective: Nucleotide oligomerization domain (NOD) proteins are cytoplasmic receptors that play an important role in host's innate immune responses to pathogen by recognizing self or non-self molecules in many autoimmune diseases like Guillain-Barré syndrome (GBS). We investigated whether NOD polymorphism (NOD1-Glu266Lys and NOD2-[Arg702Trp; Gly908Ar]) in patients with GBS contributes towards the susceptibility and severity of GBS.

Methods: We determined single nucleotide polymorphisms of NOD gene (NOD1-Glu266Lys and NOD2-[Arg702Trp; Gly908Ar]) in 303 patients with GBS and 303 healthy controls by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and

Sanger-sequencing. Genotype and allele frequencies were compared by performing Chi-square or Fisher's exact test with Yates' continuity correction.

Results: We did not find that any of the NOD variants were associated with susceptibility, severity and subgroups of GBS. Homogenous distribution of NOD1 genotypes (GG, 17% vs. 18%; GA, 53% vs. 47%; AA, 30% vs. 35%) and alleles (G allele, 43% vs. 42% and A allele, 57% vs. 58%) were observed in patients and in healthy individual. Moreover, NOD2 polymorphism showed wild type NOD2 C2104 and NOD2 G2722 respectively, with homozygous Arg/Arg genotype of NOD2 (Arg702Trp) polymorphism and homozygous Gly/Gly genotype of NOD2 (Gly908Arg) for all study subjects.

Interpretation: NOD variants confer no risk to the susceptibility and severity of GBS. Moreover, NOD2 polymorphism is rare in patients with GBS as well as in the healthy individuals of Bangladesh.

Keywords

Nucleotide oligomerization domain; Guillain-Barré syndrome; Polymorphism; Allele; Genotype.

Introduction

Guillain-Barré syndrome (GBS) is a common immune-mediated neurological disorder characterized with flaccid paralysis after global eradication of poliomyelitis. Molecular mimicry between the outer core lipooligosaccharide (LOS) on *Campylobacter jejuni* (*C. jejuni*) and the host's gangliosides is thought to induce cross-reactive autoimmune responses.[1–3] Besides molecular mimicry, genetic host factors are thought to be responsible for the induction of the disease[4] and polymorphisms within these host immune response genes are one of the candidates.[5] In spite of the established association of *C. jejuni* infection and GBS,[4,6] the rare occurrence (1 in 1000 to 5000)[7–10] and induction of a specific subtypes of GBS by a single infection is still mysterious. Thus, microbial recognition and host defense has become one of the most important areas of investigation of host genetic predisposition for disease pathogenesis of GBS.

Nucleotide-binding oligomerization domain (NOD) protein is a family of evolutionarily conserved pattern-recognition receptors (PRRs) that play an effective role in first line defense of innate immunity by recognizing the pathogen-associated molecular patterns (PAMPs) of microorganisms.[11,12] Among the NOD-like receptors (NLRs), NOD1 and NOD2 are the most common and located on chromosome 7p14-15 and 16q12, respectively.[13,14]NOD1 and NOD2 are composed of a series of C-terminal leucine-rich repeats (LRRs), a centrally located nucleotide-binding oligomerization domain[15] and N-terminal caspase-activating and recruitment domain (CARD). Both the CARD domain and the nucleotide-binding domain are required for the transcription factor NF-$_K$B (Nuclear Factor of Kappa Light Chain Gene Enhancer in B cells inhibitor) activation, whereas the leucine-rich repeats interact with PAMPs derived from gram-negative bacteria.[16,17] A single nucleotide polymorphism (SNP) in NOD1 from G to A substitution at position 796 (G796A) and in NOD2, C to T substitution in exon 4 at position 2104 (C2104T) and G to C substitution in exon 8 at position 2722 (G2722C) resulted in an amino acid substitution of Glu266Lys, Arg702Trp and Gly908Arg respectively.[14,18,19] Variability in NOD gene may play an important role in regulation and activation of inflammatory responses and bacterial clearance and thus subsequently in survival of host following infection with enteric pathogens.[20]

Association studies of NOD1 (Glu266Lys) and NOD2 (Arg702Trp and Gly908Arg) with disease susceptibility and severity of GBS is limited. However, genetic variability in these genes can alter a number of cellular processes and influence evolution of several human diseases including autoimmune diseases, chronic inflammatory disorders and cancer.[21,22] Several studies other than GBS have indicated the association of NOD gene variability with defectiveness of innate immune system with atopic dermatitis,[23] inflammatory bowel disease,[24] sarcoidosis[25] and Crohn disease.[21] A single report was published concerning NOD1 and NOD2 polymorphisms with the risk of developing GBS in Indian population.[26] In Bangladesh, Jahan et al. has reported the association of Toll-like receptor-4 299Gly allele with increased risk of GBS[27] which is another important receptor molecule in the mammalian innate immune system. As majority of our patients were triggered by *C. jejuni*, genetic variability in NOD1 and NOD2 can deregulate the sensing of microbial pathogens and might hindered the homeostasis of innate immunity and thus be involved in disease pathogenesis. Therefore, we investigated the role of NOD1 and NOD2 polymorphisms in the susceptibility and severity of the disease and thus unveiled their contribution in the risk of developing GBS in Bangladesh.

Methods and material

Patients and controls

Three hundred and three patients (n = 303) with GBS (208 males and 95 females, with a median age of 29 years [interquartile range, 17-42]) participated in this prospective case-control study with six months of follow-up from Dhaka Medical College and Hospital (DMCH), Dhaka, Bangladesh following informed consent. Patients were diagnosed based on National Institute of Neurological Disorders and Stroke (NINDS) criteria by Asbury and Cornblath, 1990.[28] Three hundred and three healthy individuals without any history of neurological disorder, diabetes and recent antecedent infection were recruited in this study (204 males and 99 females, with a median age of 34 years [interquartile range, 28-46]). Control individuals were geographically matched and genetically unrelated to patients with GBS. Healthy controls (HC) were enrolled during patient recruitment time period following informed consent. Clinical data including age, sex, antecedent events before developing

neurological sign and symptoms of GBS, electrophysiological data and serological data were obtained from patients after recruitment. Blood specimen was collected at entry before medication and at 2 weeks, 4 weeks and after 6 months for follow-up study. Mild disease and severe disease were assessed based on patients' Medical Research Council (MRC) sum score at nadir (maximum muscle weakness) during entry.[29] Severely affected patients with GBS had MRC sum score < 40 and mildly affected patients with GBS had MRC sum score ≥ 40-60.[30] The GBS disability score (GBS-DS) was used to assess the prognosis of disease after six months of follow-up.[31] At six months follow up, patients were classified as 'good prognosis' based on their ability to walk independently with GBS-DS 0, 1, 2 and as 'poor prognosis' with GBS-DS 3, 4, 5 and 6 (unable to walk independently or death).[30] The study procedure was reviewed and approved by the Institutional Review Board (IRB) and ethical committees of icddr, b, Dhaka, Bangladesh.

Antecedent event and electrophysiological studies

Patients with GBS had history of various (75%) antecedent illness; diarrhoea (43%, 129/303), respiratory infection (15%, 45/303) and fever (8%, 25/303) were most common. Nine percent had other types of infection including Varicella-zoster, measles, flu like infection and 25% had unidentified infection or no infection.

Electrophysiological studies[32] of 82% (247/303) of patients with GBS revealed 59% (146/247) were axonal subtype of GBS including acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN), 27% (68/247) were demyelinating subtype (acute inflammatory demyelinating polyradiculoneuropathy [AIDP]) and 13% (33/247) were unclassified cases of GBS with inexcitable nerves or equivocal findings respectively.

Serology for C. jejuni and anti-ganglioside antibodies

Serum samples separated from pre-treated blood were used for serological study of recent *C. jejuni* infection and determination of common anti-ganglioside antibodies (e.g. GM1, GD1a and GQ1b). Serology was done in duplicate using previously described enzyme-linked immunosorbent assay (ELISA) technique and absorbance was read at 450 nm using a micro-

titer plate reader.[6,33,34]

Isolation of genomic DNA

Isolation of genomic DNAs of 606 study subjects from lithium heparin anticoagulated blood samples was performed using the QIAamp® DNA Blood Midi Kit (100) (Qiagen, Hilden, Germany) according to the manufacturer's protocol and DNAs were dissolved in 1× TE-buffer (10 mM Tris-Cl, pH 8.0 & 1 mM EDTA). All the samples were stored at -80°C for use until SNP detection.

Detection of NOD1 and NOD2 polymorphisms

Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) was used for the detection of NOD1 (Glu266Lys, rs2075820) and NOD2 (Arg702Trp, rs2066844 and Gly908Arg, rs2066845) polymorphisms.[26] PCR-RFLP analysis was performed followed by PCR reaction using specific primer sequences and restriction endonuclease as per manufacturer instructions (***Table 5.1***). Primers were designed and collected from established paper.[26] PCR analysis was performed using master mix of 25 µL containing 20 ng of genomic DNA, 10 pmol of each primer, 0.1 mM dNTPs (Promega), 1 U of GoTaq® Flexi DNA Polymerase (Promega), 5× Green GoTaq® Flexi Buffer, 25 mM MgCl2 and Milli-Q water. PCR program was performed with an initial denaturation at 95 °C for 5 min, followed by 35 cycles with denaturation at 94 °C for1 min, annealing at 61 °C or 58 °C for 1 min for NOD1 or NOD2 respectively, extension at 72 °C for 1 min and 10 min at 72 °C for final extension using a MJ Research PTC-200 Thermal Cycler. Template free PCR water was used as negative control. After digestion with specific restriction endonuclease the digested PCR products were visualized in 2-3% agarose gel pre-stained with ethidium bromide (EtBr) for detection of NOD1 and NOD2 polymorphisms using a Molecular Imager® Gel Doc™ XR+system (Bio-Rad Laboratories Inc, USA). Sanger-sequencing was performed by Genetic Analyzer ABI 3500 automated DNA sequencer according to the manufacturer's instructions for the detection of *NOD2* polymorphisms using the same primer sequences used in the PCR amplification.

Table 5.1: Primer sequences and restriction endonucleases used for the detection of *NOD1* and *NOD2* gene polymorphisms

NOD Polymorphisms	Primer sequence (5′→ 3′)	PCR product size(bp)	Length	Restriction Endo-nuclease
1.NOD1_Glu266 Lys	F 5′-AAGTGACAGGCTGTGTCTGC-3′ R 5′-CTTCCCACTGAGCAGGTTG-3′	232	20 mer 19 mer	Bsob1
2.NOD2_Arg702Trp	F 5′- CTG GCA GGG CTG TTG TCC-3′ R 5′- TGGCGGGATGGAGTGGAA-3′	141	18 mer 18 mer	Msp1
3.NOD2_Gly908 Arg	F 5′- ACATATCAGGTACTCACTGA-3′ R 5′-GATCACCCAAGGCTTCAG-3′	113	20 mer 18 mer	Hha l

NOD, nucleotide-binding oligomerization domain; bp, base pair.

Statistical analysis

The associations of the genotype and allele frequencies with GBS susceptibility and the subgroups between patients and healthy subjects were assessed using Fisher's exact test with Yates' continuity correction. Chi-square test was applied whether the SNPs in control group were in Hardy–Weinberg equilibrium. A *P*-value less than 0.05 was taken to be statistically significant. Genotype/ allelic frequency was estimated by simple counting method and the data were processed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), GraphPad prism (version 5.01, GraphPad software, Inc., La Jolla, CA, USA) and SPSS (16.0 version, Chicago, IL, USA). Sequencing analysis was performed using Chromas 2.6.6 and MEGA6 software.

Results

We did not find any significant association between *NOD1* polymorphism and susceptibility to GBS when NOD1 genotypes and alleles were compared with healthy controls (***Table 5.2***). However, we found almost homogenous distribution of genotypes (GG, 17% vs. 18%; GA, 53% vs. 47%; AA, 30% vs. 35%) and alleles (G allele, 43% vs. 42% and A allele, 57% vs. 58%) in patients and in healthy individuals (***Table 5.2***). Subgroup of GBS (Axonal type vs. demyelinating type; Axonal type vs. HC; demyelinating type vs. HC) analysis with *NOD1* polymorphisms also revealed no significant association (***Table 5.3***). NOD1 genotypes and alleles did not influence *C. jejuni* infection, anti-GM1 antibody, severity of the disease and disease outcome (***Table 5.4***).

Table 5.2: Distributions of *NOD1* and *NOD2* genotypes and alleles in patients with GBS and healthy controls

Genotype/allele	GBS patients *n = 303(%)*	HC *n = 303(%)*	*P* -value	OR (95% CI)
NOD1-Glu266 LYS				
Glu/Glu	50 (17)	55 (18)		Reference
Glu/Lys	162 (53)	142 (47)	0.365	1.25 (0.81-1.96)
Lys/Lys	91 (30)	106 (35)	0.904	0.94 (0.59-1.52)
Glu Allele	262 (43)	252 (42)		Reference
Lys Allele	344 (57)	354 (58)	0.603	0.93 (0.74-1.17)
NOD2-Arg702 Trp				
Arg/Arg	303	303	-	Reference
Arg/Trp	0	0	-	*nc*
Trp/Trp	0	0	-	*nc*
Arg-Allele	606	606	-	Reference
Trp-Allele	0	0	-	nc
NOD2-Gly908 Arg				
Gly/Gly	303	303	-	Reference
Gly/Arg	0	0	-	*nc*
Arg/Arg	0	0	-	*nc*
Gly-Allele	606	606	-	Reference
Arg-Allele	0	0	-	*nc*

GBS, Guillain-Barré syndrome; HC, healthy control; NOD, nucleotide-binding oligomerization domain; 95% CI, 95% confidence interval.

Table 5.3: Distribution of NOD1 genotypes and alleles among control, axonal and demyelinating cases

NOD1	**Subtypes**			**a and c**		**b and c**		**a and b**	
Genotype/ Allele	Axonal (a) *n* = 146 (%)	Demyelinating (b) *n* = 68 (%)	HC (c) *n* =303 (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
Glu/Glu	22 (15.1)	10 (14.7)	55 (18.1)		Reference		Reference		Reference
Glu/Lys	80 (54.8)	35 (51.5)	142 (46.9)	0.288	1.41 (0.80-2.48)	0.724	1.36 (0.63-2.92)	0.860	0.96 (0.41-2.24)
Lys/Lys	44 (30.1)	23 (33.8)	106 (35.0)		1.04 (0.57-1.90)		1.19 (0.53-2.69)		1.15 (0.47-2.83)
Glu allele	124 (42.5)	55 (40.4)	252 (41.6)		Reference		Reference		Reference
Lys allele	168 (57.5)	81 (59.6)	354 (58.4)	0.829	0.96 (0.73-1.28)	0.848	1.05 (0.72-1.53)	0.752	1.08 (0.72-1.64)

NOD, Nucleotide-binding oligomerization domain; OR (95% CI), odds ratio (95% confidence interval); HC, healthy controls.

Table 5.4: Distribution of NOD1 genotypes and alleles in *C. jejuni* positive and negative serology, anti-GM1 antibody positivity, disease prognosis and mildly and severely affected patients with GBS (*n* = 303)

NOD1 Genotype/ Allele	Association variables		*P* value	Odds ratio (95% CI)
	C. jejuni positive serology *n* = 186 (%)	*C. jejuni* negative serology *n* = 117 (%)		
Glu/Glu	33 (17.7)	17 (14.5)		Reference
Glu/Lys	101 (54.3)	61 (52.1)	0.548	1.17 (0.60-2.28)
Lys/Lys	52 (28)	39 (33.3)		1.46 (0.71-2.98)
Glu allele	167 (44.9)	95 (40.6)		Reference
Lys allele	205 (55.1)	139 (59.4)	0.313	1.19 (0.86-1.66)
	Anti-GM1 antibody sero-positive patients *n* = 118 (%)	Anti-GM1 antibody sero-negative patients *n* = 185 (%)		
Glu/Glu	18 (15.2)	32 (17.3)		Reference
Glu/Lys	69 (58.5)	93 (50.3)	0.368	0.76 (0.39-1.46)
Lys/Lys	31 (26.3)	60 (32.4)		1.09 (0.53-2.24)
Glu allele	105 (44.5)	157 (42.4)		Reference
Lys allele	131 (55.5)	213 (57.6)	0.674	1.09 (0.78-1.51)
	Severely affected patients *n* = 232 (%)	Mildly affected patients *n* = 71(%)		
Glu/Glu	35 (15.1)	15 (21.1)		Reference
Glu/Lys	126 (54.3)	36 (50.7)	0.486	0.67 (0.33-1.36)
Lys/Lys	71 (30.6)	20 (28.2)		0.66 (0.33-1.36)
Glu allele	196 (42)	66 (46)		Reference
Lys allele	268 (58)	76 (54)	0.384	0.84 (0.58-1.23)
	Good outcome *n* = 209 (%)	Poor outcome *n* = 94 (%)		
Glu/Glu	36 (17.2)	13 (13.8)		Reference
Glu/Lys	111(53.1)	51 (54.3)		1.27 (0.62-2.60)
Lys/Lys	62 (29.7)	30 (31.9)	0.746	1.34 (0.62-2.89)
Glu allele	183 (43.8)	77 (41)		Reference
Lys allele	235 (56.2)	111 (59)	0.535	1.12 (0.79-1.590

NOD, nucleotide oligomerization domain; *C. jejuni, Campylobacter jejuni*; 95% CI, 95% confidence interval; patients at nadir with MRC-sumscore < 40 were defined as severely affected patients and with MRC-sumscore ≥ 40 were defined as mildly affected patients.[30]

We also assessed the association of *NOD2 (Arg702Trp and Gly908Arg)* polymorphisms with disease susceptibility in patients with GBS and healthy controls (***Table 5.2***). Noteworthy, we found all the patients with GBS and the healthy controls present the same allelic pattern indicating the absence of *NOD2 (Arg702Trp and Gly908Arg)* polymorphisms in patients with GBS and in healthy controls. We found homozygous Arg/Arg genotype of NOD2 (Arg702Trp) polymorphism and homozygous Gly/Gly genotype of NOD2 (Gly908Arg) for all study subjects. In addition, we confirmed our findings by sequencing as shown in ***Figure 5.2 (a1, a2, b1 and b2)***, which present wild type NOD2 C2104 and NOD2 G2722, respectively and signifies no mutation in patients and controls.

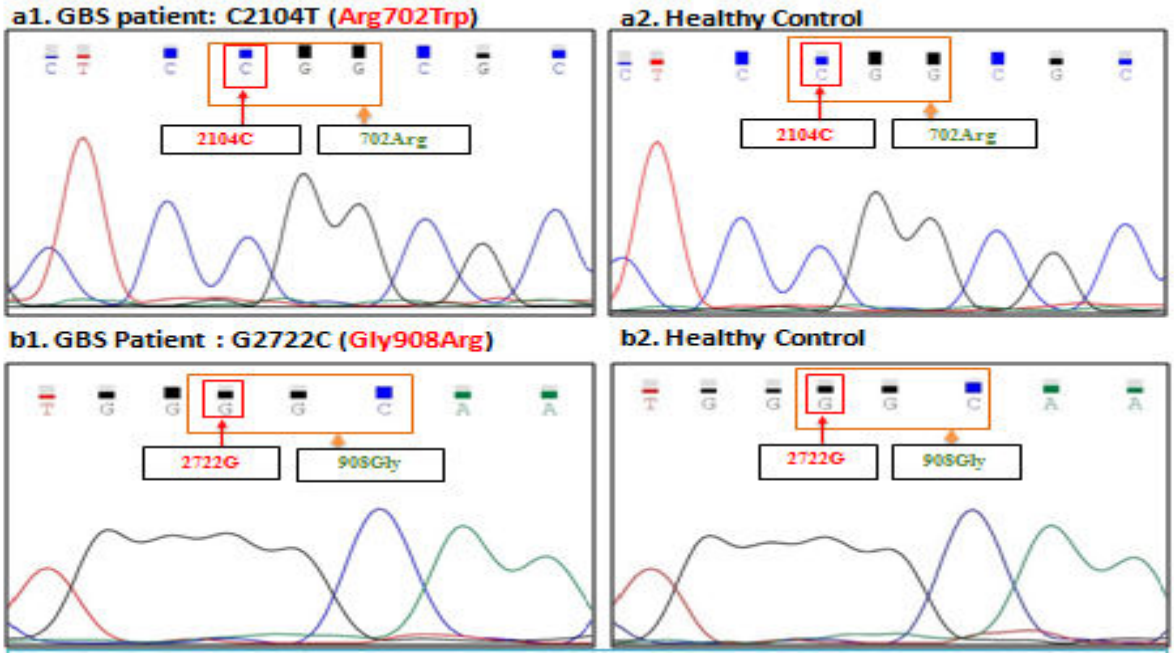

Figure 5.2 Sequence analyses of NOD2 polymorphisms. GBS patients as well as healthy controls in Bangladesh found completely negative for both C2104T (Arg702Trp) and G2722C (Gly908Arg) polymorphisms using Sanger DNA sequencing method**.**

Discussion

In this study, we investigate the association of *NOD1* (Glu266Lys) and *NOD2* (Arg702Trp and Gly908Arg) polymorphisms with the development of GBS as well as other serological and clinical features among the patients of Bangladesh and healthy controls. No significant association was observed between *NOD1* (Glu266Lys) gene polymorphism and susceptibiluty to GBS; but, we found wildtype genotype of *NOD2* (Arg702Trp and Gly908Arg) in both patients and healthy individuals of Bangladeshi population.

Previously, Kharwar et al. have reported a significant association of *NOD1* polymorphism with high risk of developing of GBS in Indian populaton.[26] This study also reported significant association of NOD1 variant allele with subgroups (AMAN and AIDP) of GBS.[26] However, we studied one of the largest GBS cohorts in the world; but, our study did not support the findings from the Indian population. Nevertheless, association of *NOD1* polymorphism with the development of several autoimmune disorders other than GBS have been reported previously, including atopic eczema, asthma and inflammatory bowel syndrome.[35–37] It is not surprising that geographical and ethnic diversity causes variation in adaptation, genetic predisposition and subsequent disease induction. Nonetheless, it has been reported that the recognition of pathogenic bacteria in intestinal cells lacking Toll-like receptors (TLRs) relies on the *NOD1* activity.[38] *NOD1* signaling is required as a 'backup mechanism' for activating NF-κB in human intestinal epithelial cells infected with Gram-negative enteric bacteria that can bypass TLR activation.[38] Previously, the association of TLR-4 299Gly allele with increased susceptibility to GBS and the axonal GBS subtype has been described in the Bangladeshi population.[39] In this study, we did not find such type of association of *NOD1* polymorphism with disease susceptibility, not even in *C. jejuni*-triggered subtype of GBS.

NOD proteins are cytosolic pattern recognition receptors that respond to bacterial substrate and induce NF-κB activation in host thereby enhancing the inflammation.[11,16,17] As most of our GBS patients are *C. jejuni*-associated AMAN subtype, we looked for a candidate gene for risk of GBS development based on the role of NOD proteins in the recognition of bacterial components in Bangladeshi population. In this present study, we demonstrate an association between polymorphisms in the coding region of the NOD2 gene and risk of GBS development in Bangladeshi population. None of the study subjects, including patients with

GBS and healthy controls, possessed any of the common NOD2 variants (Arg702Trp and Gly908Arg) that are present in the Western population.[40] Our study firmly supports the study findings of the Asian countries including Japan, Korea and China who also describe absence of the common NOD2 variant in their population.[14,41,42] However, Kharwar et al reported a significant association of NOD2 (Arg702Trp and Gly908Arg) polymorphisms was observed among patients with GBS in the Indian population.[26] Our study did not support their findings even though, geographically India is our neighboring country. The current study strongly provides the evidence for a significant difference between the genetic variability of the NOD2 gene in Bangladeshi patients and in that of non-Bangladeshi Asian and Western populations. Remarkably, our study implies that NOD2 polymorphisms are rare or nonexistent in the Bangladeshi population. These findings suggest that NOD2 polymorphism is not a significant risk marker for susceptibility to GBS.

It is generally accepted that multiple genetic defects may contribute to the phenotype of complex diseases and SNP-associated diseases. However, the absences of the NOD2 polymorphisms in this population signifies that the effects of these SNP in disease pathogenesis remain veiled.[22] Association studies of NOD polymorphisms along with TLRs in the same population could be more helpful to harness the expanding base of knowledge about *NOD1* and *NOD2* polymorphisms for GBS pathogenesis.

Declaration of interest

ZI has received funding/grant support from Fogarty International Center, Department of Health and Human Services, National Institutes of Health, and Annexon Biosciences, USA. SH, AA, MA, IM and ZHH are free from all sort of conflict of interest.

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Chapter 6

FcγR polymorphisms and GBS in Bangladesh

Fc-gamma IIIa-V158F receptor polymorphism contributes to the severity of Guillain-Barré syndrome

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Abstract

Objective: Guillain-Barré syndrome (GBS) is a rare, life-threatening disorder of the peripheral nervous system. Immunoglobulin G Fc-gamma receptors (FcγRs) mediate and regulate diverse effector functions and are involved in the pathogenesis of GBS. We investigated whether the FcγR polymorphisms FcγRIIa H/R131 (rs1801274), FcγRIIIa V/F158 (rs396991) and FcγRIIIb NA1/NA2 and their haplotype patterns affect the affinity of IgG-FcγR interactivity and influence GBS susceptibility and severity.

Methods: We determined FcγR polymorphisms in 303 patients with GBS and 302 ethnically matched healthy individuals from Bangladesh by allele-specific polymerase chain reaction. Pairwise linkage disequilibrium and haplotype patterns were analyzed based on D′statistics and the genotype package of R statistics, respectively. Logistic regression analysis and Fisher's exact test with corrected *P* (*P*c) values were employed for statistical comparisons.

Results: FcγRIIIa-V158F was associated with the severe form of GBS compared to the mild form ($P = 0.005$, OR = 2.24, 95% CI = 1.28-3.91; $Pc = 0.015$); however, FcγR genotypes and haplotype patterns did not show any association with GBS susceptibility compared to healthy controls. FcγRIIIa-V/V158 and FcγRIIIb-NA2/2 were associated with recent *Campylobacter jejuni* infection ($P \leq 0.001$, OR = 0.36, 95% CI = 0.23-0.56; $Pc \leq 0.003$ and $P = 0.004$, OR = 1.70, 95% CI = 1.18-2.44; $Pc \leq 0.012$, respectively). Haplotype 1 (FcγRIIa-H131R- FcγRIIIa-V158F- FcγRIIIb-NA1/2) and the FcγRIIIb-NA2/2 genotype were more prevalent among anti-GM1 antibody-positive patients ($P = 0.031$, OR = 9.61, 95% CI = 1.24-74.77, $Pc = 0.279$; $P = 0.027$, OR = 1.62, 95% CI = 1.06-2.5, $Pc = 0.081$; respectively).

Interpretation: FcγR polymorphisms and haplotypes are not associated with susceptibility to GBS, though the FcγRIIIa-V158F genotype is associated with the severity of GBS.

Introduction

Guillain-Barré syndrome (GBS) is a post-infectious autoimmune disorder of the peripheral nervous system that can lead to significant morbidity, long-term disability or death. Cross-reactive immune responses induced by molecular mimicry between the outer core structure of infectious agents that trigger GBS and host nerve gangliosides[1] result in a blockade of nerve conduction.[1,2] *Campylobacter jejuni* has been identified as the predominant causative microbial infectious agent in GBS.[3–5] In addition to multifarious microorganism-derived factors, host immunogenic factors are likely to affect GBS susceptibility as only a subset of *C. jejuni*-infected individuals (1 in 1000-5000 cases) develop GBS.[6–9] Natural variations in genetic host susceptibility factors have become a focus of research on the susceptibility and severity of disease pathogenesis in GBS.

Immunoglobulin G Fc-gamma receptors (FcγRs) are important immune-response modulating molecules that link the cellular and humoral immune system by interacting with IgG subtypes (IgG1-4). The most common autoantibodies in GBS are produced against GM1, GD1a and GQ1b gangliosides.[5,10,11] These autoantigens may influence nerve disruption, demyelination or axonal degeneration via diverse mechanisms[12], including induction of inflammatory immune responses, by interacting with Fc receptors. FcγR polymorphisms can determine the vigor of inflammatory responses, affect downstream functions such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators, and have been implicated in the development of autoimmune disease.[13,14] Thus, FcγRs may represent important effector molecules in the pathogenesis of GBS.[15] Three subclasses of FcγRs, namely FcγRIIa, FcγRIIIa and FcγRIIIb, exhibit allelic variation.[13,16] The most widely distributed receptor, FcγRIIa, is expressed on all types of white blood cells and has two allelic forms: FcγRIIa-H131 and FcγRIIa-R131. These alleles differ by the replacement of histidine by arginine at position 131 due to an A→G single nucleotide exchange at position 494.[17,18] FcγRIIa-H131 is reported to bind human IgG2 with a higher affinity than FcγRIIa-R131.[19] FcγRIIIa is expressed on macrophages, dendritic cells, γ/δ T-cells and natural killer (NK) cells.[20] A functional polymorphism at nucleotide 559 results in either a valine (V) or phenylalanine (F) at amino acid position 158, which affects the receptor binding capacity of IgG1, IgG3, and IgG4.[21] Fcγ RIIIb is expressed on neutrophils

and exhibits two allelic forms, neutrophil antigen 1 (NA1) and neutrophil antigen 2 (NA2). NA1 and NA2 differ by five base substitutions (nucleotides 141, 147, 227, 277 and 349) that lead to four amino acid changes (at positions 36, 65, 82 and 106) within exon 3.[18,22] However, these allelic forms of FcγR (NA1/NA2) have different affinities for IgG1 and IgG3. Thus, the various allelic forms of FcγR may possibly determine the extent of inflammatory responses and thereby influence autoimmune diseases, including GBS.

Several studies have already evaluated the relationship between FcγR polymorphisms and the pathogenesis of GBS.[23–27] FcγRIIa-H/H131 was significantly associated with susceptibility to GBS and was also a potent risk factor for the development of GBS in a Dutch population.[23] These findings were consistent with a study of Indian patients with GBS, but not with a report on Norwegian Caucasian patients.[24,26] One meta-analysis indicated that every FcγRIIIb-NA2 allele cumulatively increases the GBS severity score, though none of the genotypes or alleles were associated with susceptibility to GBS.[25] However, consensus regarding the role of FcγR polymorphisms in the pathogenesis of GBS has not yet been established due to the inadequate statistical power of studies with small sample sizes and differences in the ethnicities of the populations tested. Thus, we aimed to evaluate whether candidate gene polymorphisms in FcγR are a major causative factor for GBS susceptibility or severity in Bangladeshi patients with *C. jejuni*-triggered GBS, which represents the world's largest cohort.

Materials and Methods

Research participants

The GBS cohort used in this study includes 303 patients with GBS (208 males, 95 females; median age, 29-years-old [interquartile range, 17-42]; ***Table 6.1***) and 302 ethnically matched healthy controls (204 males, 98 females; median age, 34-years-old [interquartile range, 28-46]). Patients with GBS were diagnosed based on the previously established diagnostic criteria described by Asbury and Cornblath[28] and enrolled from Dhaka Medical College and Hospital (DMCH), Dhaka, Bangladesh. No preference was given to race, religion or socioeconomic status during study subject selection. Genetically unrelated healthy individuals who did not have neurological diseases, antecedent infections, recent surgery or

Table 6.1: Demographic and clinical characteristics of the patients with GBS

Characteristic		Number of patients, *n* = 303 (%)
Sex	Male/Female	208/95
Age	Median (IQR)	29 (17-42)
Preceding illness, *n* = 303	Diarrhea	129/303 (43)
	Respiratory tract infections	45/303 (15)
	Fever	25/303 (8)
	Other	28/303 (9)
	None/Unknown	76/303 (25)
Electrophysiological classification, *n* = 247		
	Axonal	146/247 (59)
	Demyelinating	68/247 (27)
	Unclassified	33/247 (13)
MRC sum score (at entry)		
	Severely affected patients	232/303 (77)
	Mildly affected patients	71/303 (23)
Serological characteristics		
	Anti-GM1-Ab seropositive	118/303 (39)
	C. jejuni seropositive	186/303 (61)
Disease prognosis at 6 months, *n* = 303		
	Good outcome	209/303 (69)
	Poor outcome	94/303 (31)

GBS, Guillain-Barré syndrome; IQR, interquartile range; MRC, Medical Research Council; Ab, antibody; *C. jejuni, Campylobacter jejuni.*

other illnesses were included in this study following informed consent and matched with patients. Clinical, electrophysiological and serological data were obtained from patients with informed consent.

Blood specimens were collected by venipuncture before patients received medication and disease outcome was evaluated by assessing clinical data at specific standard time-points (at entry, 2 weeks, 4 weeks and 6 months). In this cohort, 75% (227/303) patients had an antecedent illness; most frequently diarrhea (43%; 129/303), followed by respiratory infection (15%, 45/303), fever (8%, 25/303) or other illnesses (9%, 28/303); 25% (76/303) of patients had history of unknown infections or no infection. Serological tests, i.e., antibodies against *C. jejuni* or GM1, GD1a and GQ1b gangliosides were measured using enzyme-linked

immunosorbent assays (ELISAs).[5,29]

Electrophysiological studies of 82% (247/303) of the GBS patients indicated 59% (146/247) of patients had an axonal subtype of GBS, including acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN), 27% (68/247) of patients had acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and 13% (33/247) of cases were unclassified with inexcitable nerves or equivocal findings.[30] Severity of disease (degree of muscle weakness) was assessed using the Medical Research Council (MRC) sum score[31,32] ranging from 0-60 at nadir (maximum muscle weakness); GBS patients at nadir with MRC-sumscore < 40 were defined as severely affected patients and with MRC-sumscore ≥ 40 were defined as mildly affected patients.[33] The outcome of the disease was measured using the GBS disability score after six months of follow-up.[34]This study was reviewed and approved by the Institutional Review Board (IRB) and ethical committees of the icddr, b, Dhaka, Bangladesh.

Genomic DNA isolation

Whole blood samples were collected from 605 study subjects into lithium heparin-coated anti-coagulation tubes for genomic DNA isolation. Genomic DNA was extracted using the QIAamp® DNA Blood Midi Kit (100) (Qiagen, Hilden, Germany), dissolved in 1× TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA), stored at -80 °C, diluted to 10 ng/µL with Milli-Q water and then stored at -20°C until SNP detection.

FcγR polymorphism detection and genotype analysis

The FcγR polymorphisms FcγRIIa H/R131 (rs1801274), FcγRIIIa V/F158 (rs396991) and FcγRIIIb NA1/NA2 were genotyped via a previously described allele-specific polymerase chain reaction (AS-PCR) method using published primer sequences and reaction conditions.[18,21] Human growth hormone (*HGH*) primers (5`-GCCTTCCCAACCATTCCCTTA-3` and 5`-CTCACGGATTTCTGTTGTGTTTC-3`) were used as an internal positive control.[18] The PCR products were visualized on 2% agarose gels using a Molecular Imager® Gel Doc™ XR+system (Bio-Rad Laboratories Inc, USA).

Statistical analysis

Statistical analysis was performed using logistic regression analysis and Fisher's exact test with Yates' continuity correction to assess associations between the FcγR polymorphisms and disease susceptibility or subgroups. In the control group, all SNPs were within Hardy-Weinberg equilibrium. *P*-values less than 0.05 were considered statistically significant. The Bonferroni method was applied to correct the *P*-values for multiple comparisons: each *P*-value was multiplied by the number of comparisons and denoted *P*c (*P*c, *P* corrected). Genotype/allelic frequencies were estimated by a simple counting method and the data were processed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), GraphPad prism (version 5.01, GraphPad software, Inc., La Jolla, CA, USA) or SPSS (version 16.0, Company, Chicago, IL, USA). Haplotype patterns and frequencies were analyzed using the genotype package of R statistics and their associations with GBS susceptibility and subgroups were assessed using logistic regression analysis.

Results

FcγRIIa, FcγRIIIa and FcγRIIIb polymorphisms and haplotype in patients with GBS and healthy individuals

No significant associations were observed between the FcγRIIa, FcγRIIIa and FcγRIIIb polymorphisms and susceptibility to GBS compared to healthy controls (***Table 6.2***). The comparison of axonal variants of GBS versus healthy controls or demyelinating subtypes versus healthy subjects showed no relation with disease susceptibility (***Table 6.3***).

.

Table 6.2: FcγR genotype and allelic distributions in Bangladeshi patients with GBS and healthy controls

FcγR genotype/allele	HC *n* = 302 (%)	GBS patients *n* = 303 (%)	*P* value	Odds ratio (95% CI)
FcγR-IIa				
H/H-131	116 (38.4)	114 (37.6)		Reference
H/R-131	136 (45)	124 (40.9)	0.283	0.93 (0.65-1.32)
R/R-131	50 (16.6)	65 (21.5)		1.32 (0.84-2.08)
R-131	236 (39.1)	254 (41.9)	0.320	0.89 (0.71-1.12)
H-131	368 (60.9)	352 (58.1)		Reference
FcγR-IIIa				
F/F-158	110 (36.4)	120 (39.6)		Reference
V/F-158	150 (49.7)	143 (47.2)	0.723	0.87 (0.62-1.23)
V/V-158	42 (13.9)	40 (13.2)		0.87 (0.53-1.45)
V-158	234 (38.7)	223 (36.8)		1.09 (0.86-1.37)
F-158	370 (61.3)	383 (63.2)	0.514	Reference
FcγR-IIIb				
NA1/1	69 (22.9)	56 (18.5)		Reference
NA1/2	126 (41.7)	125 (41.2)	0.311	1.22 (0.79-1.88)
NA2/2	107 (35.4)	122 (40.3)		1.41 (0.91-2.18)
NA1	264 (43.7)	237 (39.1)	0.115	1.21(0.96-1.52)
NA2	340 (56.3)	369 (60.9)		Reference

GBS, Guillain-Barré syndrome; HC, healthy controls; 95% CI, 95% confidence interval; NA, neutrophil antigen.

Table 6.3: Distribution of FcγR genotypes and alleles among axonal and demyelinating cases of GBS compared to healthy controls

FcγR	Subtype			Axonal vs. HC		Demyelinating vs. HC	
Genotypes/ Alleles	**Axonal *n* = 146 (%)**	**Demyelinating *n* = 68 (%)**	**Healthy control (HC) *n* = 302 (%)**	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**
FcγR IIa							
H/H -131	50 (34.2)	28 (41.2)	116 (38.4)		Reference		Reference
H/R-131	63 (43.2)	24 (35.3)	136 (45)	0.289	1.1(0.69-1.68)	0.242	0.7(0.40-1.33)
R/R -131	33 (22.6)	16 (23.5)	50 (16.6)		1.5(0.88-2.66)		1.3 (0.66-2.67)
R-131	129 (44.2)	56 (41.2)	236 (39.1)		Reference		Reference
H-131	163 (55.8)	80 (58.8)	368 (60.9)	0.147	1.2 (0.93-1.64)	0.698	1.1 (0.75-1.59)
FcγR IIIa							
F/F-158	57 (39)	33 (48.5)	110 (36.4)		Reference		Reference
V/F-158	74 (50.7)	27 (39.7)	150 (49.7)	0.542	0.9(0.6-1.4)	0.178	0.6(0.3-1.0)
V/V-158	15 (10.3)	8 (11.8)	42 (13.9)		0.7(0.4-1.3)		0.6(0.3-1.5)
V-158	104 (35.6)	43 (31.6)	234 (38.7)		Reference		Reference
F-158	188 (64.4)	93 (68.4)	370 (61.3)	0.378	0.9 (0.65-1.17)	0.141	0.7 (0.49-1.09)
FcγR IIIb							
NA1/1	27 (18.5)	17 (25)	69 (22.8)		Reference		Reference
NA1/2	61 (41.8)	25 (36.8)	126 (41.7)	0.506	0.8 (0.5-1.4)	0.753	1.2 (0.6-2.4)
NA2/2	58 (39.7)	26 (38.2)	107 (35.4)		0.7 (0.4-1.2)		1.0 (0.5-2.0)
NA1	115 (39.4)	59 (43.4)	264 (43.7)		Reference		Reference
NA2	177 (60.6)	77 (56.6)	340 (56.3)	0.248	0.8 (0.6-1.1)	1.0	1.0 (0.7-1.4)

OR, odds ratio; 95% CI, 95% confidence interval.

The haplotype distributions of the three loci were compared between patients with GBS and healthy individuals. Haplotype analysis revealed 27 possible different patterns for the FcγRIIa, FcγRIIIa and FcγRIIIb polymorphic loci (***Figure 6.1***). The nine most predominant patterns (haplotypes 1-9; frequency > 5%), representing 61.5% of total variation, were selected for further haplotype analysis (***Figure 6.2***). No significant association was observed between any haplotype and GBS susceptibility when each haplotype was analyzed individually.

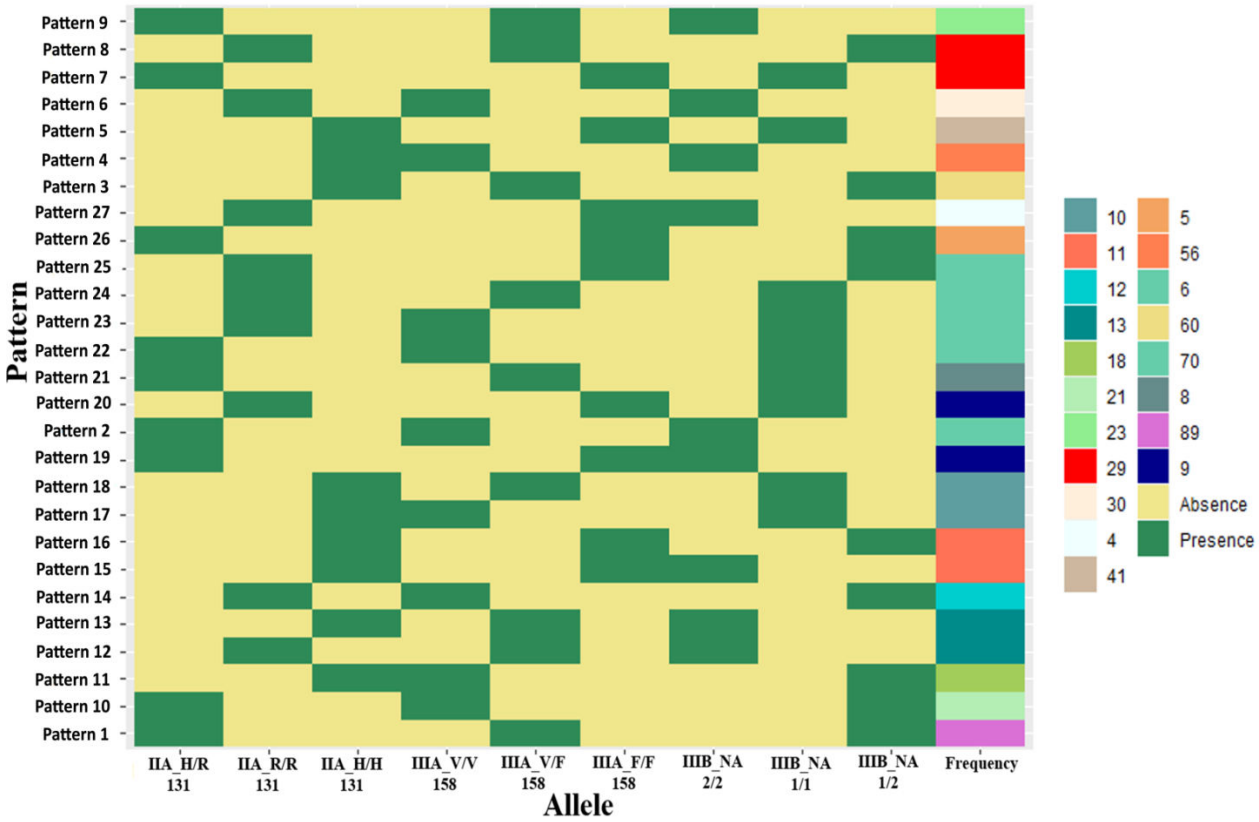

Figure 6.1 Haplotype analysis of FcγRIIa, FcγRIIIa and FcγRIIIb polymorphic loci for the study subjects from Bangladesh. Twenty-seven different haplotype patterns were observed; pattern 1 was the most common (pink). Green indicates the presence and yellow indicates the absence of specific FcγR polymorphisms for each of the three loci. The polymorphism frequencies are presented as a color gradient on the right.

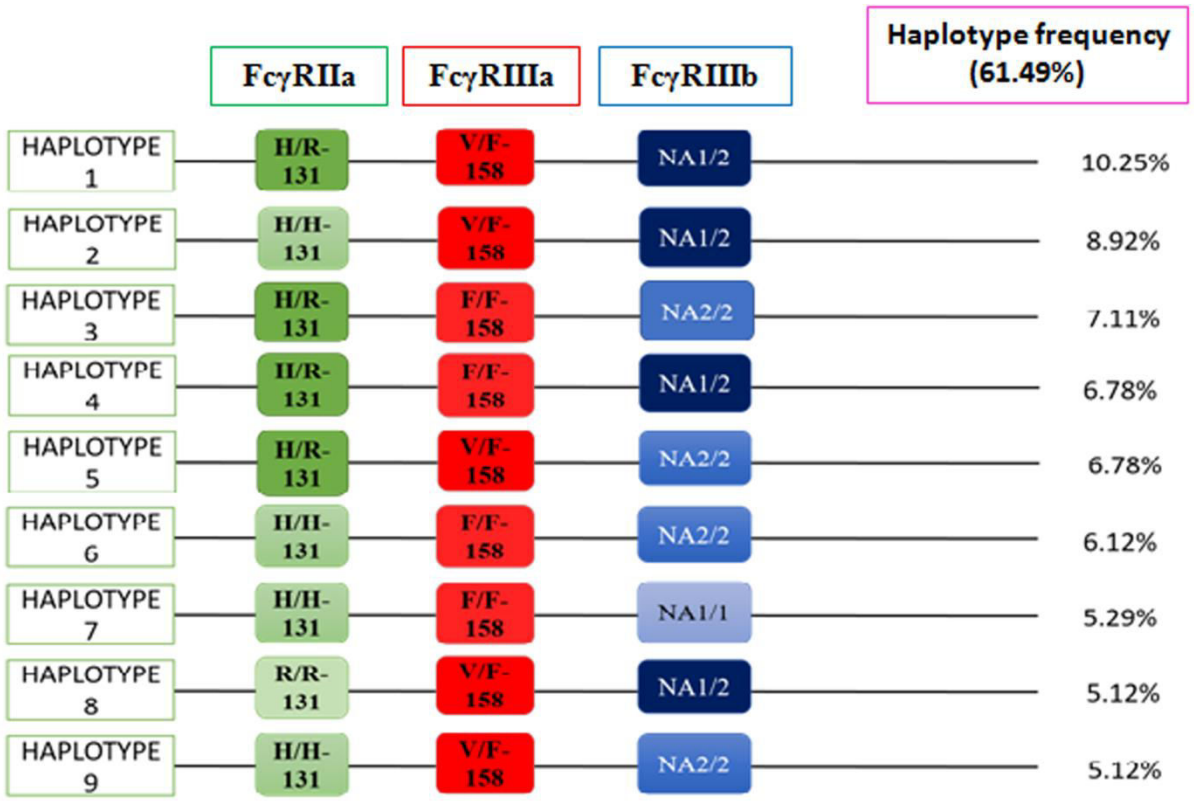

Figure 6.2 Haplotype frequencies for FcγRIIa, FcγRIIIa and FcγRIIIb (FcγRs) polymorphisms for the study subjects from Bangladesh. The nine most predominant patterns (haplotypes 1-9; frequency > 5%) represented 61.49% of total variation and were selected for haplotype analysis. The frequencies of specific haplotypes are presented on the left.

FcγRIIa, FcγRIIIa and FcγRIIIb polymorphisms and haplotypes in anti-GM1 antibody-positive GBS

The frequency of FcγRIIIb-NA2/2 genotypes was predominant among anti-GM1 antibody-positive patients compared to healthy individuals but association was not significant (P = 0.051, OR = 1.93, 95% CI = 1.03-3.62; ***Table 6.4***). Haplotype 1 (FcγRIIa-H131R- FcγRIIIa-V158F- FcγRIIIb-NA1/2) and the FcγRIIIb-NA2/2 genotype were significantly prevalent among anti-GM1 antibody-positive patients than antibody-negative patients with GBS; however, these associations were lost after Bonferroni correction (P = 0.031, OR = 9.61, 95% CI = 1.24-74.77; Pc = 0.279 and P = 0.027, OR = 1.62, 95% CI = 1.06-2.47; Pc = 0.081; respectively; ***Table 6.5***). The homozygous FcγRIIIb NA1/1 genotype was predominant in

healthy individuals compared to anti-GM1 antibody-positive patients (22.9% vs. 14.2%; ***Table 6.4***) and significantly present in anti-GM1 antibody-negative patients with GBS than antibody-positive patients (*P* = 0.002, OR = 0.43, 95% CI = 0.25-0.73; *P*c = 0.006; ***Table 6.5***). Except haplotype 1, no other haplotypes (haplotype 2-9) were associated with anti-GM1 antibody positivity (***Table 6.5***).

Associations of FcγRIIa, FcγRIIIa and FcγRIIIb polymorphisms and haplotype patterns with disease severity and outcome

FcγRIIa, FcγRIIIa and FcγRIIIb genotypes and haplotype patterns were investigated in patients with severe and mild form of GBS (***Table 6.5***). The haplotype patterns were not associated with disease severity, though homozygous FcγRIIIa-F158 was significantly associated with the mild form of disease before Bonferroni correction (*P* = 0.03, OR = 0.55, 95% CI = 0.32-0.94; *P*c = 0.09; ***Table 6.5***). Heterozygous FcγRIIIa-V158F was significantly associated with the severe form of disease (compared to the mild form) after correcting the *P*-value (*P* = 0.005, OR = 2.24, 95% CI = 1.28-3.91; *P*c = 0.015; ***Table 6.5***). FcγRIIIa-NA1/NA1 was significantly predominant in the mild form of GBS than the severe form (*P* = 0.007, OR = 0.41, 95% CI = 0.22-0.77; *P*c = 0.021; ***Table 6.5***). FcγRIIIa-NA1/NA2 tended to be more common in severe GBS (*P* = 0.054, OR = 1.75, 95% CI = 0.99-3.08; *P*c = 0.162; ***Table 6.5***). However, the FcγRIIa-H131 and FcγRIIa-R131 alleles and genotypes were not associated with the severity of GBS. Individual FcγR genotypes were not associated with disease outcome at six-month follow-up.

FcγRIIa, FcγRIIIa and FcγRIIIb genotypes in patients with recent C. jejuni infection

The homozygous FcγRIIIb-NA2 and heterozygous FcγRIIIb-NA1/2 genotypes were associated with recent *C. jejuni* infection in patients with GBS; however, the association for the heterozygous FcγRIIIb-NA1/2 genotype lost significance after Bonferroni correction (*P* = 0.004, OR = 1.70, 95% CI = 1.18-2.44; *P*c = 0.012 and *P* = 0.026, OR = 1.48, 95% CI = 1.05-2.10; *P*c = 0.078; respectively; ***Table 6.5***). Frequency of homozygous FcγRIIIb-NA2 and

heterozygous FcγRIIIb-NA1/2 genotypes were significantly prevalent in *C. jejuni* infected patients with GBS compared to healthy controls. But *P*-value lost its significance after Bonferroni correction (*P* = 0.041, OR = 1.74, 95% CI = 1.03-2.94; *P*c = 0.123 and *P* = 0.048, OR = 1.74, 95% CI = 1.02-2.98; *P*c = 0.144; respectively; ***Table 6.4***). The FcγRIIIa-V/V158 genotype was less frequent in *C. jejuni* seropositive patients (*P* ≤ 0.001, OR = 0.36, 95% CI = 0.23-0.56; *P*c ≤ 0.003; ***Table 6.5***); however, the FcγRIIIa-F/F158 and FcγRIIIa-V/F158 genotypes were significantly prevalent among *C. jejuni* seropositive patients than seronegative patients before correcting the *P*-values (*P* = 0.038, OR = 1.47, 95% CI = 1.02-2.11; *P*c = 0.114 and *P* = 0.025, OR = 1.49, 95% CI = 1.05-2.10; *P*c = 0.075, respectively; ***Table 6.5***).

Table 6.4: Distribution of FcγR genotypes and alleles between healthy controls versus *C. jejuni* seropositive patients and healthy controls versus anti-GM1 antibody seropositive patients with GBS

FcγR genotype /allele	Healthy controls (a) *n* = 302 (%)	*C. jejuni* seropositive patients (b) *n* = 186 (%)	Anti-GM1-Ab seropositive patients (c) *n* = 119 (%)	a vs. b *P* value	a vs. b *P*c	Odds ratio (95% CI)	a vs. c *P* value	a vs. c *P*c	Odds ratio (95% CI)
FcγR-IIa									
H/H-131	116 (38.4)	67 (36.0)	42 (35.3)			Reference			Reference
H/R-131	136 (45)	81 (43.6)	53 (44.5)	0.917	*na*	1.03 (0.69-1.55)	0.809	*na*	1.08 (0.67-1.73)
R/R-131	50 (16.6)	38 (20.4)	24 (20.2)	0.351	*na*	1.32 (0.78-2.21)	0.354	*na*	1.33 (0.73-2.42)
R-131	236 (39.1)	157 (42.2)	101 (42.4)			0.88 (0.68-1.14)			0.87 (0.64-1.18)
H-131	368 (60.9)	215 (57.8)	137 (57.6)	0.347	*na*	Reference	0.391	*na*	Reference
FcγR-IIIa									
F/F-158	110 (36.4)	70 (37.6)	44 (37.3)			Reference			Reference
V/F-158	150 (49.7)	90 (48.4)	55 (46.6)	0.839	*na*	0.94 (0.63-1.40)	0.722	*na*	0.92 (0.57-1.46)
V/V-158	42 (13.9)	26 (14.0)	20 (16.1)	1.0	*na*	0.97 (0.55-1.73)	0.623	*na*	1.20 (0.63-2.25)
V-158	234 (38.7)	142 (38.2)	95 (39.9)			1.02 (0.78-1.34)			0.95 (0.70-1.29)
F-158	370 (61.3)	230 (61.8)	143 (60.1)	0.892	*na*	Reference	0.754	*na*	Reference
FcγR-IIIb									
NA1/1	69 (22.9)	27 (14.3)	17 (14.2)			Reference			Reference
NA1/2	126 (41.7)	86 (46.2)	51 (42.9)	0.041	0.123	1.74 (1.03-2.94)	0.134	*na*	1.64 (0.88-3.06)
NA2/2	107 (35.4)	73 (39.3)	51 (42.9)	0.048	0.144	1.74 (1.02-2.98)	0.051	*na*	1.93 (1.03-3.62)
NA1	264 (43.7)	140 (37.6)	85 (35.7)			1.29 (0.98-1.68)			1.40 (1.02-1.91)
NA2	340 (56.3)	232 (62.4)	153 (64.3)	0.071	*na*	Reference	0.036	0.072	Reference

OR, odds ratio; 95% CI, 95% confidence interval; *C. jejuni, Campylobacter jejuni;* Anti-GM1 Ab, Anti-GM1 antibody*; na,* not applicable; *Pc, P* corrected.

Table 6.5: Associations between FcγR genotypes and haplotypes with disease severity, anti-GM1 antibody seropositivity and *C. jejuni* seropositivity among patients with GBS

Variables	FcγR genotype/haplotype	*P* value	Odds ratio	95% CI	*P* corrected (*P*c)
Mildly affected (*n* = 71) vs. severely affected (*n* = 232) patients	FcγRIIIa				
	F/F-158	0.03	0.55	0.32-0.94	0.09
	V/F-158	0.005	2.24	1.28-3.91	0.015
	V/V-158	0.25	0.68	0.32-1.41	-
	FcγRIIIb				
	NA1/1	0.007	0.41	0.22-0.77	0.021
	NA1/2	0.054	1.75	0.99-3.08	0.162
	NA2/2	0.891	1.06	0.62-1.82	-
Anti-GM1-Ab seropositive (*n* = 118) vs. seronegative (*n* = 185)	FcγRIIIb				
	NA1/1	0.002	0.43	0.25-0.73	0.006
	NA1/2	0.482	1.16	0.76-1.77	-
	NA2/2	0.027	1.62	1.06-2.47	0.081
	Haplotype 1	0.031	9.61	1.24-74.77	0.279
C. jejuni seropositive (*n* = 186) vs. seronegative (*n* = 117)	FcγRIIIa				
	F/F-158	0.038	1.47	1.02-2.11	0.114
	V/F-158	0.025	1.49	1.05-2.10	0.075
	V/V-158	≤ 0.001	0.36	0.23-0.56	≤ 0.003
	FcγRIIIb				
	NA1/1	≤ 0.001	0.32	0.21-0.49	≤ 0.003
	NA1/2	0.026	1.48	1.05-2.10	0.078
	NA2/2	0.004	1.70	1.18-2.44	0.012

OR, odds ratio; 95% CI, 95% confidence interval; MRC sum scores < 40 at nadir were defined as severely affected; MRC sum scores ≥ 40 were defined as mildly affected; Anti-GM1-Ab, Anti-GM1-antibody; *P*c, Bonferroni-corrected *P* values.

Discussion

This study investigated the association of three functionally relevant polymorphisms in FcγR and the resulting haplotype patterns with the susceptibility and severity of GBS among patients compared to healthy controls in a large cohort of GBS in Bangladesh. We found no significant associations between individual FcγR alleles or genotypes and susceptibility to GBS; however, the FcγRIIIa-V/F158 genotype influenced the severity of disease. Moreover, associations between the FcγRIIIa and FcγRIIIb genotypes and haplotype patterns were evident in patients with an antecedent *C. jejuni* infection and anti-GM1 antibody-positive patients, respectively.

Associations between FcγR polymorphisms and susceptibility to GBS have previously been studied in patients with different ethnic backgrounds (***Table 6.6***).[23–26] We observed no significant differences in the FcγR allele or genotype frequencies and haplotype patterns between Bangladeshi patients with GBS and healthy controls. These findings confirm previous a meta-analysis of British, Dutch and Norwegian GBS cases[25], which suggested FcγR polymorphisms were not related to disease susceptibility, regardless of ethnic variation.

In addition, we found the FcγRIIIa-F/F158 genotype was associated with the mild form of GBS based on MRC sum score at nadir, while the FcγRIIIa-V/F158 genotype was associated with the severe form of GBS. As phagocytosis, cellular cytotoxicity, cytokine production and other immune responses depend on efficient FcγR-IgG interactions, the higher frequency of FcγRIIIa-F/F158 among patients with the mild form of GBS may indicate this genotype reduces the affinity of IgG binding and in turn impairs immune complex clearance and decreases subsequent inflammation.[13,35,36]

Table 6.6: Summary of population-association studies of Fc-gamma receptor polymorphisms with GBS disease susceptibility and severity in various ethnicities

Study (Author, year)	Ethnic origin/ population	Country	Participants (*n*) (GBS vs. controls)	Reported association
van der Pol WL, 2000	Caucasian	Netherlands	31 vs. 187	FcγRIIa-H/H131 more frequent in patients than controls (OR, 2.45; P = 0.037). FcγRIIa-H/H131 associated with disease severity (OR, 18.57; P = 0.007).
Vedeler, 2000	Caucasian	Norway	62 vs. 89	FcγRIIIb-NA1/NA1 associated with mild GBS (P = 0.027).
van Sorge, 2005	Caucasian	Netherlands	192 vs. 514	FcγRIIIb-NA2/2 more frequent in severe GBS (OR, 2.03; P = 0.03).
van Sorge, 2005	British	United Kingdom	91 vs. 111	FcγRIIa-H/H131 more frequent in patients than controls (OR, 2.48; P = 0.02). FcgRIIIa-F158 allele more frequent in patients than controls (OR, 1.56; P = 0.03).
Sinha, 2010	Asian	India	80 vs. 80	FcγRIIa-H/H131 and FcγRIIa-H131 more frequent in patients than controls ($P \leq 0.0001$ and $P \leq 0.0001$). FcγRIIIa-V/V158 more frequent in patients than controls ($P \leq 0.0001$).
Hayat, 2020	Asian	Bangladesh	303 vs. 302	FcγRIIIa-V/F158 associated with severe GBS (OR, 2.24; P=0.015). FcγRIIIb NA1/NA1 associated with mild GBS (OR, 0.41; P = 0.02).

GBS, Guillain-Barré syndrome; OR, odds ratio.

Patients with FcγRIIIa-V/F158 genotypes may better able to clear immune complexes (ICs) via degranulation and phagocytosis more efficiently, resulting in more severe disease.[36] We observed a higher frequency of FcγRIIIb-NA1/NA1 genotypes in patients with the mild form of GBS, similarly to a previous study of Norwegian patients with GBS.[24] The NA1/NA1 genotype has a high affinity for IgG1 and IgG3,[37] which are the most common among the anti-GM1 and anti-GQ1b antibodies.[38] Autoantibodies such as anti-ganglioside antibodies are neutralized in the circulation, thus cross-reaction of these auto-antibodies with the peripheral nerves may be partially prevented in patients with GBS who are homozygous for FcγRIIIb-NA1.[24]

Ganglioside-specific IgG have been reported to damage nerve tissues by activating effector functions (e.g., phagocytosis and/or degranulation) via FcγR.[35,39] Homozygous FcγRIIIb-NA1 was less frequent among both *C. jejuni*-seropositive patients and anti-GM1 antibody-positive patients with the mild form of the disease. In contrast, FcγRIIIb-NA2/2 was associated with recent *C. jejuni* infection and anti-GM1 antibody production. In addition, *C. jejuni*-seropositive patients had higher frequencies of the FcγRIIIa-F/F158 and FcγRIIIa-V158F genotypes. These findings indicate *C. jejuni*-seropositive patients with higher frequency of the FcγRIIIa-V158F genotype may suffer severe muscle weakness.

One limitation of this study is that polymorphisms of FcγRIIIb receptor gene, FcγRIIIb-SH alleles were not investigated; however, it is not yet known whether FcγRIIIb-SH polymorphisms influence the function of FcγRIIIb or not.[16,40]

The present study strengthens the evidence that FcγR polymorphisms and haplotypes influence the clinical and serological subgroup of GBS, as well as the strength of the immune responses that ultimately trigger the development of GBS and affect disease severity. In addition, the FcγRIIIa-V158F genotype was more frequent among patients with recent *C. jejuni* infection and was found to contribute to disease severity. Variation in the FcγR gene differs greatly between populations of different ethnicities, thus it will be important and interesting to confirm our findings in a multiethnic population, such as the International GBS Outcome Study (IGOS) population.[41]

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Conflict of interest

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Chapter 7

IL-10 polymorphisms in patients with GBS

Interleukin-10 promoter polymorphisms in patients with Guillain–Barre´ syndrome in Bangladesh

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Abstract

Objective: Interleukin-10 (IL-10) is a multifunctional cytokine with both pro- and anti-inflammatory effects on immune system as well as in the pathogenesis of Guillain-Barré syndrome (GBS). In this study, we assessed whether the three common polymorphisms -1082 G/A (rs1800896), -819 C/T (rs1800871) and -592 C/A (rs1800872) in the promoter region of IL-10 influence the susceptibility and severity of GBS in Bangladesh.

Methods: Genotyping of the IL-10 gene promoter polymorphism was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) and allele specific oligonucleotide -polymerase chain reaction (ASO-PCR) in 152 patients with GBS and 152 ethnically matched healthy controls in Bangladesh. Verification of Hardy–Weinberg equilibrium and comparison of genotype and allele frequencies were performed using Pearson's chi-square test or Fisher's exact test (two-sided) when appropriate. P values <0.05 were considered to be statistically significant.

Results: The homozygous -819 TT genotype was the most prevalent in axonal variant of GBS compared to demyelinating subtypes and healthy controls (P = 0.042, OR = 8.67, 95% CI = 1.03-72.97; Pc = 0.123 and P = 0.005, OR = 4.2, 95% CI = 1.55-11.40; Pc = 0.015, respectively). Moreover, the -819 TT genotype tended to be associated with disease susceptibility when patients were compared with healthy controls as P value lost its significance after Bonferroni correction for multiple comparisons (P = 0.029, OR = 2.73, 95% CI = 1.15-6.45; Pc = 0.08). No other genotypes or haplotypes of IL-10, -1082 G/A, -819 C/T and -592 C/A polymorphisms showed significant association with disease susceptibility. The high IL-10 expression haplotype combinations (GCC/GTA, GCC/ATA and GCC/GCA) may influence severity of the disease (P = 0.008, OR = 3.22, 95% CI = 1.4-7.43; Pc = 0.024).

Interpretation: The -819 TT genotypes may influence axonal variant of GBS, and high frequency of IL-10 expression haplotype combination (GCC/GTA, GCC/ATA and GCC/GCA) may play a pivotal role in disease severity.

Introduction

Guillain–Barré syndrome (GBS) is an autoimmune-mediated disorder affecting the axons and myelin sheath of the peripheral nervous system (PNS) with high clinical disability.[1] GBS is considered an excellent paradigm of molecular mimicry in which infectious agents induce cross-reactive antibodies against host nerve gangliosides.[2] These pathogenic antibodies result in aberrant immune system and subsequent peripheral nerve damage.[3–5] Based on recent evidence, molecular mimicry alone is not enough to explain the etiology of GBS but also immune response host susceptibility may also play an essential role for the induction of the disease.[6,7] Both strain properties and host properties are crucial in determining the risk of development of GBS.[8] Host factors like Interleukin-10 (IL-10) and their genetic predisposition to GBS is very important to decipher their role in disease pathogenesis.

IL-10 is an important cytokine in the regulation of inflammatory and immune responses and has been implicated in autoimmunity.[9] This cytokine, which is produced by B cells, T cells and macrophages, is also considered as the 'cytokine synthesis inhibitory factor' (CSIF) that inhibits the release of T-helper (Th) 1-type cytokines such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and IL-2,[10–13] and downregulates major histocompatibility complex (MHC) class II expression on macrophages.[14] IL-10 may also contribute towards pro-inflammatory actions such as the activation of B cells, along with the production of auto-antibodies and inhibition of T cell apoptosis. All these effects are considered very important in the pathogenesis of GBS.[15]

IL-10 gene is located in chromosome 1q31-1q32[16,17] and the production of IL-10 is strongly influenced by genetic factors.[18] Several polymorphic sites have been described in the promoter region of IL-10 gene including the bi-allelic polymorphisms at -1082 G/A (rs1800896), -819 C/T (rs1800871) and -592 C/A (rs1800872) locus of the transcriptional start site which were most common and important in autoimmune disease pathogenesis.

Several studies were performed to observe whether these single nucleotide polymorphisms (SNPs) of IL-10 gene encoding macrophage mediators are responsible for the severity and susceptibility of GBS.[15,19] Press et al. showed that high levels of IL-10-secreting blood mono-nuclear cells (MNCs) correlated with serum levels of anti-ganglioside antibodies and axonal damage suggesting the up-regulation of IL-10 in the early phase of GBS

development.[1,20] In a Norwegian population, -592 CC and -819 CC genotypes were associated with increased IL-10 response in GBS.[15] One Dutch study reported no associations between the single nucleotide polymorphisms (SNPs) in IL-10 promoter region and disease susceptibility or subgroups.[19] However, very few data are available on IL-10 polymorphism from low-income countries. Therefore, we intended to investigate the distribution of IL-10 promoter polymorphisms and their influence on disease susceptibility, severity and prognosis in patients with GBS from a well-documented cohort of Bangladesh.

Materials and Methods

Study subjects

This study included 152 patients with GBS (103 males and 49 females; median age, 29 years [interquartile range, 17-42 years]) and 152 healthy individuals of Bangladesh (78 males and 74 females; median age, 35 years [interquartile range, 28-40 years]). Healthy controls were genetically unrelated to patients and ethnically matched with no history of previous GBS or other neurological disorders. Study subjects were selected randomly from a study cohort and consisted of 303 patients with GBS and 303 healthy subjects of Bangladesh. Patients were enrolled from Dhaka Medical College and Hospital (DMCH) after the onset of neuropathic symptoms that fulfilled the diagnostic criteria for GBS as described by Asbury and Cornblath[21] during 2010-2016. Healthy controls were enrolled during patient enrolment period. Written informed consent was obtained from each study subject before clinical examination, specimen collection and data collection. This study was reviewed and approved by the Institutional Review Board (IRB) and ethical committees of icddr, b, Dhaka, Bangladesh. Data were collected on the basis of age, sex, antecedent events, detailed neurological signs and symptoms, treatment, days to nadir, complications, duration of admission, GBS disability score (GBS-DS) [22] and the Medical Research Council (MRC) [23] sum score at standard points (entry, 2 weeks, 4 weeks, and 6 months after enrollment). Both the GBS-DS and MRC sum score indicated the severity of disease. Patients with an MRC sum score at nadir of < 40 were defined as severely affected and of 40-60 as mildly affected.[19] Patients with GBS-DS of 0, 1 and 2 (independent walking) within six months represented good outcome and GBS-DS of 3, 4, 5 and 6 (unable to walk or death) presented poor outcome.[19,22]

Electrophysiological studies on 68% (104/152) patients with GBS revealed, 57% (59/104; 55, AMAN and 4, AMSAN) were axonal variants of GBS; 26% (27/104; AIDP) were demyelinating type and 17% (18/104) were unclassified GBS cases with inexcitable nerves or equivocal findings. Among the patients, 86% (130/152) had antecedent events of infection with 55% (71/130) of diarrhea, 18% (24/130) of respiratory infection and 27% (35/130) of other preceding illness.

Detection of C. jejuni infection and anti-ganglioside antibodies

Serology of *C. jejuni* infection and auto-antibodies against GM1, GD1a and GQ1b were determined based on enzyme linked immunosorbent assays (ELISAs).[24–26]

Genomic DNA isolation and detection of IL-10 polymorphisms

Genomic DNA of 304 study subjects were extracted from whole blood using a QIAamp® DNA Blood Midi Kit (100; Qiagen, Hilden, Germany) as described by the manufacturer. The DNA samples were dissolved in 1X TE-buffer (10mM Tris-Cl, pH 8.0 & 1 mM EDTA) and eventually diluted with Milli-Q water to a final concentration of 10ng/µl and stored at -20°C for polymorphism study. IL-10 SNPs including -1082 G/A (rs1800896) and -592 C/A (rs1800872) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay consisting of an initial PCR followed by specific restriction endonuclease *Mnll* and *Rsal digestion,* repectively.[17] Allele specific oligonucleotide-polymerase chain reaction (ASO-PCR) assay was performed to detect -819 C/T (rs1800871) polymorphisms. For ASO-PCR, primers were designed using NCBI public database and OligoAnanlyzer 3.1.[27,28] Details of the primers and enzymes used in the study for SNPs detection were given in ***Chapter 3; Table 3.2d***. Master mix (25 µL) was prepared containing 10 ng of genomic DNA, 10 pmol of each specific primer, 0.1 mM dNTPs (Promega), 1 U of GoTaq® Flexi DNA Polymerase (Promega), 5× Green GoTaq® Flexi Buffer, 25 mM MgCl2 and Milli-Q to perform PCR analysis. After digestions, the digested products and PCR products were visualized on 2% agarose gels using a Molecular Imager® Gel Doc™ XR+system (Bio-Rad Laboratories Inc, USA).

Statistical analysis

Genotypes (combination of alleles of a given SNP) and allele frequencies were analyzed using chi-square (χ^2) test and Fisher's exact test with Yates correction. Hardy-Weinberg equilibrium was analyzed for healthy individuals by chi-square (χ^2) test. For a value less than 5 in any cell of the 2 x 2 table, Yates correction was performed and considered significant at a *P*-value of < 0.05. The Bonferroni method was conducted to correct the *P*-values for multiple comparisons where each *P*-value was multiplied by the number of comparisons and represented as *P*c (*P*c, *P* corrected). The results were represents as odds ratio (OR) showing 95% CI as well. Haplotypes and allele frequencies were estimated by simple gene counting and the data was processed in Microsoft Excel 2007. Statistical analyses were performed using the GraphPad Prism (version 5.01, GraphPad Software, Inc. La Jolla, CA 92037 USA) and SPSS (20.0 version, Chicago, IL, USA) computer software programs.

Results

The frequency of the -819 TT genotype was increased in patients of GBS compared to the healthy controls when comparisons were made prior to the Bonferroni correction of *P* values (*P* = 0.029, OR = 2.73, 95% CI = 1.15-6.45; *P*c = 0.08; ***Table 7.1***). The homozygous -819 TT genotypes were most prevalent in the axonal variant of GBS than the demyelinating subtypes (22% vs. 3.7%) and healthy controls (22% vs. 5.3%).

Table 7.1: Frequency distribution of IL-10 promoter polymorphisms in patients with GBS and healthy controls

Gene polymorphisms	GBS Patients *n* = 152 (%)	Healthy Control *n* = 152 (%)	*P* value	Odds ratio (95% CI)
-1082(G/A)				
GG	103(67.8)	97 (63.8)		Reference
GA	45 (29.7)	49 (32.2)	0.617	0.86 (0.53-1.41)
AA	4 (2.6)	6 (4.0)	0.532	0.63 (0.17-2.29)
-819(C/T)				
CC	71(46.7)	62(40.8)		Reference
CT	56 (36.8)	82(53.9)	0.038[a]	0.59 (0.37-0.96)
TT	25 (16.5)	8 (5.3)	0.029[b]	2.73 (1.15-6.45)
-592(C/A)				
CC	35 (23)	37(24.3)		Reference
CA	80 (52.6)	83(54.6)	1.00	1.01 (0.58-1.78)
AA	37 (24.3)	32(21.1)	0.614	1.22 (0.63-2.37)

GBS, Guillain-Barré syndrome; 95% CI, 95% confidence interval; a, *P*c = 0.114; b, *P*c = 0.08 (*P*c, *P* corrected).

However, the *P* value lost its significance between axonal vs. demyelination following Bonferroni correction (*P* = 0.042, OR = 8.67, 95% CI = 1.03-72.97; *P*c = 0.123 and *P* = 0.005, OR = 4.2, 95% CI = 1.55-11.40; *P*c = 0.015, respectively; ***Table 7.2***). Patients were categorized into different haplotype combinations including high (GCC/GTA, GCC/ATA and GCC/GCA; frequency ≥ 10.7%), medium (GCC/GTC, GCC/GCC, GCC/ACC, GCC/ACA, GCA/GTA, GCA/GCA and GCA/ACA) and low prevalence (rest of the haplotype combinations; frequency ≤ 2.7%) ***(Table 7.3).*** Haplotype analysis revealed no significant association between patients and healthy controls and presented distribution of major haplotype combinations as GCC/GTA (17.9% vs. 23.3%), GCC/ATA (10.6% vs. 15.3%) and GCC/GCA (13.9% vs. 10.7%) respectively in the study cases and healthy controls ***(Table not shown).***

The genotype frequencies of -1082G/A, -819C/T and -592C/A were analyzed to investigate the association between IL-10 polymorphisms with *C. jejuni* infection and anti-ganglioside antibody production ***(Table 7.4).*** The genotype distributions did not significantly differ between *C. jejuni* positive vs. *C. jejuni* negative GBS patients and anti-GM1 antibody positive vs. anti-GM1 antibody negative patients ***(Table 7.4).*** The homozygous -819 TT

genotypes were prevalent in *C. jejuni* serology positive patients compared to negative patients (21.1% vs. 8.8%) but association was not significant (P = 0.088, OR = 0.36, 95% CI = 0.12-1.07; ***Table 7.4***).

Table 7.2: Distribution of IL-10 promoter polymorphisms in axonal and demyelinating cases compared to healthy controls

Genotypes	Axonal *n* = 59 (%) (a)	Demyelinating *n* = 27 (%) (b)	Healthy Control *n* =152 (%) (c)	*P* value a vs. b	Odds ratio (95% CI)	*P* value a vs. c	Odds ratio (95% CI)	*P* value b vs. c	Odds ratio (95% CI)
-1082(G/A)									
GG	43 (72.9)	15 (55.6)	97 (63.8)		Reference		Reference		Reference
GA	15 (25.4)	12 (44.4)	49 (32.2)	0.132	0.44 (1.66-1.14)	0.319	0.69 (0.34-1.36)	0.282	1.58 (0.69-3.64)
AA	1 (1.7)	0	6 (4.0)	*nc*		0.675	0.38 (0.04-3.22)	*nc*	
-819(C/T)									
CC	24 (40.7)	16 (59.3)	62 (40.8)		Reference		Reference		Reference
CT	22 (37.3)	10 (37)	82 (53.9)	0.47	1.47 (0.55-3.90)	0.309	0.69 (0.36-1.35)	0.091	0.47 (0.20-1.11.)
TT	13 (22)	1 (3.7)	8 (5.3)	0.041[a]	8.67 (1.03-72.97)	0.005[b]	4.2 (1.55-11.4)	0.682	0.48 (0.06-4.16)
-592(C/A)									
CC	12 (20.3)	6 (22.2)	37 (24.3)		Reference		Reference		Reference
CA	30 (50.9)	17 (63)	83 (54.6)	1.0	0.88 (0.28-2.78)	0.847	1.11 (0.51-2.41)	0.805	1.26 (0.46-3.46)
AA	17 (28.8)	4 (14.8)	32 (21.1)	0.515	2.12 (0.49-9.2)	0.376	1.64 (0.68-3.94)	0.748	0.77 (0.20-2.97)

nc, not calculated; 95% CI, 95% confidence interval; a, *P*c = 0.123; b, *P*c = 0.015 (*P*c, *P* corrected).

Table 7.3: IL-10 expression haplotype among patients with GBS and healthy controls

IL-10 expression haplotype	GBS *n* = 152 (%)	Healthy control *n* = 152 (%)
High (frequency ≥ 10.7%)	64 (42.1)	74 (48.7)
Medium (frequency ≥ 4.7-< 10.6)	58 (38.2)	61 (40.1)
Low (frequency ≤ 2.7)	30 (19.7)	17 (11.2)

GBS, Guillain-Barré syndrome; GCC/GTA, GCC/ATA and GCC/GCA represent high frequency; GCC/GTC, GCC/GCC, GCC/ACC, GCC/ACA, GCA/GTA, GCA/GCA and GCA/ACA represent medium frequency; frequency ≤ 2.7 represent low haplotype combinations.

Genotype distribution of IL-10 polymorphisms did not differ between the subgroups of patients with mild and severe GBS or among the patients with good outcome and poor outcome (***Table 7.5***). However, the haplotype distributions of these SNPs significantly differed between mildly and severely affected patients. The high IL-10 expression (frequency ≥10.7%) haplotype combination GCC/GTA, GCC/ATA and GCC/GCA were predominantly present (48.7% vs. 26.8%) in severely affected patients with GBS compared to mild form and reached statistical significance (P = 0.008, OR = 3.22, 95% CI = 1.4-7.43; Pc = 0.024; ***Table 7.5***).

Table 7.4: Distribution of IL-10 promoter polymorphisms in *C. jejuni* serology positive or negative patients and *anti-GM1 antibody* positive and negative patients with GBS

Genotypes	*C. jejuni* sero-positive *n* = 95 (%)	*C. jejuni* sero-negative *n* = 57 (%)	*P* value	Odds ratio (95% CI)	*Anti-GM1-Ab* positive patients *n* = 58 (%)	*Anti-GM1-Ab* negative patients *n* = 94 (%)	*P* value	Odds ratio (95% CI)
-1082(G/A)								
GG	70 (73.7)	33 (57.9)		Reference	38 (65.5)	65 (69.2)		Reference
GA	25 (26.3)	20 (35.1)	0.192	1.7 (0.83-3.48)	18 (31)	27 (28.7)	0.717	0.88 (0.43-1.78)
AA	0 (0)	4 (7)	*nc*	*nc*	2 (3.5)	2 (2.1)	0.629	0.58 (0.08-4.32)
-819(C/T)								
CC	42 (44.2)	29 (50.9)		Reference	31 (53.4)	40 (42.6)		Reference
CT	33 (34.7)	23 (40.3)	1.0	1.0 (0.49-2.05)	18 (31)	38 (40.4)	0.203	1.6 (0.79-3.4)
TT	20 (21.1)	5 (8.8)	0.088	0.36 (0.12-1.07)	9 (15.5)	16 (17)	0.638	1.38 (0.54-3.53)
-592(C/A)								
CC	24 (25.3)	11 (19.3)		Reference	14 (24.1)	21 (22.3)		Reference
CA	47 (49.4)	33 (57.9)	0.405	1.53 (0.66-3.55)	28 (48.3)	52 (55.4)	0.676	1.24 (0.55-2.80)
AA	24 (25.3)	13 (22.8)	0.806	1.18 (0.44-3.16)	16 (27.6)	21 (22.3	0.815	0.88 (0.34-2.24)

C. jejuni, Campylobacter jejuni; 95% CI, 95% confidence interval; *Anti-GM1-Ab, anti-GM1 antibody;* 95% CI, 95% confidence interval.

Table 7.5: Distribution of IL-10 promoter polymorphisms and haplotype in severity of the disease and disease prognosis *in* patients with GBS

Genotypes	Disease severity				GBS disability at 6 months			
	Severely affected *n* =111 (%)	**Mildly affected *n* = 41 (%)**	***P* value**	**Odds ratio (95% CI)**	**Good outcome *n* = 96 (%)**	**Poor outcome *n* = 56 (%)**	***P* value**	**Odds ratio (95% CI)**
-1082(G/A)								
GG	77 (69.4)	26 (63.4)		Reference	70 (72.9)	33 (58.9)		Reference
GA	30 (27)	15 (36.6)	0.324	1.48 (0.69-3.18)	25 (26)	20 (35.7)	0.192	1.7 (0.83-3.48)
AA	4 (3.6)	0 (0)	*nc*	*nc*	1 (1.1)	3 (5.4)	0.11	6.37 (0.64-63.55)
-819(C/T)								
CC	51 (46)	20 (48.8)		Reference	44 (45.8)	27 (48.2)		Reference
CT	42 (37.8)	14 (34.1)	0.84	0.85 (0.38-1.89)	35 (36.5)	21 (37.5)	1.0	0.98 (0.47-2.02)
TT	18 (16.2)	7 (17.1)	1.0	0.99 (0.35-2.70)	17 (17.7)	8 (14.3)	0.637	0.77 (0.29-2.02)
-592(C/A)								
CC	23 (20.7)	12 (29.3)		Reference	24 (25)	12 (21.4)		Reference
CA	63 (56.8)	17 (41.4)	0.164	0.55 (0.21-1.24)	49 (51)	30 (53.6)	0.681	1.22 (0.53-2.8)
AA	25 (22.5)	12 (29.3)	1.0	0.92 (0.34-2.45)	23 (24)	14 (25)	0.808	1.22 (0.47-3.18)

	IL-10 expression haplotype				IL-10 expression haplotype	
	Mild form *n* = 41 (%)	**Severe form *n* = 111 (%)**	***P* value**	**Odds ratio (95% CI)**	**Independent locomotion *n* = 96 (%)**	**Unable to walk *n* = 56 (%)**
High (frequency ≥ 10.7%)	11 (26.8)	54 (48.7)			38 (39.6)	26 (46.4)
Medium (frequency ≥ 4.7-<10.6)	23 (56.1)	35 (31.5)	0.008	3.2 (1.4-7.43)	39 (40.6)	19 (33.9)
Low (frequency ≤ 2.7)	7 (17.1)	22 (19.8)	0.41	1.6 (0.53-4.5)	19 (19.8)	11 (19.7)

Patients at nadir with MRC-sumscore <40 were defined as severely affected patients and with MRC-sumscore ≥ 40 were defined as mildly affected patients; ability to walk independently at six months of follow-up was classified as good outcome (with GBS-DS of 0, 1, 2); unable to walk independently (with GBS-DS of 3, 4and 5) or death (with GBS-DS of 6) as poor outcome; GCC/GTA, GCC/ATA and GCC/GCA represent high frequency; GCC/GTC, GCC/GCC, GCC/ACC, GCC/ACA, GCA/GTA ,GCA/GCA and GCA/ACA represent medium frequency; frequency ≤ 2.7 represent low haplotype combinations.

Discussion

In this study, we investigated the association of the three common polymorphic sites in the promoter region of IL-10 gene -1082 G/A (rs1800896), -819 C/T (rs1800871) and -592 C/A (rs1800872) with the risk of developing GBS in Bangladesh. This study indicates that the homozygous -819 TT genotype is associated with the axonal variant of GBS with respect to healthy controls and high IL-10 expression haplotype combination GCC/GTA, GCC/ATA and GCC/GCA may influence disease severity.

The homozygous -819TT genotype was found to be prevalent in patients with GBS thus indicating its role in the development of the GBS. However, after Bonferroni correction this association was no longer significant thereby indicating only a probable link between -819TT genotype and GBS. One of the previous studies claimed that the -592CC and -819CC genotypes are significantly predominant in Norwegian patients with GBS compared to controls.[15] On the other hand, Geleijns et al. did not find any such association between Dutch patients and healthy controls.[19] These incoherent findings might be a result of the ethnic variation among the various populations involved in the association studies. This is a very important factor to consider when studying the link between genetic makeup and disease susceptibility. Furthermore, we found that the -819TT genotype was predominant in the axonal variant compared to the demyelinating form of GBS and/or the healthy individuals indicating a high impact of this genotype with the axonal form. Previous studies also supported our findings, suggesting a correlation between increased IL-10 –secreting blood mononuclear cells and axonal damage.[1,20] In addition, a strong influence of genetic factors on the production of IL-10 was also described by Kasamatsu et al.[18]

We did not find any significant association between IL-10 polymorphisms and anti-ganglioside antibody positivity that confirms/ the similar findings of Geleijns et al.[19] However, Press et al. showed inconsistency with our findings with high levels of pathogenic autoantibodies with increased IL-10 –secreting blood mononuclear cells.[1] Our study also supports the previous findings of Myhr et al. and Geleijns et al. that reports no associations of promoter polymorphisms with recent *C. jejuni* infections.[15,19]

Our study findings reveal a significant association of high (GCC/GTA, GCC/ATA and GCC/GCA) IL-10 expression haplotype with disease severity. These results were inconsistent with previous findings[15,19] where no influence of high IL-10 expression haplotype on GBS severity was reported. We previously described that the -819TT genotypes were prevalent in axonal variant of GBS which is the most severe form of GBS. The high IL-10 expression haplotype combinations may somehow influence severe muscle weakness of patients since IL-10 has proinflammatory functions via activating B cells and inhibiting T cell apoptosis. Moreover, the polymorphisms of IL-10 could affect the transcription, translation and secretion of IL-10.[29]

One of our limitations is that we did not analyze two additional IL-10 polymorphisms at -1082 G/T (rs3024491) and -1082 G/T (rs3024491).Nevertheless, the production of IL-10 is mainly/controlled by the three studied polymorphisms of our research.

In conclusion, the IL-10 gene promoter polymorphisms -1082 G/A, -819 C/T and -592 C/A are not associated with susceptibility to GBS. However, homozygous -819 TT genotypes may have an impact on the axonal variant of GBS and high IL-10 expression haplotype combinations (GCC/GTA, GCC/ATA and GCC/GCA) may play a crucial role in disease severity. Large-scale studies using a well-designed cohort with populations of different ethnicities are required to confirm this relation and to get a clear understanding of the underlying genetic makeup concerning GBS pathogenesis.

Declarations of interest

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Chapter 8

MMP9 -1562 C/T polymorphism in severity of GBS

Association of matrix metalloproteinase-9 polymorphism with severity of Guillain-Barré syndrome

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Abstract

Guillain-Barré syndrome (GBS) is an immune-mediated neurological disorder with a multifaceted nature. Infectious agents and immune-response genetic host factors may contribute to the development of GBS. The matrix metalloproteinase-9 (MMP-9), an enzyme is upregulated by pro-inflammatory cytokines and might play an important role in the pathogenesis of GBS. This study investigated the association of a single nucleotide polymorphism (-1562C/T, rs3918242) in the *MMP9* gene with the susceptibility and severity of GBS in Bangladesh. The allele and genotype distributions of the *MMP9* polymorphism were not significantly different between 303 patients with GBS and 303 healthy controls. Serum concentrations of MMP-9 were significantly elevated in patients with GBS compared to healthy controls ($P \leq 0.0001$). No significant association of MMP-9 (-1562C/T) polymorphism was observed with disease prognosis. The frequencies of the *MMP9* -1562 CT genotype and T allele ($P = 0.01$, OR = 2.28, 95% CI = 1.22-4.22; $Pc = 0.03$ and $P = 0.012$, OR = 2.0, 95% CI = 1.14-3.38; $Pc = 0.024$, respectively) were significantly increased in patients with severe form of GBS, indicates the *MMP9* polymorphism plays a role in the disease severity of GBS.

Keywords: Matrix metalloproteinase-9; Polymorphism; Genotype; Allele.

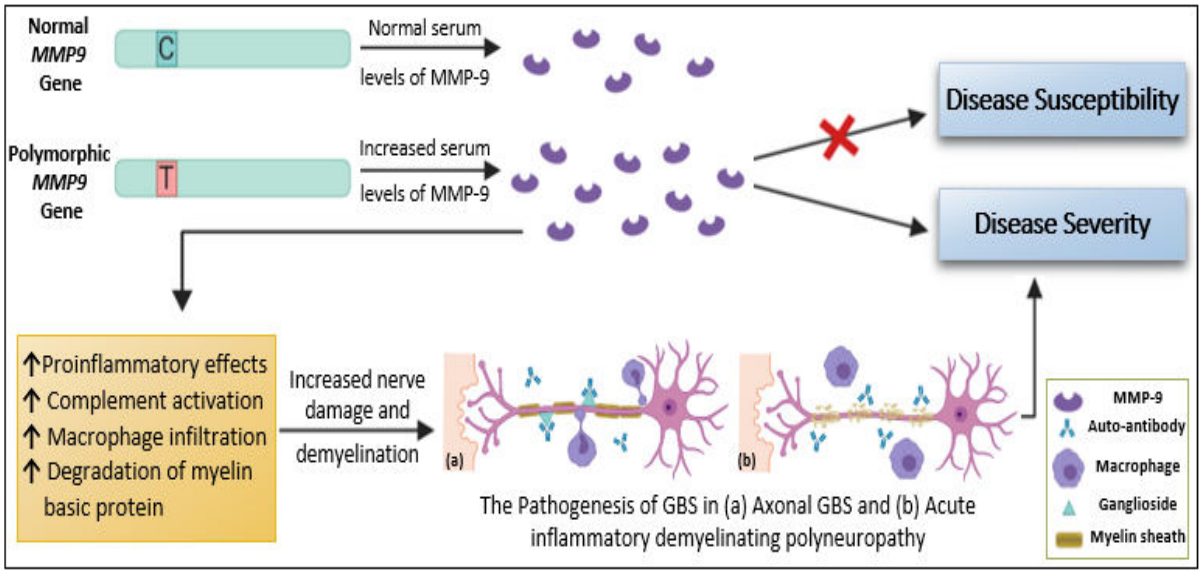

Figure 8.1 Graphical abstract

Introduction

Guillain-Barré syndrome (GBS) is a progressive, immune-mediated disorder of the peripheral nervous system. It can be characterized into a wide spectrum of subtypes according to clinical, electrophysiological and etiological features.[1–5] Infection by *Campylobacter jejuni* (*C. jejuni)* has been identified as the predominant cause for triggering GBS.[5] *C. jejuni*-triggered GBS involves the induction of cross-reactive antibodies as a result of the molecular mimicry between *Campylobacter* lipo-oligosaccharides (LOS) and host nerve gangliosides.[6–8] In addition to pathogen-derived factors, immune-response genetic host factors may also play an important role in the pathogenesis of GBS.[9]

Matrix metalloproteinase-9 (MMP-9) is an inflammatory mediator that regulates the composition of the extracellular matrix by degrading components such as collagens, proteoglycans and elastins.[10] MMP-9 is activated by pro-inflammatory cytokines or peptides and participates in recruitments of macrophages and infiltration of the blood-nerve barrier.[9] In addition, higher expression of MMP-9 along with TNF-a and IL-1b during disease progression and subsequent down regulation of MMP-9 with proinflammatory cytokines in recovery in patients with GBS [11,12] clearly indicate the involvement of MMP-9 in disease pathogenesis of GBS. A single nucleotide polymorphism (SNP) in the promoter region of the *MMP9* gene, in which the cytosine at the -1562 position is replaced by a thymine base, prevents a repressor protein to bind to the promoter region, and thus increases the promoter activity.[10] In a previous study, in which most patients had the demyelinating form of GBS, an association was found between the -1562 C/T *MMP9* polymorphism and the severity of GBS but no relation with susceptibility was established.[9]

Therefore, further in-depth study of the role of the *MMP-9* (-1562 C/T) polymorphism is necessary to determine whether this polymorphism is associated with GBS susceptibility or severity, and may potentially help to identify suitable treatments for GBS. Thus, we investigated the association of the *MMP9* -1562 C/T polymorphism with GBS susceptibility or severity in the population of Bangladesh, where the *Campylobacter jejuni* triggered axonal subtype is common, most patients are severely affected and the mortality rate is high.[13,14]

Material and methods

Study subjects

Blood samples were collected from 303 patients with GBS neither received IVIg nor plasma exchange therapy (males/females, 208/95; median age, 29-years-old [interquartile range, 17-42]; ***Table 8.1***) admitted at Dhaka Medical College and Hospital (DMCH) and 303 genetically unrelated healthy individuals (males/females, 204/99; median age, 34-years-old [interquartile range, 28-46]). The individuals in the control group were ethnically matched and had no history of neurological disease, recent infection or chronic medical illnesses. GBS was diagnosed using the National Institute of Neurological Disorders and Stroke (NINDS) criteria [15] and clinical and electrophysiological data were obtained for all patients (***Table 8.1***). Written informed consent was obtained from all participants prior to data collection, clinical examination and specimen collection. This study was approved by the Institutional Review Board (IRB) and the ethics committees at the icddr, b, Dhaka, Bangladesh.

Clinical and serological characteristics

Most patients with GBS had a history of antecedent illnesses 75% (227/303); with recent diarrhea 43% (129/303); respiratory infection 15% (45/303); fever 8% (25/303); other illnesses 9% (28/303) and 25% (76/303) had no history of infection. Serum antibodies against *C. jejuni* and GM1 ganglioside were quantified using enzyme-linked immunosorbent assay (ELISA). [16,17]

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.[17,18]
Serum levels of total MMP-9 in all the study participants were determined in duplicate by ELISA using commercially available kits (R & D Systems) as per the manufacturer's instructions and results were expressed as nanograms of MMP-9 per milliliter (ng/mL).

Electrophysiological studies were conducted for 82% (247/303) of the patients with GBS; 59% (146/247) of these patients had an axonal subtype, including acute motor axonal

Table 8.1: Demographic and clinical characteristics of the patients with GBS

Characteristic		Number of patients, *n* = 303 (%)
Sex	Male/Female	208/95
Age	Median (IQR, full range)	29 (17-42)
Preceding illness, *n* = 303	Diarrhea	129/303 (43)
	Respiratory tract infections	45/303 (15)
	Fever	25/303 (8)
	Other	28/303 (9)
	None/Unknown	76/303 (25)
Electrophysiological classification, *n* = 247		
	Axonal (AMAN & AMSAN)	146/247 (59)
	AMAN	139/247 (56)
	AMSAN	7/247 (3)
	Demyelinating	68/247 (27)
	Unclassified	33/247 (13)
MRC sum score (at entry), *n* = 303		
	Severely affected patients	232/303 (77)
	Mildly affected patients	71/303 (23)
Serological characteristics, *n* = 303		
	Anti-GM1-Ab seropositive	118/303 (39)
	C. jejuni seropositive	186/303 (61)
Disease prognosis at 6 months, *n* = 303		
	Good outcome	209/303 (69)
	Poor outcome	94/303 (31)

GBS, Guillain-Barré syndrome; IQR, interquartile range; Axonal, acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN); Demyelinating, acute inflammatory demyelinating polyradiculoneuropathy (AIDP); MRC, Medical Research Council; Ab, antibody; *C. jejuni, Campylobacter jejuni.*

neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN); 27% (68/247) had a demyelinating type (acute inflammatory demyelinating polyradiculoneuropathy; AIDP); and 13% (33/247) were unclassified with inexcitable nerves

or equivocal findings (***Table 8.1***).[19] Severity of the disease was assessed using Medical Research Council (MRC) sum scale (ranging from 0-60) at nadir (maximum level of weakness in GBS patients).[20] GBS patients at nadir with MRC sumscore < 40 were defined as severely affected patients and with MRC sumscore ≥ 40 were defined as mildly affected patients.[9,21] The outcome of the disease was measured using the GBS disability score (GBS-DS) after six months of follow-up.[22] According to GBS-DS patients with their ability to walk independently (with GBS-DS of 0, 1 or 2) at six months of follow-up were classified as good outcome and patients who were unable to walk independently (with GBS-DS of 3, 4 or 5) as poor outcome.[9]

DNA isolation and detection of polymorphisms

Whole blood samples from 606 study subjects were collected in lithium heparin-coated tubes. The QIAamp® DNA Blood Midi Kit (100) (Qiagen, Hilden, Germany) was used to isolate genomic DNA according to the manufacturer's instructions and stored at -80 °C. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) was performed using restriction endonuclease *SphI to* detect the MMP-9 (1562 C/T, rs3918242) gene polymorphisms. PCR analysis was performed using master mix of 25 µL containing 10 ng of genomic DNA, 10 pmol of each primer (MMP-9 specific forward primer 5'-AAATGGCAGAGCCGGGAT-3' and reverse primer 3'-ACCAGCAGCCTCCCTCACT-5'), 0.1 mM dNTPs (Promega), 1 U of GoTaq® Flexi DNA Polymerase (Promega), 5× Green GoTaq® Flexi Buffer, 25 mM MgCl2 and Milli-Q water. After digestions, the products were visualized on 2% agarose gels using a Molecular Imager® Gel Doc™ XR+system.

Statistical analysis

The genotype distribution among healthy controls was within Hardy-Weinberg equilibrium. Genotype and allele frequencies were analyzed using the chi-square or Fisher's exact test with Yates' continuity correction. The data of serum levels of MMP-9 were expressed as median with interquartile range (IQR). The differences in the serum concentrations of MMP-9 (ng/mL) between healthy controls and GBS or subgroups of GBS

were analyzed using the Mann–Whitney U test and one way ANOVA. A *P* value of < 0.05 was considered statistically significant. Odds ratios (ORs) were calculated using a dominant model due to the low frequency of polymorphic homozygous variants. Genotype and allele frequencies were estimated by simple gene counting and processed using Microsoft® Excel 2007. The Bonferroni method was applied to correct *P* values for multiple comparisons, whereby the *P* value was multiplied with the number of comparisons and denoted as *P*c (*P*c, *P* corrected). Statistical analyses were conducted using GraphPad Prism (version 5.01, GraphPad Software, Inc. La Jolla, CA, USA) and SPSS (version 16.0, Company, Chicago, IL, USA).

Results

Association of MMP9 (-1562 C/T) promoter polymorphism with disease susceptibility and subtypes of GBS

We determined the genotype and allele frequencies of the *MMP9* promoter polymorphism among patients with GBS and healthy controls and no significant association was found (*P* = 0.665 and *P* = 0.479, respectively; ***Table 8.2***). The C-allele was prevalent in both patients with GBS (80%) and healthy controls (78%). No significant association was observed between MMP9 polymorphism and susceptibility to any subtype of GBS (***Table 8.3***).

Table 8.2: Distribution of *MMP9* (-1562 C/T) genotypes and alleles in patients with GBS and healthy controls and their associations with GBS susceptibility

Genotype/allele	Healthy controls *n* = 303 (%)	GBS patients *n* = 303 (%)	*P* value	OR (95% CI)
CC	181 (59.7)	189 (62.4)		Reference
CT	112 (37)	107 (35.3)		1.09 (0.78-1.52)
TT	10 (3.3)	7 (2.3)	0.665	1.49 (0.56-4.04)
C allele	474 (78.2)	485 (80)		Reference
T allele	132 (21.8)	121 (20)	0.479	0.89 (0.68- 1.18)

GBS, Guillain-Barré syndrome; OR, odds ratio; 95% CI, 95% confidence interval.

Serum levels of MMP-9 were significantly elevated in patients with GBS compared to healthy controls (median, 203 ng/mL, IQR, (145-266) vs. median, 96 ng/mL, IQR, (66-132), $P \leq 0.0001$; ***Figure 8.2A***). Analysis based on the subtype of GBS (axonal [AMAN & AMSAN] vs. controls; demyelinating vs. controls; (axonal [AMAN & AMSAN] vs. demyelinating) revealed significant increased concentrations of MMP-9 in axonal and demyelinating subtypes compared to healthy controls (median, 210 ng/mL, IQR, (147-267) vs. median, 96 ng/mL, IQR, (66-132), $P \leq 0.0001$ and median, 188 ng/mL, IQR, (145-264) vs. median, 96 ng/mL, IQR, (66-132), $P \leq 0.0001$; ***Figure 8.2B***).

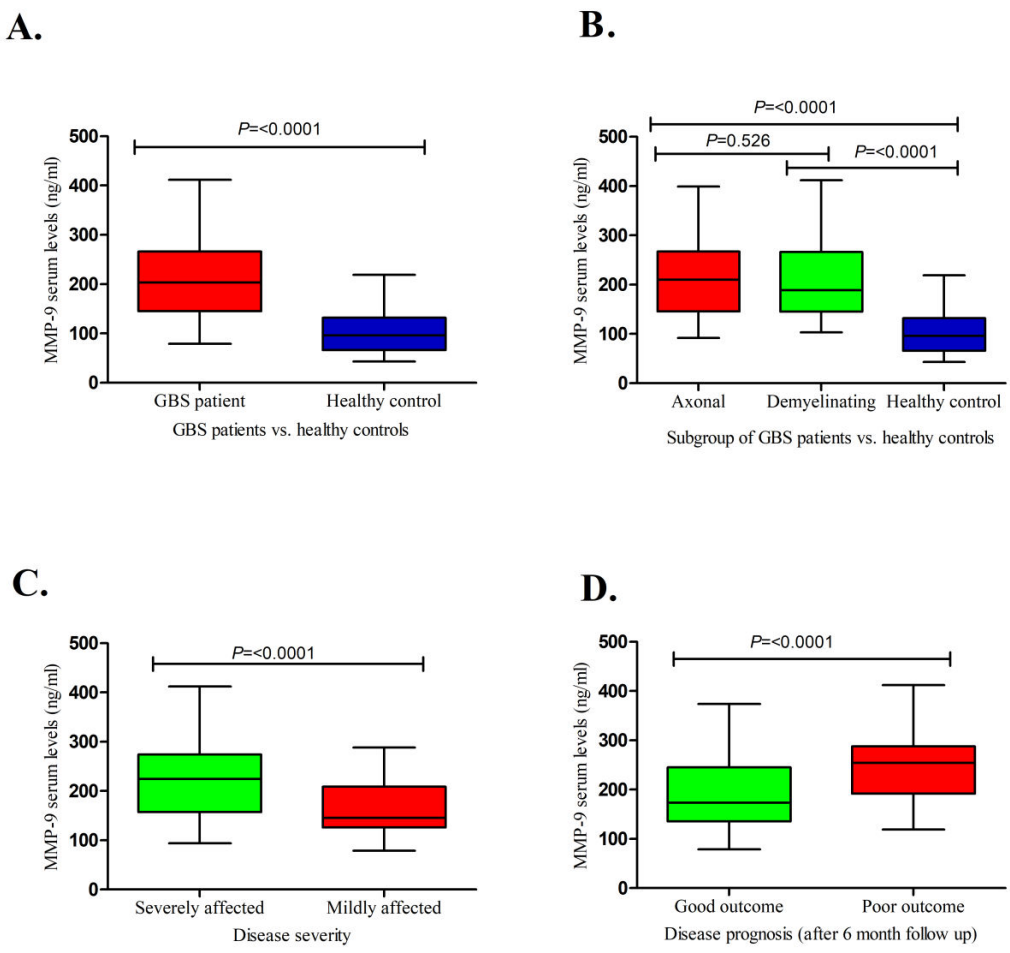

Figure 8.2 Serum levels of MMP-9 (ng/mL) in study participants. Boxes represent lower and upper quartile and median. A. GBS patients vs. healthy controls. B. Axonal variant vs. healthy controls; Demyelinating subtype vs. healthy controls; Axonal vs. demyelinating subtype. C. Severely affected patients vs. mildly affected patients. D. Patients with good outcome vs. poor outcome.

Table 8.3: Distribution of MMP-9 (-1562 C/T) genotypes and alleles among axonal (AMAN & AMSAN) and demyelinating (AIDP) cases of GBS and healthy controls

MMP-9	Subtype				(a) and (c)		(b) and (c)		(a) and (b)
Genotype /allele	**Axonal variant *n* = 146 (%) (a)**	**Demyelinating type *n* = 68 (%) (b)**	**Healthy control *n* = 303 (%) (c)**	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**
CC	91 (62.3)	41 (60.3)	181 (59.7)		Reference		Reference		Reference
CT	53 (36.3)	26 (38.2)	112 (37)		1.1 (0.7-1.6)		1.0 (0.6-1.7)		1.1 (0.6-2.0)
TT	2 (1.4)	1 (1.5)	10 (3.3)	0.47	2.5 (0.5-11.7)	0.72	2.3 (0.3-18.2)	0.96	1.0 (0.1-12.6)
C allele	235 (80.5)	108 (79.2)	474 (78.2)		Reference		Reference		Reference
T allele	57 (19.5)	28 (22.8)	132 (21.8)	0.48	1.1 (0.8-1.6)	0.82	1.1 (0.7-1.7)	0.80	1.0 (0.6-1.8)

Axonal variant, acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN); Demyelinating type, acute inflammatory demyelinating polyradiculoneuropathy (AIDP); OR, odds ratio; 95% CI, 95% confidence interval.

The frequency distribution of the CC, CT, and TT genotypes was 62.3%, 36.3%, 1.4% for axonal cases and 60.3%, 38.2% and 1.5% for demyelinating cases, respectively (***Table 8.3***).

Association of MMP9 (-1562 C/T) promoter polymorphism with clinical and serological subgroups of GBS

We assessed the contribution of the candidate gene in various clinical and serological subgroups of GBS based on muscle weakness, antecedent *C. jejuni* infection, the presence of anti-GM1 antibodies (Abs) and disease prognosis (***Tables 8.4, 8.5 and 8.6***). Most of the patients with GBS were severely affected based on MRC sum score and 23% were mildly affected patients (77%, 232/303 vs. 23%, 71/303; ***Table 8.1***).

Table 8.4: Associations between *MMP9* (-1562 C/T) genotypes and alleles and disease severity in GBS

Genotype/allele	Severely affected patients *n* = 232 (%)	Mildly affected patients *n* = 71(%)	*P* value	Corrected *P* value (*Pc*)	Odds ratio (95% CI)
CC	135 (58.2)	54 (76.1)			Reference
CT	91 (39.2)	16 (22.5)	0.01	0.03	2.28 (1.22-4.22)
C Allele	361 (77.8)	124 (87.3)			Reference
T Allele	103 (22.2)	18 (12.7)	0.012	0.024	2.0 (1.14-3.38)

OR, odds ratio; 95% CI, 95% confidence interval; patients at nadir with MRC-sumscore <40 were defined as severely affected patients and with MRC-sumscore ≥ 40 were defined as mildly affected patients.

Serum levels of MMP-9 in severely affected patients with GBS were significantly elevated compared to mild form (median, 225 ng/mL, IQR, (158-273) vs. median, 145 ng/mL, IQR, (126-206), $P \leq 0.0001$; ***Figure 8.2C***). Genetic analysis revealed that *MMP9* (-1562) heterozygous genotype (CT) and T allele were more frequent in patients with severe form of GBS compared to mild form of the disease ($P = 0.01$, OR = 2.28, 95% CI = 1.22-4.22; *P*c =

0.03 and *P* = 0.012, OR = 2.0, 95% CI = 1.14-3.38; *P*c = 0.024, respectively; ***Table 8.4***). There was no significant difference in the distribution of *MMP9* (-1562) alleles and genotypes among *C. jejuni* seropositive and seronegative patients (***Table 8.5***). The TT genotype was less frequent among *C. jejuni* seropositive patients with GBS than *C. jejuni* seronegative patients and healthy controls (1.6% vs. 3.4% and 1.6% vs. 3.3%; ***Tables 8.5 & 8.6***). The serum levels of MMP-9 were significantly increased in patients with poor outcome compared to good outcome (median, 254 ng/mL, IQR, (193-287) vs. median, 173 ng/mL, IQR, (136-245), $P \leq 0.0001$; ***Figure 8.2D***). TT genotypes were predominant in patients with poor prognosis compared to good prognosis at 6 months after disease onset (4.2% vs. 1.4%; ***Table 8.5***) but association was not statistically significant. No significant association was evident between other genotypes and disease prognosi safter 6 months of follow-up (*P* = 0.076; ***Table 8.5***).

Table 8.5: Associations between *MMP9* (-1562 C/T) polymorphisms and *C. jejuni* seropositivity, anti-GM1 antibody positivity and disease outcome in patients with GBS

Genotype/ Allele	Association variables		*P*-value	Odds ratio (95% CI)
	C. jejuni sero-positive patients *n* =186 (%)	*C. jejuni* sero-negative patients *n* =117 (%)		
CC	116 (62.4)	73 (62.4)		Reference
CT	67 (36.0)	40 (34.2)		1.05 (0.65-1.72)
TT	3 (1.6)	4 (3.4)	0.582	0.47 (0.10-2.17)
	Anti-GM1 Ab-positive patients *n* = 118 (%)	*Anti-GM1 Ab*-negative patients *n* = 185 (%)		
CC	72 (61.0)	116 (62.7)		Reference
CT	45 (38.1)	63 (34.1)		1.15 (0.71-1.86)
TT	1 (0.9)	6 (3.2)	0.379	0.27 (0.03-2.28)
	Good outcome *n* = 209 (%)	Poor outcome *n* = 94 (%)		
CC	138 (66.0)	51(54.3)		Reference
CT	68 (32.5)	39(41.5)		1.55 (0.93-2.58)
TT	3 (1.4)	4 (4.2)	0.076	0.28 (0.06-1.28)

OR, odds ratio; 95% CI, 95% confidence interval; *C. jejuni* seropositive, *Campylobacter jejuni* seropositive; *C. jejuni* seronegative, *Campylobacter jejuni* seronegative; Anti-GM1 Ab, Anti-GM1 antibody. Ability to walk independently at six months of follow-up was classified as good outcome (with GBS-DS of 0, 1 and 2); unable to walk independently (with GBS-DS of 3, 4 and 5) or death (with GBS-DS of 6) as poor outcome.

Table 8.6: Genotype and allele distribution of the *MMP9* gene among healthy controls as well as patients with axonal and demyelinating GBS and *C. jejuni*-seropositive and -seronegative GBS

Genotype and allele distribution	Controls *n* = 303 (%)	*C. jejuni* sero +ve GBS patients *n* = 186 (%)	Axonal *n* = 146		Demyelinating *n* = 68	
			C. jejuni sero +ve *n* = 108 (%)	*C. jejuni* sero –ve *n* = 38 (%)	*C. jejuni* sero +ve *n* = 26 (%)	*C. jejuni* sero –ve *n* = 42 (%)
CC	181 (59.7)	116 (62.4)	69 (63.9)	23 (60.5)	18 (69.2)	23 (54.8)
CT	112 (37.0)	67 (36)	37 (34.2)	15 (39.5)	8 (30.8)	18 (42.8)
TT	10 (3.3)	3 (1.6)	2 (1.9)	0 (0)	0 (0)	1 (2.4)
C allele	474 (78.2)	299 (80.4)	175 (81.0)	61 (80.3)	44 (84.6)	64 (76.2)
T allele	132 (21.8)	73 (19.6)	41 (19.0)	15 (19.7)	8 (15.4)	20 (23.8)

GBS, Guillain-Barré syndrome; Ve, positive or negative*; C. jejuni* sero +ve, *Campylobacter jejuni*-seropositive; *C. jejuni* sero –ve, *Campylobacter jejuni* seronegative; Axonal variant, acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN); Demyelinating type, acute inflammatory demyelinating polyradiculoneuropathy (AIDP).

Discussion

This study demonstrates that *MMP9* (-1562C/T) promoter polymorphism is a candidate risk factor for disease severity, but is not associated with susceptibility to GBS among the Bangladeshi population. We did not observe any associations between the *MMP9* (-1562C/T) promoter polymorphism and any clinical or serological subgroups, including GBS subtypes, *C. jejuni* infection and GM1 auto-antibodies, or disease outcome in GBS. However, serum levels of MMP-9 were significantly elevated in patients with GBS compared to healthy controls.

To the best of our knowledge, this study involved the largest cohort of GBS patients to have ever been studied. Elevated levels of MMP-9 (ng/mL) in serum among patients with GBS or subtypes of GBS with respect to healthy individuals have been reported previously.[11,12,23] This study also confirms the previous findings of the association of high serum concentration of MMP-9 with GBS compared with controls. However, the allele and genotype distribution of the *MMP9* (-1562C/T) polymorphism among healthy controls and patients with the axonal subtypes and demyelinating subtypes of GBS were not significantly different, in accordance with a previous study of Dutch patients.[9]

MMPs are zinc metalloproteases involved in the degradation of collagen in the extracellular matrix, and thus play crucial roles during tissue remodeling and repair during development and inflammation. Our finding for disease severity is in agreement with the study conducted in the Netherlands, which reported a higher frequency of T allele and CT genotype among patients with severe form compared to mild form of GBS.[9] This might be a result of the upregulation of the *MMP9* gene rendered by the T allele.[10] MMP-9 may also degrade myelin basic protein and thereby aggravate demyelination in GBS. Increased serum levels of MMP-9 have previously been associated with severe GBS.[10–12] The current study finding reaffirms the previous report and strengthens the evidence of increased serum concentration of MMP-9 in GBS disease severity regardless of geographical variation. In addition, the CC genotype has been reported to decrease transcriptional activity of the *MMP9* promoter [10] and the higher frequency of T allele and CT genotype in disease severity is consistent with the Dutch study.[9]

A previous report demonstrated an association between *MMP-9* expressions and *C. jejuni* infection in mice.[24] Our study could not support these findings, most patients in this study had an antecedent *C. jejuni* infection and a larger proportion of patients with the axonal subtype were *C. jejuni* seropositive compared to patients with the demyelinating subtype. The frequency of the TT genotype and the T allele was lower in patients positive for GM1 auto-antibodies.

Overall, this study indicates that the -1562C/T *MMP9* promoter polymorphism confers no risk for GBS susceptibility but intensifies the severity of disease in the population of Bangladesh. The high levels of MMP-9 in severe form of GBS and poor disease prognosis indicate the importance of MMP-9 in GBS disease pathogenesis. Further research should be warranted to determine the role of the -1562C/T *MMP9* polymorphism with a large cohort of GBS such as International GBS outcome study (IGOS). Moreover, the combined effects of the -1562C/T *MMP9* polymorphism and other polymorphisms (e.g. in genes encoding TNF-α ,TLR-4) that have previously been associated with GBS pathogenesis in Bangladesh [25,26] need to be studied. Future research on the involvement of MMP-9 and the pathways downstream of MMP-9 will help to make progress towards disentangling the enigma of GBS.

Declarations of interest

None

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Chapter 9

General Discussion

General Discussion

Following the eradication of poliomyelitis in low-income countries, Guillain-Barré syndrome (GBS) has become the emerging threat, with a dramatic onset and catastrophic nature. Widespread exposure to infection, poor nutrition, and the natural resemblance between microbial agents and host nerve gangliosides, coupled with immune-response host genetics that convert the homeostatic immune system to an aberrant condition, ultimately trigger the severe and often tragic pathology of GBS. However, only a subset of individuals develops GBS after *C. jejuni* infection (1 in 1000-5000 people),[1–3] indicating that molecular mimicry alone is insufficient to trigger the disease. Individuals of different ethnicities exhibit varied responses to infectious agents: the genetic variations observed in different ethnic groups can alter the expression of molecules and control the physiological response to infection, and thereby play a key role in the immune response and susceptibility of the host. Polymorphisms within these host immune-response genes are one of the strong candidates underlying susceptibility to GBS.[4] Considering the role of host-pathogen interactions in GBS, and the current need to identify biomarkers and establish their pathophysiological roles in GBS in various regions, this study aimed to identify the contribution of several immune-response genetic host factors to the pathogenesis of GBS in Bangladesh.

The highly polymorphic *HLA-DQB*1 allele complex, SNPs in *NOD*, immunoglobulin G *FcγRs* and *IL-10,* and *MMP9-1562C/T* promoter polymorphisms were selected to study on the basis of their roles in the central immune system. Allelic variation in such genes may alter the expression of the corresponding proteins, and consequently, their functions. The majority of publications concerning these genetic host factors and their associations with the pathogenesis of GBS reported on cohorts of patients from high-income countries (***Tables 1-3***). However, in Bangladesh, the incidence and mortality rate of GBS are higher than in any other part of the world.[5–7] Thus, the current study was designed to use one of the largest cohorts of patients with GBS in the world, with the patients suffering mostly from a *C. jejuni*-associated axonal variant of GBS.[5]

Chapters 1 and ***2*** of this thesis provided a general introduction to the pathogenesis of GBS, and then summarized the current state of knowledge regarding the historical background of GBS. The literature review section **(*Chapter 2*)** included reflections from the

initial discovery of the syndrome by Jean-Baptiste Octave Landry (1859), Georges Guillain, Jean Alexandre Barré, and Andre Strohl (1916), and then moved through the timeline of the epidemiology, clinical manifestations and pathogenesis of GBS. Current knowledge on the clinical epidemiology, preceding infections and antibody responses of patients with GBS in Bangladesh was also summarized to emphasize the rationale and objectives of this thesis. The current study describes the contribution of polymorphisms in host immune-response factors and assesses their relationship with the pathogenesis of GBS in Bangladesh. The following sections of the final chapter (***Chapter 9)*** of this thesis will discuss the study cohort (***Chapter 3)*** and the major findings of the studies (***Chapters 4-8).***

Study Population

The genetic studies of this thesis were conducted in one of the largest and well-documented cohorts of patients with GBS in the world,[8] and a group of healthy individuals were included as controls ***(Chapter 3).*** Patients with GBS and healthy controls were enrolled in the study on the basis of exclusion and inclusion criteria. Selection of 303 patients with GBS from Dhaka Medical College and Hospital (DMCH) was based on the National Institute of Neurological Disorders and Stroke (NINDS) criteria [9] along with detailed documentation regarding clinical and electrophysiological information. Serology for recent infection with *C. jejuni* and the presence of anti-ganglioside antibodies were well documented.[5,10,11] Medical Research Council (MRC) sum score (ranging from 0-60) is the basis for defining the severity of the disease.[12] Patients with a MRC sum score <40 at nadir were defined as 'severely affected', while patients with an MRC sum score of 40-60 at nadir were defined as 'mildly affected'.[13] Patients with a good outcome (able to walk independently) at the six-month follow-up had a GBS-DS score of 0, 1 or 2, while patients with a poor outcome (unable to walk independently or death) had a GBS-DS score of 3, 4, 5 or 6.[14]

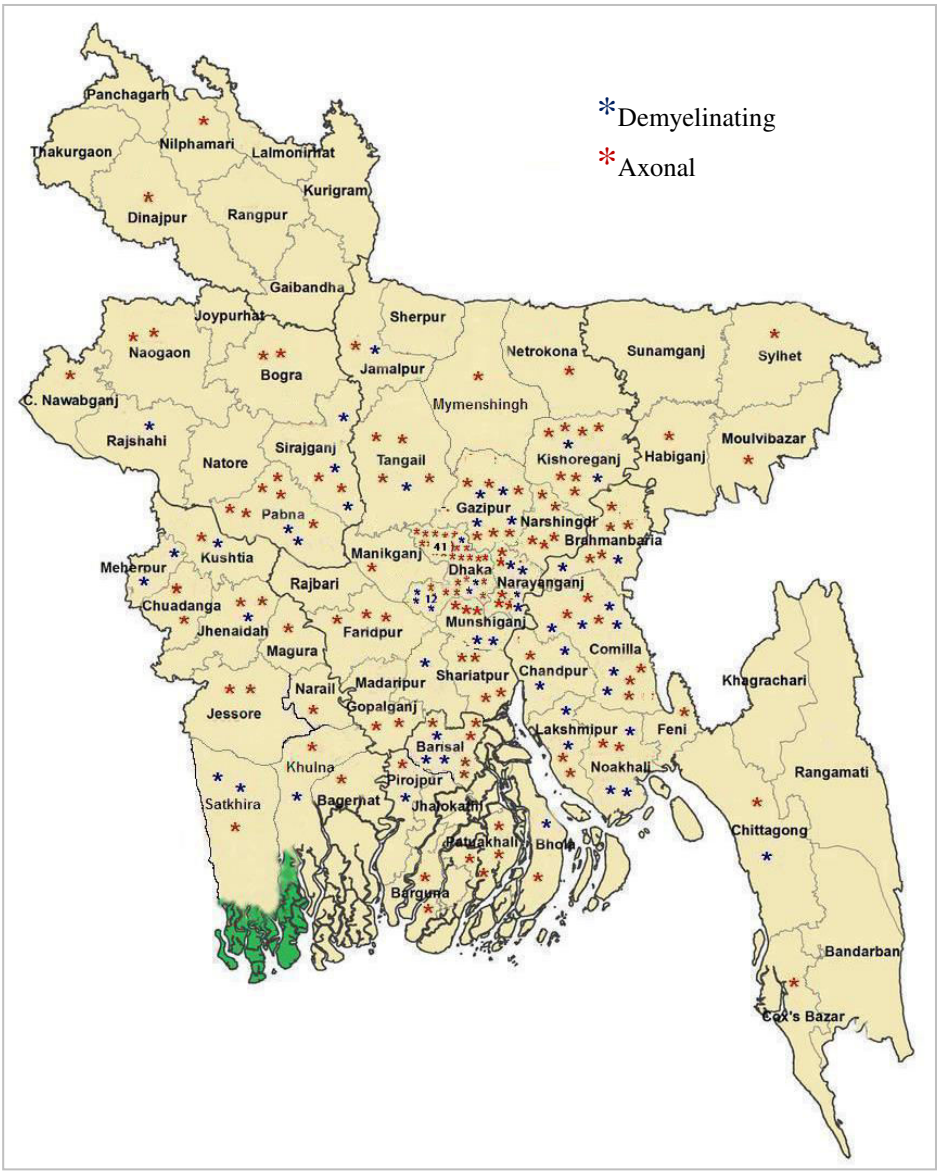

Figure 9.1 Patients from different parts of Bangladesh with the demyelinating (27%) and axonal (59%) variant of GBS.

Three hundred and three individuals from a well-defined healthy Bangladeshi population participated in these population-based genetic association studies; these control individuals did not have any history of GBS or any other neurological disorders. Healthy individuals were also free from recent antecedent history of infections, major surgery, and other serious comorbid conditions. The serological and electrophysiological studies described in ***Chapter 3*** confirmed the presence of recent *C. jejuni* infection-triggered axonal variants of GBS with high titers of anti-GM1 and anti-GD1a antibodies (***Figures 9.1 & 9.2***). The majority of the

patients was young adults (71%), presented with a severe form of GBS (77%) at entry to the study, and came from rural areas of Bangladesh (72%).

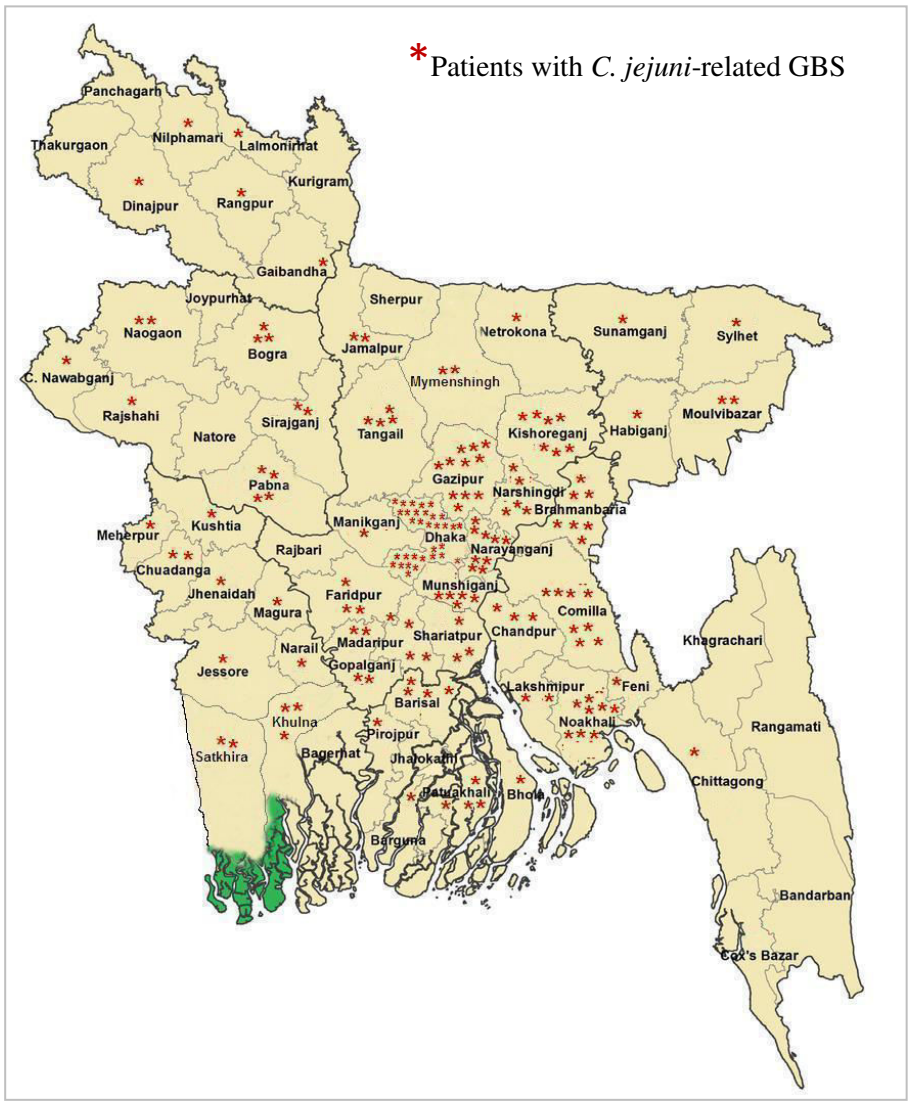

Figure 9.2 Patients with *C. jejuni*-associated GBS (61%) from Bangladesh.

Associations between the alleles or genotypes and susceptibility to GBS and subgroups of GBS were assessed using Fisher's exact test with Yates' continuity correction and logistic regression analysis ***(Chapters 4-8).*** Estimated *P*-values, odds ratios (ORs), and 95% confidence intervals (CIs) were used to investigate the associations between the candidate gene polymorphisms and the risk of developing GBS. The Bonferroni method was used to correct the *P*-values when appropriate. Pairwise linkage disequilibrium (LD) was analyzed based on D´statistics, and haplotype patterns and frequencies were estimated from genotypic

data, and their associations with GBS susceptibility and the clinical and serological subgroups were assessed using logistic regression analysis.

Chapters 4-8 describe the major findings regarding the associations of the highly polymorphic *HLA-DQB*1 allele complex, SNPs in the *NOD*, immunoglobulin G *FcγRs*, and *IL-10* genes, and *MMP9-1562C/T* promoter polymorphisms with disease susceptibility and severity, and clinical or serological subgroups of GBS. Corresponding discussions are included in individual chapters. The chapters also describe the limitations of the studies and suggestions for future aspects.

Human leukocyte antigen-DQB1 polymorphisms and haplotype patterns in GBS (Chapter 4)

In ***Chapter 4*** we aimed to determine whether polymorphisms and the haplotype structures of the *HLA-DQB1* gene are related to the autoimmune response to infection and affect the development of GBS. The *HLA-DQB1* gene is a strong candidate gene for regulation as well as identification of self and non-self-antigens in the immune system.[15] Associations between HLA complex genes and the risk of developing GBS have been reported among populations with different genetic backgrounds, but the findings were inconclusive (***Table 1***).[3,16–21] Our study described the disassociation of *DQB1* alleles or haplotype patterns and susceptibility to GBS, indicating that *DQB1* alleles and haplotype patterns have no influence on the risk of disease development or the clinical and serological subgroups of GBS in Bangladesh. Our population represents 136 patterns for the 13 *HLA-DQB1* alleles, with 10 predominant haplotype variations (haplotypes 1-10; frequency > 4%; representing 64% of variation; ***Figure 4.1, Chapter 4***). Among the predominant variations, haplotype 9 (*HLA-DQB1**0601-*0303) was less frequent among patients with GBS compared to healthy controls. LD analysis indicated the *DQB1**0601 and *0303 alleles exert a reciprocal effect towards the development of GBS in the Bangladeshi population. A number of candidate genes were expected to be involved with very small effects on disease severity, outcome, and clinical or serological features. Subgroup analysis was performed to detect these minor effects on disease pathogenesis. The *DQB1**0303 alleles were significantly associated with the severe form of GBS, based on MRC sum score, compared with the mild form of GBS, before correcting for

multiple comparisons. This implied that *HLA-DQB1* polymorphisms may possibly influence disease severity and the extent of the inflammatory response at the peripheral nerves. A Dutch study reported no association between *HLA-DQB1* alleles and disease severity, although the *HLA-DRB1**01 allele was associated with the need for mechanical ventilation in patients with GBS.[20]

Rees et al. reported that the *DQB1*03* allele was significantly associated with *C. jejuni*-positive patients with GBS compared with *C. jejuni*-negative patients.[16] Our study did not support these findings: a decreased frequency of the *DQB1*0303* and **0601* alleles and a slightly higher frequency of the **0502* alleles were found in patients who were seropositive for *C. jejuni* compared with healthy controls. However, our findings were consistent with other studies on Asian and Dutch populations with GBS (***Table 9.1***).[17,18,20,22] Local evolutionary pressure among infectious agents in different ethnic populations may be one of the reasons for this discrepancy. There was also no significant association between *HLA-DQB1* alleles and anti-GM1 antibody positivity in our study, in support of previous studies .[17,18,20,22] Our study additionally revealed that haplotype 5 (**0501-*0602*) is common in the *C. jejuni*-associated axonal variant of GBS. Human ancestry and race lead to natural variation in the immune systems of individuals in different regions, such that local strains of *C. jejuni* interact with the immune system and trigger different subtypes of GBS in different populations.[23] Across the human genome, the LD pattern varies markedly between different ethnicities and geographical locations .[24–26]

In this study, HLA alleles other than DQB1 were not investigated for their association with GBS pathogenesis. Moreover, the sample size was not large enough for investigation of a substantial number of haplotypes in patients with GBS, even though we used one of the largest cohorts of patients with GBS from developing countries. These factors can be considered as limitations of this study.

We conclude that *HLA-DQB1* gene polymorphisms and haplotype were not associated with susceptibility to GBS. In addition, these genetic markers did not influence the clinical features or serological subgroup in patients with the *C. jejuni*-triggered axonal variant of GBS in Bangladesh. However, microbial recognition and host defense are very important for

Table 9.1: Summary of population-association studies of HLA-DQB1 polymorphisms with disease susceptibility, severity, and clinical and serological features of GBS in various ethnicities

Study (Author, year)	Ethnic origin /population	Country	Participants (*n*) (GBS vs. controls)	Major findings of *HLA-DQB1* alleles
Rees, 1995	Caucasian	England/Wales	97 vs. 100	Not associated with susceptibility & severity of GBS; *DQB1*03* allele associated with recent *C. jejuni* infection (*P*=0.003; *P*c=0.05).
Koga, 1998	Asian	Japan	35 vs. 112	Not associated with susceptibility & severity of GBS; *DQB1*03* alleles not associated with *C. jejuni* after correction (*P*=0.05; *Pc*=0.24); not associated with anti-ganglioside antibodies.
Ma, 1998	Asian	Japan	81 vs. 87	Not associated with susceptibility & severity of GBS; *DQB1*03* alleles not associated with *C. jejuni;* not associated with anti-GM1 antibodies.
Li, 2000	Asian	China	47 vs. 50	Not associated with susceptibility & severity, recent *C. jejuni* infection, or the presence of anti-GM1 antibodies.
Magira, 2003	Asian	China	72 vs. 97 (AMAN=47; AIDP=25)	*DQB1*0401* alleles increased in AIDP before correction (*P*=0.03); the *DQβRLD* $^{55\text{-}57}$ */ED* $^{70\text{-}71}$epitope positively associated (*P*=0.009) with AIDP & *DQβRPD* $^{55\text{-}57}$ with protection from AIDP (*P*=0.05).
Geleijns, 2005	Caucasian	Netherlands	164 vs. 207	Not associated with susceptibility, severity, antecedent infections, presence of anti-ganglioside antibodies, or clinical characteristics.
McCombe, 2006	Caucasian & Asian	Australia	74 vs. 158	*DQB1*050x & DQB1*060x* were studied, no particular HLA associations were notified.
Sinha, 2010	Asian	India	54 vs. 202	*DQB1*060x* was associated with susceptibility of GBS.
Fekih Mrissa, 2014	Arabic	Tunisia	38 vs. 100	Haplotypes *DRB1*14/DQB1*05* and *DRB1*13/DQB1*03* were associated with susceptibility to GBS; *DRB1*07/DQB1*02* and *DRB1*03/DQB1*02* haplotypes confer protection (*P*c= 0.007 and *P*c< 10^{-3}, respectively).
Hayat, 2019	Asian	Bangladesh	151 vs. 151	Not associated with susceptibility, presence of anti-ganglioside antibodies, or clinical characteristics; haplotype 5 (**0501-*0602*) was associated with *C. jejuni*-triggered axonal variant; *DQB1*0303* was associated with severity before correction (*P*=0.025; *P*c=0.32).

AMAN, acute motor axonal neuropathy; AIDP, acute inflammatory demyelinating polyneuropathy.

homeostasis of the innate immune system, and have become one of the most important sectors of genetic susceptibility for the pathogenesis of many inflammatory diseases such as GBS. Two of the most important receptors, Toll-like receptor-4 and the nucleotide-binding oligomerization domain (NOD) have crucial roles in pathogen recognition in innate immunity. Previously, our group described the association of Toll-like receptor-4 with an increased risk of GBS in a Bangladeshi population.[27] Here, we focused on the contribution of *NOD* polymorphisms in the pathogenesis of GBS in low-and middle-income countries (LMIC).

NOD polymorphisms in GBS (Chapter 5)

NOD proteins are cytoplasmic pattern-recognition receptors (PRRs) that play an effective role in the first line of defense of the immune system by recognizing microorganisms .[28,29] Genetic variation in NOD-encoding genes, particularly *NOD1* and *NOD2*, can deregulate the sensing of microbial pathogens, and might hinder the natural phenomena of innate immunity. ***Chapter 5*** reported that none of the NOD variants [*NOD1* (Glu266Lys) and *NOD2* (Arg702Trp and Gly908Arg)] are associated with susceptibility, severity, or subgroups of GBS. Moreover, polymorphisms in *NOD2* are rare in patients with GBS as well as in healthy individuals from Bangladesh. Kharwar et al. reported a significant association between *NOD1* polymorphisms and the risk of developing GBS in an Indian population (***Table 9.2***);[30] however, our data did not support these findings. Kim et al. previously reported that recognition of human intestinal Gram-negative bacteria relies on a 'backup mechanism' of NOD1 to activate NF-κB in infected cells that lack Toll-like receptors (TLRs).[31] Furthermore, several studies described the association of *NOD1* polymorphisms with the development of various autoimmune diseases other than GBS, including atopic eczema, asthma, inflammatory bowel syndrome.[32–34] With the exception of the development of GBS, we did not find such an association in our population. Moreover, we identified only the wild-type genotype of *NOD2* (Arg702Trp and Gly908Arg) in patients with GBS and healthy individuals from Bangladesh, which does not support the findings of Kharwar et al. in an Indian population,[30] and is very much similar to studies from other parts of Asia, including Japan, China and Korea.[35–37] This strongly implies that *NOD2* polymorphisms are rare or nonexistent in the Bangladeshi population. In the Western world, *NOD2* polymorphisms (Arg702Trp and Gly908Arg) were

associated with various autoimmune diseases, including Crohn's disease.[38] In Europe, the *NOD2* variants associated with susceptibility to Crohn's disease were reported to be less prominent in Finland, Sweden, Iceland, Scotland, and Ireland than in other populations.[35]

Table 9.2: Association studies of NOD polymorphisms with susceptibility to GBS

Study (Author, year)	Ethnic origin/ population	Country	Participants (*n*) (GBS vs. controls)	Methods	Major findings of NOD polymorphisms
*Kharwar, 2016	Asian	India	105 vs. 100	PCR-RFLP	Lys/Lys variants of *NOD1* were associated with the risk of GBS (*P*=0.013, OR=2.89), 266Lys variant allele was associated with a 1.63-fold higher risk for GBS (*P*=0.016, OR=1.63) and associated with AMAN (*P*=0.001) and AIDP susceptibility. *NOD2 Arg702Trp* was protective for AMAN, AMSAN and AIDP. *NOD2702Trp* allele associated with AMAN (*P*=0.001), AMSAN (*P*=0.039) and AIDP (*P*=0.001). *NOD2* Gly908Arg genotype was protective for GBS (*P*=0.003), and Gly908Arg was associated with the risk of AIDP.
This study	Asian	Bangladesh	303 vs. 303	PCR-RFLP and sequencing	No association of *NOD1* with GBS susceptibility. *NOD2* polymorphisms were absent.

NOD, nucleotide-binding oligomerization domain; PCR-RFLP, polymerase chain reaction and restriction fragment length polymorphism.
*No other studies on NOD polymorphisms and pathogenesis of GBS were identified.

Anti-ganglioside antibodies play a key role in the pathophysiology of GBS [39] by inducing immune response nerve damage via diverse mechanisms including interaction with FcγR. Polymorphisms in *FcγRs* influence the efficacy of this binding and induce inflammatory immune responses.[40] Thus, natural variations in *FcγRs* and their association with GBS pathogenesis is the focus of the next part of this discussion.

FcγR polymorphisms and GBS (Chapter 6)

The study of three common *FcγR* polymorphisms described in ***Chapter 6*** confirms the disassociation of polymorphisms in disease susceptibility of GBS,[40,41] and also the association of *FcγRIIIa* polymorphisms with the severity of GBS.[40] In addition, this chapter also described the associations of the FcγRIIIa and FcγRIIIb genotypes and haplotype patterns with recent *C. jejuni* infection and the presence of anti-GM1 antibodies. Our findings are in agreement with earlier studies on British, Dutch and Norwegian populations that examined *FcγR* polymorphisms and the risk of developing GBS,[40] although the majority of those patients had the AIDP subtype of GBS. Our study contradicts that of an Indian population with GBS, in which the FcγRIIa-H/H131 genotype is classed as highly risky for development of GBS.[21] Nevertheless, variability in genetic makeup and disease susceptibility is possible due to ethnic variation.

Our findings on subgroup analysis, based on MRC sum score, revealed an association of the FcγRIIIa-F/F158 genotype with the mild form of GBS, while the FcγRIIIa-V/F158 genotype was associated with the severe form. Efficient interaction of IgG-FcγR is an essential prerequisite for immune complex clearance and subsequent inflammation.[42–44] In the mild form of GBS, the FcγRIIIa-F/F158 genotype may reduce the affinity of IgG binding, which would impair immune complex (IC) clearance and thereby reduce subsequent inflammation. In severe GBS, the FcγRIIIa-V/F158 genotypes have a higher affinity for IgG and are better able to clear ICs, resulting in severe nerve damage through activation of effector functions.[44] Our study also describes the predominance of FcγRIIIb-NA1/NA1 genotypes in mild forms of GBS, consistent with a study from Norway.[41] Autoantibodies, such as anti-ganglioside antibodies, are neutralized in the circulation and possible cross-reaction with peripheral nerves may be partially prevented in patients with GBS that are homozygous for FcγRIIIb-NA1.[41] Homozygous FcγRIIIb-NA1 was less common in patients that were seropositive for *C. jejuni* and in patients that were positive for anti-GM1 antibodies, whereas FcγRIIIb-NA2/2 genotypes were associated with recent *C. jejuni* infection and anti-GM1 antibody production. This indicates that FcγRIIIb-NA1 homozygotes are less sensitive to infection with *C. jejuni* that leads to production of autoantibodies, and appear to suffer less with mild muscle weakness. In addition, the increased frequency of FcγRIIIa-F/F158 and

FcγRIIIa-V158F genotypes in patients that are seropositive for *C. jejuni* indicates the influence of these genotypes in *C. jejuni* infection.

FcγRIIIb-SH polymorphisms were not investigated and are mentioned as a limitation of our study. Thus, our study supports the influence of *FcγR* polymorphisms and haplotypes on the clinical and serological subgroup of GBS and demonstrates that the FcγRIIIa-V158F genotype plays a pivotal role in disease severity. Further studies on multi-ethnic populations (e.g. International GBS Outcome Study [IGOS]) are required to confirm the findings of this study, as genetic variability in the FcγR gene differs greatly between races. However, genes involved in cytokine-mediated inflammation and macrophage recruitment are potential candidates in the pathogenesis of GBS.[13] A plethora of research has been conducted on cytokine-mediated neuroinflammation following nerve injury.[45] The association of the *TNFα*-863C/A polymorphism with disease susceptibility and severity has been studied in Bangladeshi patients with GBS.[46] ***Chapter 7*** describes the role of the three most common *IL-10* promoter polymorphisms in the pathogenesis of GBS.

Il-10 promoter polymorphisms in patients with GBS in Bangladesh (Chapter 7)

The study described in ***Chapter 7*** supports the previous findings of Press et al., which showed increased frequencies of the -819TT genotype in the promoter region of the IL-10 gene were associated with the axonal variant of GBS.[47] In addition, our study suggested that the *IL-10* expression haplotype combinations GCC/GTA, GCC/ATA and GCC/GCA, may have an impact on disease severity. Previously, Myhr et al. reported higher frequencies of the -592CC and -819CC genotypes in Norwegian patients with GBS.[48] However, these findings were not supported in Dutch patients with GBS[13] or this study (***Table 9.3***). Our study implies a link between the -819TT genotype and GBS, but this association was not significant after correcting the *P*-values. The association of the -819TT genotype with the axonal variant of GBS firmly supports the findings of Kasamatsu et al. and Press et al., who described strong association between this genetic factor and the production of IL-10 and subsequent axonal damage.[47,49,50]

Table 9.3: Association studies of IL-10 promoter polymorphisms with GBS susceptibility

Study (Author, year)	Ethnic origin/ population	Country	Participants (*n*) (GBS vs.controls)	Methods	Major findings of IL-10 promoter polymorphisms
Press, 2001 & 2002	Swedish/ Caucasian	Sewden	41 vs. 55	ELISA	High levels of IL-10-secreting blood MNCs correlated with serum levels of anti-ganglioside antibodies and axonal damage
Myhr, 2003	Caucasian	Norway	87 vs. 87	PCR	GBS patients had higher frequency of -592 CC and -819 CC genotypes compared to controls
Geleijns, 2007	Caucasian	Netherlands	263 vs. 210	PCR	No association of IL-10 polymorphisms with disease susceptibility and severity
Current study	Asian	Bangladesh	152 vs. 152	PCR-RFLP	-819 TT genotype was prevalent in axonal variant compared to AIDP and healthy controls

IL-10**,** interleukin-10; ELISA, enzyme linked immunosorbent assay; PCR-RFLP, polymerase chain reaction and restriction fragment length polymorphism.

Our study also confirms the reports of Myhr et al. and Geleijns et al., with no associations between *IL-10* polymorphisms and recent infection with *C. jejuni*.[13,48] Our data were inconsistent with the findings of Press et al. who found a significant association between anti-ganglioside antibodies and increased IL-10–secreting blood mononuclear cells.[47] We also observed that high *IL-10* expression haplotype combinations may somehow influence disease severity in patients with GBS. Proinflammatory effects of IL-10 have previously been reported,[51] and under certain conditions, the stimulatory effects of IL-10 on $CD4^+$, $CD8^+$ T cells, and/or NK cells may result in increased IFN-γ production.[52] In summary, the -1082 G/A, -819 C/T and -592 C/A polymorphisms of *IL-10* are not associated with susceptibility to GBS. However, the -819 TT genotypes may have a link to the axonal variant of GBS, and high IL-10 expression haplotype combinations may influence disease severity. The crucial roles of pro- or anti- inflammatory cytokines in the pathogenesis of GBS are evident.[53] Pro-inflammatory cytokines also activate MMP-9,[54] a member of the zinc-metalloproteinase

family that participates in the recruitment of macrophages[13] and subsequent inflammatory-mediated nerve damage in GBS.[55]

MMP9 -1562 C/T polymorphism in GBS (Chapter 8)

The study described in ***Chapter 8*** confirms that a polymorphism in the *MMP9* promoter (-562 C/T) is a candidate risk factor for the severe form of GBS with high expression of serum MMP9 levels. Geleijns et al. reported the association of this SNP with severe muscle weakness in Dutch patients with GBS (Geleijns et al., 2007).[13] Our data support the findings of Geleijns et al., with higher frequencies of the T allele and CT genotype in patients with the severe form of GBS compared with patients with a mild form of GBS. This indicates that the polymorphism at position -1562 of the MMP-9 gene causes upregulation of *MMP9* promoter activity rendered by the T allele, potentially resulting in increased expression of MMP-9.[13,56] In accordance with current study, increased levels of the MMP-9 enzyme have previously been reported in the serum of patients with severe GBS.[56–58] Our study also confirmed the findings of the Dutch study, with no significant associations detected between the *MMP9* polymorphism and susceptibility to GBS or any clinical subgroups of GBS, including the demyelinating and axonal variants. The allele and genotype distribution of the *MMP9* (-1562 C/T) polymorphism did not influence infection with *C. jejuni*, production of anti-GM1 antibodies, or disease prognosis, in accordance with the study of Dutch patients.[13] Overall, our study demonstrates the *MMP9* (-1562 C/T) polymorphism is not a risk factor for the development of GBS, but is associated with disease severity. Investigating the additive effects of the *MMP9* polymorphism with other polymorphisms that have been associated with GBS pathogenesis (e.g. TNF-α and TLR-4 genes) will assist in clarifying the role of the *MMP9* (-1562 C/T) polymorphism in GBS pathogenesis.

General conclusion

GBS is a post-infectious auto-immune disease associated with an aberrant immune system. The studies described in this thesis investigated the contribution of polymorphisms in immune-response host factors with the development of GBS in a well-documented cohort of patients from Bangladesh. Detailed explorations have provided clear insights that genetic polymorphisms in the *HLA-DQB*1 allele complex, *NOD*, immunoglobulin G *FcγRs*, *IL-10* and

MMP9 do not affect susceptibility to GBS, but do contribute to the clinical and serological subgroup of GBS, including recent infection with *C. jejuni*, the presence of anti-ganglioside antibodies, severe or mild disease, and disease prognosis. Thus, the genetic polymorphisms examined in this study play crucial roles in the pathogenesis of GBS. Our findings regarding genetic polymorphisms in immune regulators enrich current knowledge on host-pathogen chemistry, and may help to direct future research concerning *C. jejuni*-related GBS and its subsequent severity. A collaborative study of a larger cohort of patients from multi-ethnic populations is required to gain a global perspective of the role of host factor polymorphisms in the pathogenesis of GBS.

Future aspects

The lack of association between immune-response gene polymorphisms and susceptibility to GBS leads to a desire to focus our future research on the additive effects of SNPs in the pathogenesis of GBS, as well as conducting a genome-wide association study (GWAS) in patients with GBS. Sequencing of the human genome has led to a new era in understanding phenotypes and diseases. However, GWAS now provide one of the best approaches to identify true associations between genetic variability and disease development. GWAS are based on a dense set of molecular markers, known as SNPs, distributed throughout the human genome. The millions of SNPs present in the genome are used in GWAS to identify genomic loci associated with complex diseases. To date, the use of GWAS in GBS is rare. Since several SNPs are likely to contribute to the pathogenesis of GBS, assessing the effect of only one SNP at a time might not accurately reflect the contribution of this SNP to the pathogenesis of GBS. Using GWAS in the Bangladeshi patients with GBS to identify the true genetic associations between SNPs and disease traits could potentially identify genes or patterns of genes that can predict the outcome of GBS at the onset of disease. This information could have clinical consequences and help to decide whether treatment is required or not for individual patients with GBS.

Besides SNP studies, another target for future research is to explore the underlying mechanisms of host-microbiota mutualism. Microbiota are known to regulate host immune responses and affect the release of cytokines, antibodies, and antimicrobial peptides that target pathogens to be recognized and removed. Therefore, the contribution of microbiota in altering

host-pathogen interactions in the pathogenesis of GBS is of interest. In addition, advanced genomics studies on enteric *C. jejuni* shows promise in answering other unresolved questions. We also desire to explore T cell-mediated or autoantibody-mediated targeting genes that regulate innate and humoral immune system in the pathogenesis of GBS. Lymphocyte phenotype and function is crucial and complicated at the onset and throughout the course of a disease. Future research will help improve our understanding of the complexity of the immune system in GBS. At present, the prognosis of GBS is determined by clinical examination and electrophysiological studies. However, some immunological parameters are now considered as effective markers for the prognosis of the disease. The expression of Th1, Th2, Th17, and regulatory T (Treg) cells in patients with GBS, with respect to healthy controls, are very important in this aspect. The cytokines IL-17, IL-21, and IL-22 are potentially released with the elevation of Th17 cells in GBS patients. To measure these cytokines, an antibody in lymphocyte supernatant (ALS) assay (48-h peripheral blood mononuclear cell [PBMC] culture) could be employed as a promising biomarker of disease pathogenesis. Our future work also aims to investigate the profiles of cytokines and Th cell subsets at disease onset. This would potentially allow us to identify dysfunctional cells in the immune network, including T cells, B cells and other immune cells, and also explore the prognosis of GBS by assaying immunological parameters, and ultimately, identify target cytokines for therapeutic approaches.

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APPENDICES I

Reagents for DNA extraction

1. QIAGEN Protease stock solution

Lyophilized QIAGEN Protease was provided with QIAamp DNA Blood Midi Kit (100). 5.5 ml distilled water was added into a vial of lyophilized QIAGEN Protease. QIAGEN protease is the optimal enzyme for use with Buffer AL for sample lysis. It is completely free of DNase and RNase activities.

It was stored at 2-8°C or -20°C.

2. Buffer AL

Buffer AL was provided with QIAamp DNA Blood Midi Kit (100). It contains chaotropic salt and guanidine hydrochloride. So, appropriate precautions were taken during handling. QIAGEN protease is strongly prohibited to add directly to Buffer AL. It is better to add QIAGEN protease first and then blood sample and then Buffer AL. To ensure adequate lysis, the sample must be mixed thoroughly with Buffer AL to yield a homogenous solution.

It was stored in room temperature, 15-25°C.

3. Absolute Ethanol

To increase yield and purity of DNA extraction, absolute alcohol was used in different steps of DNA extraction. Using other alcohol may result in reduced yield and purity. It was strongly prohibited to use denatured alcohol which contains other substances such as methanol or methylethylketone.

4. Seventy percent (70%) Ethanol

Seventy percent (70%) ethanol was used to wash DNA precipitate. Washing was done twice to ensure high purity. It was prepared from absolute ethanol. 70ml ethanol was added with distilled water making final volume 100ml.

5. Buffer AW1

Buffer AW1 was provided with QIAamp DNA Blood Midi Kit (100). Ethanol was added making final volume following QIAGEN protocol. It was a washing buffer provided with QIAamp DNA Blood Midi Kit (100). DNA bound to the QIAamp membrane was first washed with Buffer AW1. It improved the purity of the eluted DNA. It contains guanidine hydrochloride. So, appropriate precautions were taken during handling.

It was stored in room temperature, 15-25°C

6. Buffer AW2

Buffer AW2 was provided with QIAamp DNA Blood Midi Kit (100). Ethanol was added making final volume following QIAGEN protocol. It is a washing buffer provided with QIAamp DNA Blood Midi Kit (100). DNA bound to the QIAamp membrane is first washed with Buffer AW1. It improves the purity of the eluted DNA. Second washing increased purity.

It was stored in room temperature, 15-25°C.

7. Buffer AE (Elution Buffer)

Buffer AE (Elution Buffer) was provided with QIAamp DNA Blood Midi Kit (100). Elution buffer was equilibrated to room temperature (15-25°C) before applying to the column. Elution with two separate volumes increased DNA yield while re-elution increased DNA concentration.

It was stored in room temperature, 15-25°C.

8. Three Molar sodium acetate

Forty point eight one (40.81) gram of $Na_2(CH_3COOH).H_2O$ was dissolved in 80ml of distilled water. The pH was adjusted to 4.5 with glacial acetic acid. The final volume was adjusted to 100ml with distilled water and the solution was sterilized by autoclaving.

It was stored at 4°C.

9. TE buffer

Ten (10) mM Tris-Cl (pH 8.0), 1 mM EDTA was prepared by diluting concentrated stock of 1M Tris-Cl (pH 8.0) and 0.5M EDTA.

The buffer was stored at 4°C.

10. QIAamp Midi column

It was provided with QIAamp DNA Blood Midi Kit (100) used in DNA extraction.

APPENDICES II

Reagents for PCR

GoTaq® Flexi DNA Polymerase

Supplied With:

Cataloge Number	GoTaq® DNA Polymerase	5X Green GoTaq® Flexi Buffer	5X Colorless GoTaq® Flexi Buffer	Magnesium Chloride Solution, 25mM
M8291	100 units (M829A)	1ml (M891A)	1ml (M890A)	0.75ml (A351B)
M8295	500 units (M829B)	4 × 1ml (M891A)	4 × 1ml (M890A)	3 × 1.2ml (A351H)
M829B-C	500 units (M829B)	---	---	---

Description: GoTaq® Flexi DNA Polymerase (a,b) is supplied in a proprietary formulation containing 50% glycerol with buffers designed for enhanced amplification. Cat.# M8291 and M8295 are provided with 5X Green and 5X Colorless GoTaq® Flexi Buffers and Magnesium Chloride Solution. The 5X Green GoTaq® Flexi Buffer contains two dyesThe 5X Green GoTaq® Flexi Buffer, contains two dyes (blue and yellow) that separate during electrophoresis to monitor migration progress. The colorless buffer is used when direct fluorescence or absorbance readings are required without prior purification of the amplified DNA from the polymerase chain reaction (PCR). The Flexi Buffers do not contain magnesium, allowing easy optimization in amplification reactions.

Biological Source: The enzyme is derived from bacteria.

Enzyme Concentration: 5u/µl.

5X Green GoTaq® Flexi Buffer (Part# M891A): Proprietary formulation supplied at pH 8.5 containing blue dye and yellow dye. The blue dye migrates at the same rate as a 3–5kb DNA fragment in a 1% agarose gel. The yellow dye migrates at a rate faster than primers (<50bp) in a 1% agarose gel. Green GoTaq® Flexi Buffer also increases the density of the sample, so it will sink into the well of the agarose gel, allowing reactions to be loaded directly onto gels without loading dye. This buffer does not contain magnesium.

5X Colorless GoTaq® Flexi Buffer (Part# M890A): Proprietary formulation supplied at pH 8.5. This buffer does not contain magnesium.

Magnesium Chloride Solution, 25mM (Part# A351B, A351H): Provided to allow users to optimize MgCl2 concentration according to their individual requirements. Vortex the MgCl2 thoroughly after thawing and prior to use.

Storage Conditions: See the Product Information Label for storage recommendations. See the expiration date on the Product Information Label.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 10 nanomoles of dNTPs into acid-insoluble material in 30 minutes at 74°C. The reaction conditions are specified below under Standard DNA Polymerase Assay Conditions.

APPENDICES **III**

Reagents for Gel Electrophoresis

Ethidium bromide solution

Ethidium bromide was dissolved in distilled water at a concentration of 10 mg/ml and stored at 4^0 C in the dark.

TBE buffer

The total content of a bag having the formula of 100 mM tris, 90 mM Boric acid, 1.0 mM EDTA was mixed with 0.99 l of distilled water to make the 1x concentrated TBE buffer. The buffer was stored at room temperature.