# Polymorphisms in glucocorticoid receptor gene: a susceptibility factor to Guillain-Barré syndrome in a Bangladeshi population


**A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY**

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## *Declaration of Authenticity*

I hereby humbly declare that this thesis is entitled “Polymorphisms in glucocorticoid receptor gene: a susceptibility factor to Guillain-Barré syndrome in a Bangladeshi population.” submitted by the undersigned has been carried out under the supervision of Zhahirul Islam, PhD, Scientist and Head, Enteric Microbiology Laboratory, Laboratory Sciences, and Services Division, Icddr,b and Mr Avizit Das, Research Officer, Enteric Microbiology Lab, LSSD, icddr,b and internal research supervisor, Nazneen Jahan, Lecturer, (Lecturer, Department of Mathematics and Natural Sciences, BRAC University) at BRAC University, Mohakhali Dhaka.

The presented dissertation is based on original research work carried out by myself and has not been submitted to any other institution for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources has been accordingly cited and referred to.

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Dedicated
*to*
**My Beloved Parents**

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# Abstract

Guillain-Barre syndrome (GBS) is an autoimmune disorder in which the body's immune system attacks part of the peripheral nervous system with a greater variable clinical progression, severity, and outcome. Infectious agent as well as host factor are responsible for developing GBS. Glucocorticoids are the steroid hormones that bind with glucocorticoid receptor (GR) gene to decrement the immune reaction through down-regulating the cytokine responsible for cellular and humoral immune reaction and thus, polymorphisms in GR gene act as a factor to develop autoimmune disease. Hence, in the population of 71 patients with GBS and healthy controls of Bangladesh , five known SNPs of glucocorticoid receptor gene along with the haplotype patterns of genetic variations has been studied. The study supports to investigate the individual SNP as well as the haplotype that are related to the susceptibility of developing GBS and their role in disease outcome. During the course of this study, which includes 5 possible SNPs in the GR gene, shows 6 different haplotype frequencies. No strong evidence has been found to conclude that polymorphism in GR gene is related in developing GBS, even though, BclI (C/G), genotype shows an association with autoantibody GD1a production (p=0.024, OR, 6.94; 95% CI, 1.28-37.58). Two associations with haplotypes have been found which includes haplotype 1 in *C. jejuni* positive acute motor axonal neuropathy (AMAN) type patient with GBS (p=0.048, OR, 5.55; 95% CI, 1.02-30.33) and haplotype 3 with disease outcome (p=0.008, OR, 0.06; 95% CI, 0.007-0.478), which means that disease improvement rate is 94% slower in the presence of haplotype 3. Additionally, pairwise Linkage disequilibrium has been performed to find the association within this 5 locus which shows an association among N363S with BclI, N363S with GR-9beta and TthIII-1 with ER22/23EK based on D'-value and P-value.

**Keywords:** Guillain-Barre´syndrome (GBS), glucocorticoid receptor (GR), Single nucleotide polymorphisms (SNPs), Haplotype frequencies, Linkage disequilibrium(LD), Acute motor axonal neuropathy (AMAN).

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# *Abbreviations*

| | |
|---|---|
| AFP | Acute Flaccid Paralysis |
| AIDP | Acute Inflammatory Demyelinating Polyradiculoneuropathy |
| AMAN | Acute Motor Axonal Neuropathy |
| AMSAN | Acute Motor and Sensory Axonal Neuropathy |
| CI | Confidence Interval |
| *C. jejuni* | *Campylobacter jejuni* |
| CSF | Cerebrospinal Fluid |
| DNA | Deoxyribonucleic acid |
| *et al.* | 'et alia' meaning 'and others' |
| GR | Glucocorticoid Receptor |
| GBS | Guillain-Barré syndrome |
| HLA | Human Leukocyte Antigen |
| IFN | Interferon |
| IL | Interleukin |
| IVIg | Intravenous Immunoglobulin |
| i.e. | That is |
| LD | Linkage Disequilibrium |
| LOS | Lip oligosaccharide |
| MFS | Miller-Fisher syndrome |
| MRC | Medical Research Council |
| MS | Multiple Sclerosis |

| | |
|---|---|
| NCBI | National Center for Biotechnology Information |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| PNS | Peripheral nervous system |
| *P*-value | Estimated probability |
| RFLP | Restriction Fragment Length Polymorphism |
| Rpm | Revolutions per minute |
| SNP | Single Nucleotide Polymorphism |
| TBE | Tris-Borate-EDTA |
| TNF | Tumor necrosis factor |
| $T_a$ | Annealing temperature |
| $T_m$ | Melting temperature |
| µl | Microliter |

Guillain-Barré syndrome is a heterogenic, autoimmune infectious disease, which most habitually causes non-polio acute flaccid paralysis in most of the countries around the globe to almost every aged people. It is witnessed that most patients have enrolled as GBS after encountering with respiratory or gastrointestinal infections. As a matter of fact, GBS has now been entitled as post-infectious disease. The nature of the disease is developing inflammation to the peripheral nervous system (Hughes et al., 2016) that involves the extent of sensory deficits and cranial nerve involvement (Geleijns, 2005). Patients usually feel symmetrical weakness in a limb, which progresses over a period of 12 to 28 days of time. The weakness ultimately affects all the voluntary muscles, eventually resulting in paralysis for the patient. To some extent, they are required to have artificial ventilation due to unable in breathing(Hughes *et al.*, 2016). In addition, it is believed that *campylobacter jejuni* is the most frequently causing infectious agent for developing GBS (Islam *et al.*, 2012). Besides, there are few more infectious agents which are also connected with GBS, for instance, cytomegalovirus and Epstein Barr virus etc. (Burns, 2008).

However, Glucocorticoids restrain numerous irritation associated molecules, for example, cytokines, chemokines, arachidonic corrosive metabolites, and adhesion molecules(Wilder *et al.*, 1993; Van Der Velden, 1998). In contrast, glucocorticoids are frequently up-regulated against inflammatory mediators (Van Der Velden, 1998). In normal populations single-nucleotide polymorphism (SNPs) of Glucocorticoid receptor gene for instance: TthIIII, ER22/23EK, N363S, BclI, GR-9 BETA are found to develop in the autoimmune disease (Dekker et al., 2009). Whereas ER22/23EK, GR-9 BETA are believed to decrease GR sensitivity and N363S, BclI increased the GR sensitivity (Dekker et al., 2009). As a result of acknowledging, the importance of GR and autoimmune response for the GR polymorphisms can play a role to disease susceptibility and the clinical course in patients as well for GBS.

In this study, we will determine whether GR haplotypes are a susceptibility factor for GBS or its precursor or subtypes or outcome.

## 1.1. HISTORY:

About a century earlier, at 1916 three French neurologists Guillain, Barré, and Stroh described an acute paralysis disease with spontaneous recovery (van Doorn, Ruts, and Jacobs, 2008). Their surveillance shows a significant level of expanding protein present in CSF with normal cell count (Geleijns, 2005). Apart from the fact, Jean Baptiste Octave Landry de Thezillat already established similar cases in 1859, which he termed as “ascending paralysis” (Paralysis, 1859). Subsequently, several cases with similar manifestations had been testified, and this scientific entity was named after Guillain and Barré. In 1927, Dragonescu and Claudian first used the term Guillain-Barré syndrome at a presentation. It was presented by Barré where

Strohl's name was mislaid from the title of the presentation and the list of authors in the reference to the 1916 paper. Later, different types of the syndrome with distinguishing clinical points had been identified. On the basis of clinical features, etiology, and electrophysiological traits, today distinguishes are viable.

## 1.2. EPIDEMIOLOGY:

### 1.2.1. Worldwide incidence:

The epidemiological study shows that Guillain Barré syndrome has been most intriguing yet challenging even though the treatments intended for this disease are developing over the years. Study shows, around 1–3/100,000 population from Europe, USA, and Australia are affected with GBS (Hughes and Cornblath, 2005). Whereas around the globe, about 1.3 cases out of 100,000 population (range, 0.4–4.0) with a median incidence happen to be GBS. It is claimed that males are more susceptible to GBS rather than females. The ratio of male commonly affected by GBS compared to female is 1.5 to 1 (Martyn and Hughes, 1997). According to 2008 data about 40,000 to 120,000 cases have diagnosed with a slightly increased frequency of GBS (1.5) in men than women (Hughes and Cornblath, 2005). However, age also has been a factor for developing GBS. It seems to play a bimodal role between elders and young adults. The frequency of developing GBS are mostly between 15-24 and 65-74 years aged people. Another study demonstrated that about 0.6/100,000/year are reportedly children less than 16 years of age in Europe and North America (Mcgrogan et al., 2009). In spite of this, about 70 % of cases illustrates an association between emerging of respiratory or gastrointestinal infections and the upsurge of age (more likely starts from 50 years of age and above)(Mcgrogan *et al.*, 2009). The incidence of GBS increased with age after 50 years are from 1.7/100,000/year to 3.3/100,000/year (Mcgrogan et al., 2009). Above all, the recovery rate for GBS reported being higher while the mortality percentage is approximately 8 %(Winer *et al.*, 2005)

### 1.2.2. GBS in Bangladesh:

In Bangladesh, more than half of the GBS patient is prompted by *campylobacter jejuni* (Islam et al., 2010).It has been claimed, the frequency of having GBS is 3.25 cases per 100,000

children less than 15 years of age. In 2006 and 2007, 608 (37%) and 855 (46%) cases of children less than 15 years of age, have fulfilled GBS symptoms. Whereas, 1,619 and 1,844 cases were enrolled as AFP (acute flaccid paralysis) patients. Moreover, it is believed that about 1.5 to 2.5 cases per 100,000 population per year among all division of Bangladesh are children less than 15 years of age, who have encountered as GBS. Perhaps, seasonal variants may affect the percentage of Guillain Barré syndrome.The incidence of GBS seems to be highest between January and March, especially in the AMAN subgroup in Bangladesh. Whereas, the majority of the GBS cases are pure motor variants (92%) with predominant axonal degeneration (67%) (Islam et., 2010).

### 1.3. CLINICAL FEATURES OF GBS:

**Table 1.1.Clinical features of Guillain-Barré syndrome (Hughes and Cornblath, 2005):**

| |
|---|
| **Motor dysfunction:** |
| **Symmetrical limb weakness: proximal, distal or global** |
| **Neck muscle weakness** |
| **Respiratory muscle weakness** |
| **Cranial nerve palsies: III-VII, IX-XII** |
| **Areflexia** |
| **Wasting of limb muscles** |
| **Sensory dysfunction :** |
| **Pain Numbness** |
| **Pain Numbness, paraesthesiae** |
| **Loss of joint position sense, Vibration,** |
| **Ataxia** |
| **Autonomic dysfunction** |
| **Sinus tachycardia and bradycardia** |
| **Other cardiac arrhythmias (both tachy and brady)** |
| **Hypertension and postural hypotension** |
| **Wide fluctuations of pulse and blood pressure** |
| **Tonic pupils** |
| **Hypersalivation** |

| |
|---|
| **Anhydrosis or excessive sweating** |
| **Urinary sphincter disturbances** |
| **Constipation** |
| **Gastric dysmotility** |
| **Abnormal vasomotor tone causing venous pooling and facial flushing** |
| **Other :** |
| **Papilloedema** |

## 1.4. DIAGNOSIS:

The significance for diagnosing GBS felt after the swine flu incidence of 1976-1977. In 1978, the explanation and criteria of GBS have published for the first time in Annals of Neurology requested by the team of National Institute of Neurological and Communicative Disorders and Stroke (NINCDS, now NINDS) (Asbury and Cornblath,1990).

Gastrointestinal infections or respiratory infections told to be noticeable as initial symptoms for developing GBS. The onset of symptoms involved symmetrical limb weakness, areflexia, mild to severe sensory dysfunctions to multiple autonomic dysfunctions. An elevated level of protein is prone to be present in CSF with normal cell count (Joshi et al., 2016). It is also demonstrated that CSF protein content is more close to being normal during the first days of the disease (Hughes and Cornblath, 2005). Perhaps, at the end of the second week, the protein level of CSF increases by more than 90%. Further, the electrophysiological and pathophysiological study is required for proper diagnosis.

**Table 1.2.DIAGNOSIS CRITERIA:**

| |
|---|
| **Features required for diagnosis:**<br>• **Progressive motor weakness of more than one limb** |

| |
|---|
| • **Low or absent reflexes**<br>• **No other identifiable cause** |
| **Features strongly support the diagnosis:** |
| **Clinical :**<br>• **Progression of symptoms over days to 4 weeks**<br>• **Symmetry of weakness**<br>• **Mild sensory signs**<br>• **Cranial nerve involvement**<br>• **The onset of recovery 2-4 weeks after progression stops**<br>• **Autonomic dysfunction**<br>• **pain** |
| **Cerebrospinal fluid(CSF) :**<br>• **high concentration of CSF protein after the first week**<br>• **Less than 50 mononuclear leukocytes per µl CSF** |
| **Electrodiagnosis:**<br>• **Conduction slowing or block conduction slowing or block** |
| **Features casting doubt on the diagnosis:**<br>• **Bladder or bowel dysfunction at onset**<br>• **Sharp sensory level**<br>• **Persistent asymmetry of weakness**<br>• **Persistent bladder or bowel dysfunction** |

## 1.5. DISEASE COURSE AND PROGNOSIS:

The study shows GBS is influenced by age, gender, season or vaccination. However, the geographical location plays a major part. The prevalence of GBS subtypes varies from western countries than Asian countries. In Asian countries, the pure motor forms, the Miller Fisher variant and axonal subtypes of GBS are higher perhaps due to the type of preceding infection and host-dependent factors (McKhann et al., 1993; Asbury, 2000; Islam et al., 2010). Despite the distinctions around the world, the severities of the disease are almost same, which has been evaluated by the GBS disability score (Hughes et al., 1978), and the Medical Research Council (MRC) sum-score (van Koningsveld et al., 2007).

The major progress starts after 1-3 weeks of the disease. The maximum level of progression, observed within four weeks. As clinical results illustrate variability, precise prospects are required for the diagnosis of GBS.

### 1.6. Preceding events:

#### 1.6.1. Antecedent infection:

GBS, a syndrome where it involves a variety of motor and sensory deficits (Islam et al., 2010). Additionally, preceding events shows that, it is considered as a post-infectious disease because of the commentary enrolment of two-third patients showing GBS illness (Jacobs, 1997). These infections may affect the immune reaction against peripheral nerve antigen in the human body, suggesting by the intermission of 1 to 4 weeks between the precursor of the infection and onset of weakness (Jacobs, 1997). However, a sustainable study shows that the root of GBS is involved with a range of infectious agents. In patients with GBS, *campylobacter jejuni* is the most common infectious pathogens(Islam et al., 2010). However, cytomegalovirus, Epstein-Barr virus, Mycoplasma pneumonia, and Haemophilus influenza are also responsible for causing GBS (van Doorn, Ruts, and Jacobs, 2008). In addition, it is reported that cytomegalovirus is the second commonest infectious pathogen causing GBS (Seneviratne, no date). Also, it has also been described in association with human immunodeficiency virus (HIV) infection (Burns, 2008). Hepatitis A, B, C, and D, typhoid, and falciparum malaria are restrained to anecdotal case reports (Seneviratne, no date). Furthermore, a study in Japan shows that most of the antecedent events in GBS and related disorders are fever (52%), cough (48%), sore throat (39%), nasal discharge (30%), and diarrhoea (27%)(van Doorn, Ruts, and Jacobs, 2008).

### 1.6.2. GBS and vaccine:

Many reports show that surgery, vaccination, and parturition have also been related to GBS (Burns, 2008). However, it is also said that vaccines for Simple rabies, oral polio, influenza, measles/mumps/rubella (MMR), tetanus toxoid and hepatitis B may have a possible association for emerging GBS, even though the association is being compared after two events. However, consideration mainly ascended after the surveillance of a slight upsurge in the occurrence of GBS after swine influenza vaccines in the USA in 1976(van Doorn, Ruts, and Jacobs, 2008). In 1992–1994 vaccine campaigns placed in the USA recognized that vaccines were related with a very minor, but major, increased risk of emerging GBS of about one GBS case per million vaccines above the background incidence. Alternative study of patients who had GBS did not show a significant development of GBS again after a vaccination. However, special caution might be obligatory when recapping a tetanus vaccination: we have encountered a relapse of GBS two times after tetanus vaccinations (van Doorn, Ruts, and Jacobs, 2008). However, this does not demonstrate that Tetanus is a GBS associated agent. However, precautions should maintain prior to any exposure.

## 1.7. Pathophysiology:

### 1.7.1. Subtypes:

**a) Acute Inflammatory Demyelinating Polyneuropathy (AIDP):**

The most common type for GBS is AIDP. However, it displays establishment in western countries rather than Asia. Immunologically, it is triggered by an auto-immune response engaged against Schwann cell membranes. It not only reduces conduction velocity but also conduction block or abnormal progressive spreading. However, the pathological changes take place by the humoral and cellular response (Seneviratne, 2000). In studies, it proves that T cell infiltration in endoneurium and infiltration in myelin sheath by macrophages causes de-myelination (Hughes, Cornblath and Willison, 2016). Additionally, Axonal damage may also arise as a secondary event, in severe cases.

**b) Acute Motor Axonal Neuropathy (AMAN) & Acute Motor Sensory Axonal Neuropathy (AMSAN):**

The major difference between AMAN and AMSAN is the Absent or reduced compound muscle in AMAN but Absent or reduced SNAP and CMAP amplitude in AMSAN (Seneviratne,2000). However, in Bangladesh, about 67% of GBS patients had an axonal variant, where 56% was AMAN and 11% was AMSAN. In addition, AMAN seemed to be found more frequently from January to March (Zhang et al., 2010). However, AMAN is differentiated by speedily progressive of weakness, often with respirational failure but usually has a good recovery, whereas AMSAN typically causes slow recovery (Seneviratne,2000). Moreover, in both cases, peri-axonal macrophages are found in peri-axonal space (Burns, 2008). However, acute motor axonal neuropathy often follows infection with *C. jejuni*, which comprises a molecular mimicry between lipo-oligosaccharides LOSs and GM1gangliosides (monosialotetrahexosylganglioside, a prototype of gangliosides) of nerve.

**c) Miller Fisher Syndrome (MFS):**

Even though MFS shares similar pathophysiological events with AMAN & AMSAN, it gets the initiation by anti-GQ1b and anti-GT1a antibodies that aim towards oculomotor and bulbar nerves in where GQ1b and GT1a ganglioside concentrations are supposed to have moderately high (Burns, 2008). However, it is a rare disorder. In this syndrome, the resolution occurs after 2-3 months.

| Subtypes | Antibodies |
|---|---|
| Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP) | Unknown |
| Acute Motor (and sensory) Axonal Neuropathy(AMAN or AMSAN) | GM1, GM1b, GD1a,<br>GalNAc-GD1a |
| MFS and GBS overlapping syndrome | GD3, GT1a, GQ1b |

**Table 1.3.: The range of GBS subtypes and serum anti-ganglioside antibodies (van Doorn, Ruts, and Jacobs, 2008)**

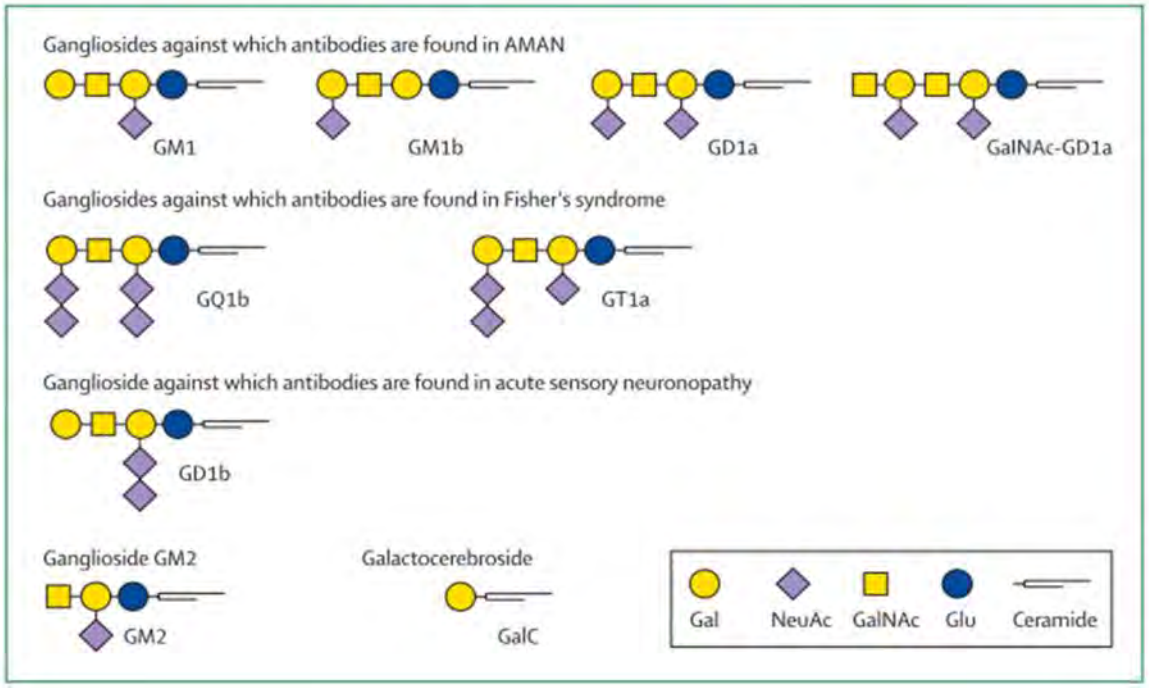



**Figure1.1. Structures of gangliosides and galactocerebroside and Guillain-Barré syndrome subtype associations (R. A. C. Hughes and Cornblath, 2005).**

### 1.8. Immunobiology:

#### 1.8.1. Anti-ganglioside antibody:

There are various types of gangliosides, which have specific distribution in tissues of the peripheral nervous system and plays a major part in maintaining cell membrane structure. On the other hand, these various types of gangliosides including LM1, GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, and GQ1b are found in half of the GBS patients (van Doorn, Ruts, and Jacobs, 2008). However, antibodies to GM1, GM1b, GD1a, and GalNAc-GD1a are found in pure motor axonal variants of GBS (Pithadia and Kakadia, 2010). However, Hostile to ganglioside antibodies that respond to self-gangliosides is found in immune system neuropathies (Pithadia and Kakadia, 2010). These antibodies were first found to respond with cerebellar cells. These antibodies demonstrate the most grounded relationship with specific types of GBS. Auto-antigenic gangliosides that are right now known are GD3, GM1, GQ3, and GT1 (Pithadia and Kakadia, 2010).

#### 1.8.2. Molecular mimicry & cross-react activity:

Patients who enrolled as GBS are found to face molecular mimicry and cross-reactivity in their body. More than half of the patients with GBS are attacked by *C jejuni* (Zhang et al., 2010), which shows that lipo-oligosaccharides (LOS) of *C. jejuni* mimics the carbohydrates of gangliosides (Pithadia and Kakadia, 2010). The sort of ganglioside mimicry in *C jejuni* appears to decide the specificity of the antiganglioside antibodies and the related variation of GBS. *C jejuni* diseased patients with pure motor or axonal GBS much of the time express a GM1-like and GD1a-like LOS, though those disengaged from patients with ophthalmoplegia or MFS normally express a GD3-like, GT1a-like, or GD1c-like LOS. Antibodies in these patients are typically cross-reactive, and perceive LOS and also gangliosides or ganglioside buildings (van Doorn, Ruts, and Jacobs, 2008). However, it also shows similar activities against mice models, which proves that it truly has molecular mimicry activity against gangliosides (van Doorn, Ruts, and Jacobs, 2008).

### 1.8.3. Complement activation:

It has been found that supplement actuation happens at the site of nerve harm after death considers have demonstrated, such as the axolemma in patients with AMAN and the Schwann cell film in patients with AIDP. However, it has been observed in a mouse model of GBS which demonstrated that some antiganglioside antibodies are extremely dangerous for peripheral nerves. An α-latrotoxin inserted in mice model can result in the arrival of acetylcholine and also transporting an exhaustion of this synapse at the nerve terminals, and blockade of nerve transmission and loss of motion of the nerve-muscle promptness, which destroys the nerve terminal and peri-synaptic Schwann cell. Antibodies to GM1 affect the sodium channels at the hubs of Ranvier of rabbit peripheral nerves. All these effects appear to be reliant on supplement actuation what's a more, arrangement of the film assault complex. The neurotoxic effects of these antibodies were repressed by immunoglobulin and the supplement inhibitor eculizumab.

### 1.8.4.Bacterial factor:

According to the study, GBS is caused by cytomegalovirus (CMV), *Mycoplasma pneumonia*, Epstein-Barr virus, and influenza virus and *campylobacter jejuni*(van Rossum *et al.*, 2004). *Campylobacter jejuni* is the most common pathogens for developing post-infectious disease named GBS (Louwen *et al.*, 2008)(Seneviratne, 2000.). It has been found that *c.jejuni* can

regulate the molecular mimicry and cross-react activity in GBS (Islam et al., 2012a). These particular bacterial strains carry a sialyltransferase gene (cst-II), which is responsible for biosynthesis of ganglioside like LOSs(Yuki, 2007). The cst–II gene is necessary for the transfer of sialic acid onto the LOS core in *C. jejuni* class A and B strains(Louwen *et al.*, 2008). However, Cst-II contains 291 amino acids, and the 51st determines its enzymatic action(Yuki, 2007). Consequently, strains with (Thr51) expressed GM1-like and GD1a-like LOS, while strains with cst-II (Asn51) stated GT1a-like and GD1c-like LOS(Yuki, 2007). As a matter of fact, the strains which has GM1-like and GD1a-like LOS encounter with anti-GM1 or anti-GD1a IgG antibodies which roots for limb weakness (Figure 1.2.). On the other hand, patients with the cst-II (Asn51) strains will encounter with anti-GQ1b IgG antibodies, and exposed to ophthalmoplegia and ataxia(Figure 1.2.). Additionally, Acute motor axonal neuropathy (AMAN) habitually follows infection with C. jejuni, which contain an epitope in their LOSs that is also present in GM1 gangliosides of nerve(Burns, 2008).

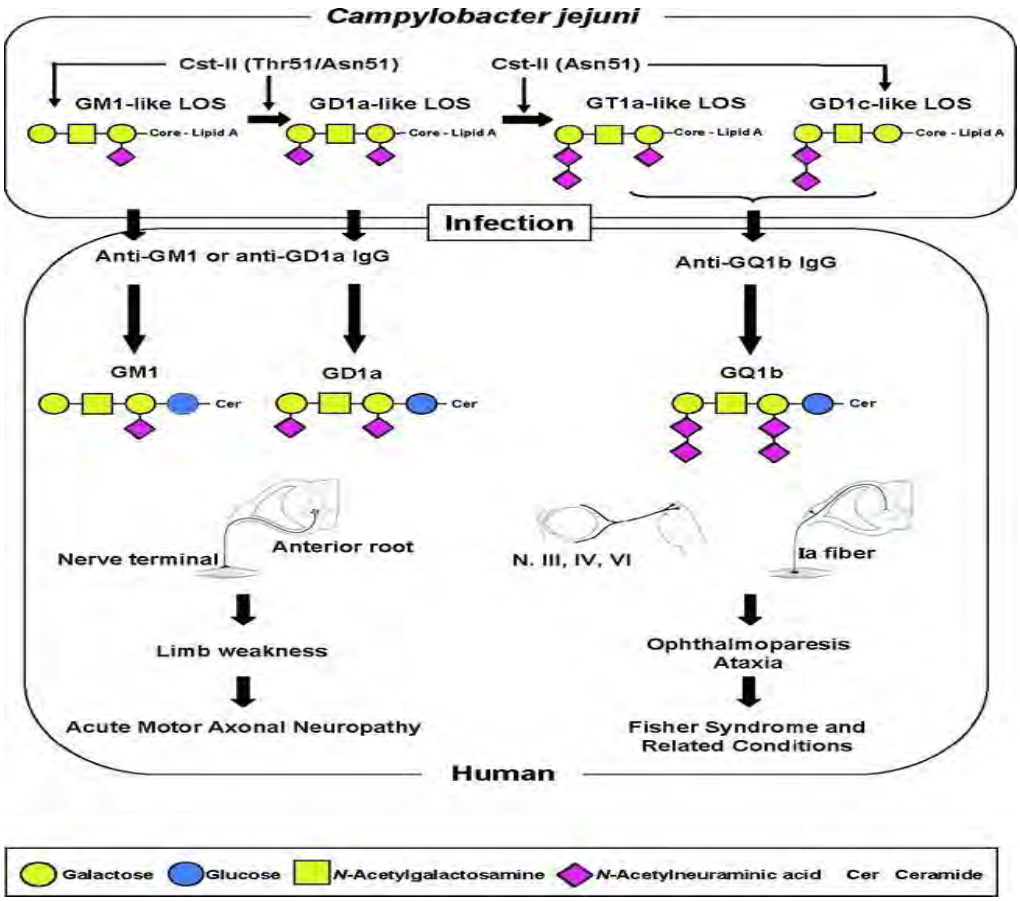



**Figure1.2.*Campylobacter jejuni* gene polymorphism as a determinant of clinical neuropathies after infection by that bacterium.**

### 1.8.5. Host factor:

Host factor involves over-all health, psychological characteristics, social status, nutrition state, race, and even gender. Therefore, host factor may influence the susceptibility to GBS or outcome or nerve damage or even severity of the disease. In addition, to find the factors involved with the pathogenesis of GBS, studying the genetic approaches in the population-based association is most common (Geleijns, 2005). As a result, SNP (Single nucleotide polymorphisms) can be compared and observed with disease severity or outcome. In addition, SNP might play a part as disease-modifying factors (van Doorn, Ruts, and Jacobs, 2008). In population-based association, the frequencies of allelic variants are compared between a group of unrelated patients with disease and healthy controls. In this case, the association can be happened because of the biological consequences of the marker studied or for a gene that is in the close proximity and inherited together with the marker (linkage disequilibrium ).

As a result, polymorphism in the glucocorticoid receptor can be one of the susceptibility factors for GBS.

#### 1.8.5.1. Glucocorticoid and Glucocorticoid Receptor:

Glucocorticoid is a hormone which regulates numerous physiological processes and supports in the treatment of inflammation, autoimmune disease, and cancer (Oakley and Cidlowski, 2011). The activities of glucocorticoids are interfered by an intracellular receptor, the glucocorticoid receptor (GR), an individual from the nuclear receptor group of ligand-dependent transcription factors (Schaaf and Cidlowski, 2003). The receptor belongs to 3-Ketosteroid receptors group, where it is in the nuclear receptor subfamily 3, group C, member 1(NR3C1). Glucocorticoid receptor (GR) is derived from a single gene which has been prevailed by cloning in 1985 (Oakley and Cidlowski, 2011). However, the human GR structure has been clarified in 1991 at chromosome 5 which consists of 9 exons. In addition, it is comprised of an amino-terminal transactivation domain (NTD), a central DNA-binding domain (DBD) connected to flexible hinge region carboxyl-terminal ligand binding domain (LBD). Whereas, DBD is the most conserved region across the superfamily and contains 2 zinc finger motifs that recognize and bind target DNA sequences termed glucocorticoid responsive elements (GREs) (Oakley and Cidlowski, 2011).

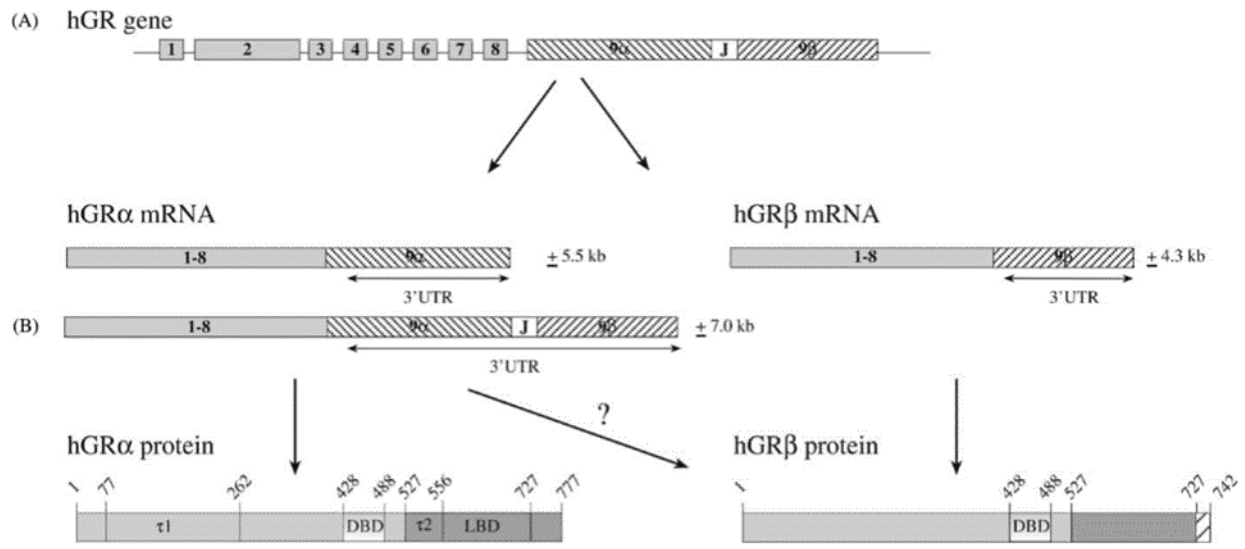



**Figure1.3. The structure of the human glucocorticoid receptor (hGR) gene, mRNA and protein.**

The hGR gene consists of nine exons. Exon 1 and the first part of exon 2 contain the 5'UTR, exons 2–9 the coding sequences, and exon 9. Three different messengers can be transcribed: they all contain exons 1–8, but contain different versions of exon 9 as a result of alternative splicing; hGR mRNA (5.5 kb) contains exon 9, whereas GR mRNA (4.3 kb) contains exon 9. The third hGR mRNA (7.0 kb) contains the entire exon 9, including exon 9, the 'J region', and exon 9. It is thought that this mRNA is translated into hGR. In addition, alternative translation initiation results in the presence of two different isoforms (A and B) of hGR. A similar mechanism of alternative translation initiation has been demonstrated for hGR. The hGR protein consists of several domains. The most N-terminal domain is called the immunogenic domain, which in hGR consists of amino acids 1–420. Amino acids 421–488 form the DNA-binding domain (DBD) of hGR, and its C-terminal ligand-binding domain (LBD) consists of amino acids 527–777. Also indicated are the two trans activational domains -1 and – 2 (Schaaf and Cidlowski, 2003).

#### 1.8.5.1.1 Mechanism:

The hypothalamic– pituitary– adrenal hub assumes a focal job in controlling motioning by the glucocorticoid receptor, which is communicated in for all intents and purposes all cells (Barnes, 2010). The glucocorticoid receptor is an individual from the steroid-hormone– receptor group of proteins. It ties with high liking to cortisol; the bound cortisol advances the separation of sub-atomic chaperones, including heat– stun proteins, from the receptor (Rhen and Cidlowski, 2005). The significant activity of corticosteroids is to turn off numerous initiated provocative qualities that encode for cytokines, chemokines, bond particles incendiary catalysts and receptors. These qualities are exchanged on in the aviation routes by pro-inflammatory translation factors, for example, atomic factor-κB (NF-κB) and activator protein-1, the two of which are initiated in asthmatic aviation routes and switch on fiery qualities by collaborating with coactivator particles, for example, CREB-restricting protein that has natural histone acetyl-transferase action. This outcome in acetylation of center histones, which opens up the chromatin structure so the quality translation is encouraged. Corticosteroid triggered GR likewise cooperates with co-activator atoms and this restrains the communication of NF-κB with co-activators, subsequently diminishing histone acetylation. The decrease of histone acetylations happens through the enlistment of histone deacetylase-2 (HDAC2) to the initiated fiery quality complex by actuated GR, in this way bringing about the powerful concealment of all enacted incendiary qualities inside the core(Barnes, 2010).

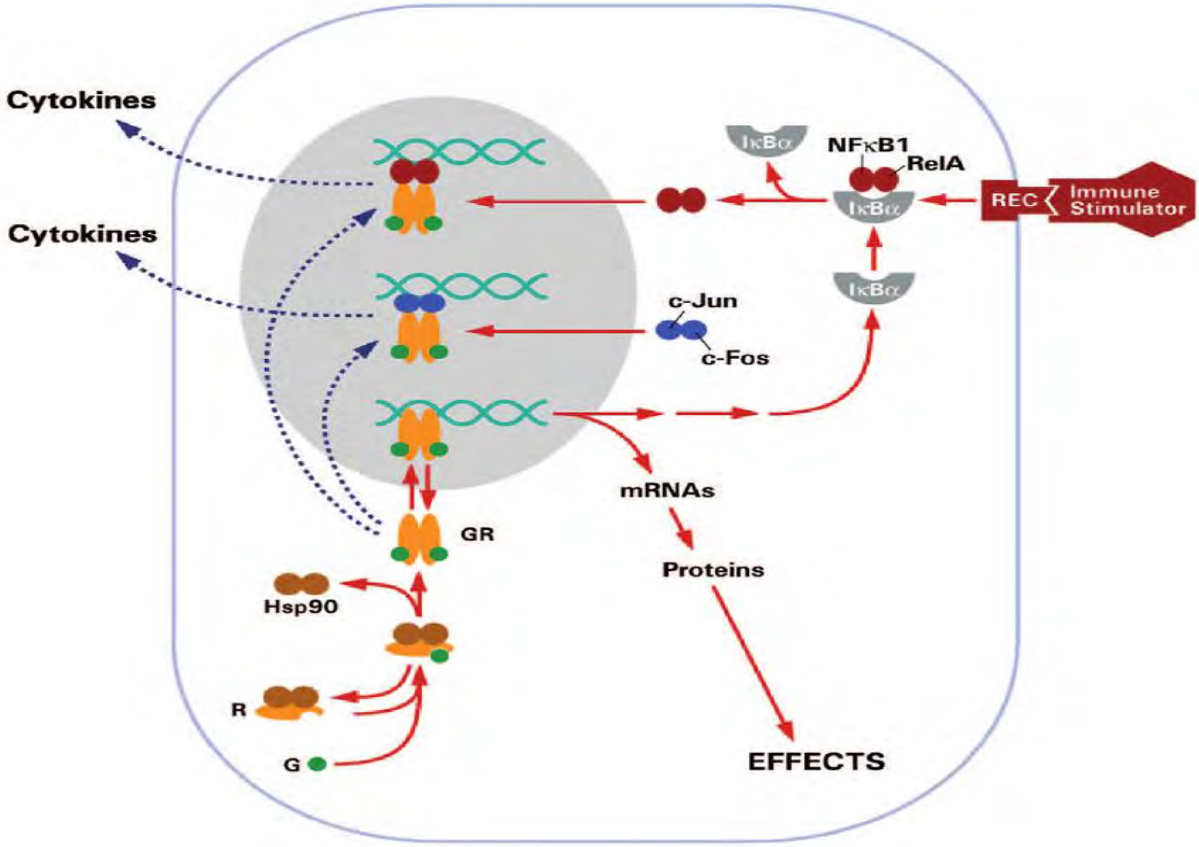



**Figure1.4. Schematic diagram illustrating the mechanism of action of the glucocorticoid receptor (GR).** Dotted lines represent negative regulatory pathways after the activation of the glucocorticoid receptor which restrained the activity of inflammation (Webster and Sternberg, 2004).

### 1.9. Treatment of GBS :

**1.9.1. Steroid:** Six qualified preliminaries have tended to the estimation of steroids in treating intense GBS. These included 195 patients. Mean inability at about a month, the extent of patients who was enhanced by one review at about a month and the enhancement in review at a year all stayed unaltered by steroids, which have all the earmarks of being protected yet insufficient. This appears differently in relation to the treatment of patients with more incessant demyelinating neuropathies, who react well to steroids. This absence of reaction to steroids isn't effectively clarified; it might be that any advantage that steroids have in decreasing irritation is exceeded by some other impending impact on the fix forms. A Cochrane investigation uncovered that a solitary pilot considers tending to consolidated treatment with methylprednisolone and intravenous immunoglobulin was not randomized. Notwithstanding,

it recommended a conceivable favorable position. A randomized report that has as of late been displayed however not yet distributed just neglects to locate a critical favorable position of the blend. Post-hoc control of the information for known hazard factors suggests leverage blend treatment; nonetheless, such examinations are known to be or maybe untrustworthy and can be misdirecting (Pithadia and Kakadia, 2010).

**1.9.2. Plasma exchange:** Plasma exchange is useful for extremely influenced patients in the main week or two of the ailment (Rac, 2009). Plasmapheresis is planned to evacuate the antibodies assaulting the nerves from your blood (Pithadia and Kakadia, 2010). Plasma exchange includes being associated with a machine that can isolate the platelets from the liquid or plasma. About 250ml of blood is evacuated at once, the plasma is disposed of and the platelets have come back to the patient with clean plasma. The method is rehashed a few times for around five days until the point when adequate plasma has been traded (Rac, 2009). The dangers of the method are to a great degree little and present-day disinfection has for functional purposes killed the danger of transmitting disagreeable diseases in the perfect plasma.

**1.9.3. Intravenous immunoglobulin (IVIg):**

Another treatment for GBS is an intravenous organization of immunoglobulins (IVIg). The antibodies utilized have been appeared to balance the humoral reaction in their capacity to repress autoantibodies and smother autoantibody creation. By hindering autoantibodies, the supplement interceded harm can be constructed (Pithadia and Kakadia, 2010). It blocks required of Fc (gamma) receptors, avoiding phagocytic damage by macrophages. In addition, The management of IVIG is modest than plasma exchange and may be the favored treatment in hospitals that have neither the plasma exchange equipment nor the expertise.

GBS is an immune system ailment influencing the peripheral nervous framework that is generally activated by an acute infectious process. It is also known an inflammatory disorder (Pithadia & Kakadia, 2010). The steroid is found to be not an appropriate drug for treating GBS (Pithadia and Kakadia, 2010). On the other hand, glucocorticoid hormone is a type of steroid hormone, which has a function of constraining the transcription of most cytokines and chemokine that are involved in immune activation. (Van Der Velden, 1998). As a result, it is

known as an anti-inflammatory agent. In this study, a total of 71 population have been taken to investigate whether polymorphism in GR can be a susceptibility factor for GBS.

## 1.10. Objective:

### 1.10.1. General objective:

- In this study, our target is to find whether polymorphisms in the GR gene are a vulnerability factor for GBS or not.

### 1.10.2. Specific objectives:

- Identifying the presence of GR polymorphisms by using RFLP-PCR and ASO-PCR in GBS patients and healthy control groups.
- Studying the correlation between polymorphisms in glucocorticoid receptors and GBS.
- Analyzing the common frequencies of a haplotype that are related to susceptibility to develop GBS.

## 2.1. Experimental design:

The purpose of this experiment is to detect the polymorphism in the receptor of glucocorticoid and its association in a patient with GBS and healthy controls. To continue the experiment about 71 patients with GBS and healthy controls of Bangladesh will be studied, using PCR-RFLP and allele-specific PCR.

In this investigation, since we will also focus on the association of polymorphisms in GR as a susceptibility factor of GBS. Therefore, it will be observed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and Allele-specific PCR.

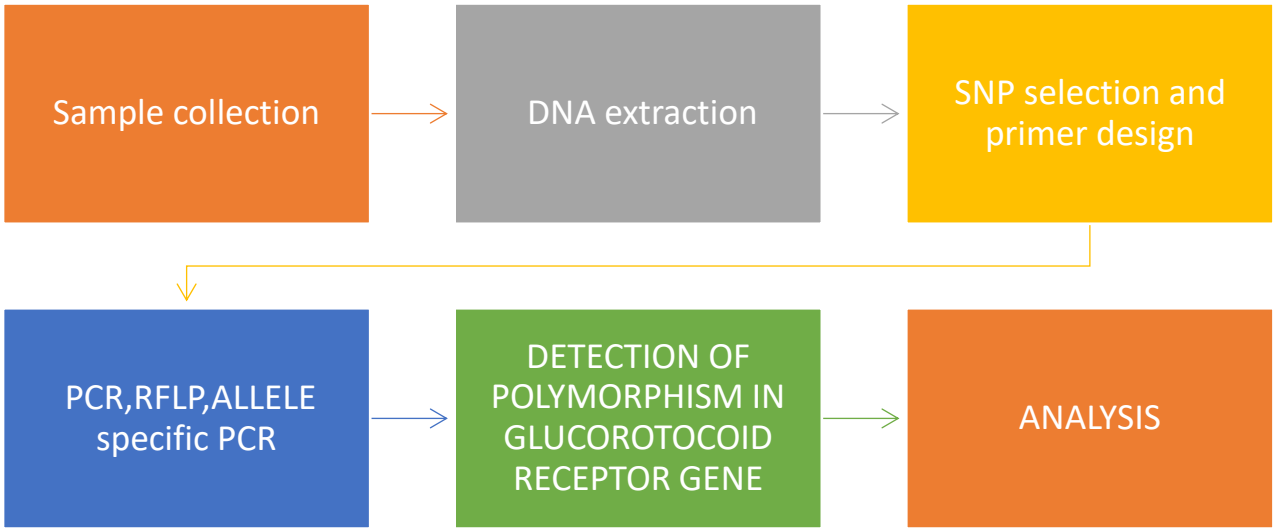
First and foremost, PCR products will be digested with particular restriction enzymes for two hours at 37°C or 50°C following the protocol for RFLP. Restriction enzymes will cut DNA from samples, which will result in separate fragments. After that, separate fragments will be witnessed by agarose gel electrophoresis. Here, we will use four types of restriction enzymes to cut the DNA, throughout the experiment.

However, another method named Allele-specific PCR will be used to witness the presence of polymorphisms in the GR gene. In this condition, one reverse primer and two forward primers with different nucleotides at the 3'-end of the forward primers complemented the base at the wild-type and variant-type DNA sample, are going to be used. In this phase, wild-type and polymorphic specific forwarding primer and single reverse primer were used to amplify the same size of PCR products, which will be observed again by gel electrophoresis.

### 2.1.1. Place of study:

This study is carried out in Enteric Microbiology Laboratory, Science and Services division (LSSD), International Center for Diarrheal Disease Research, Bangladesh (icddr, b).

## 2.2. Work outline:




### 2.2.1. Sample collection:

A potential study has been conducted by collecting samples from Dhaka Medical college and hospital , National Institute of Neurosciences & Hospital (NINS) including patients and healthy control as well.

### 2.2.2. DNA extraction:

Segregation of genomic DNA from Lithium Heparin anti-coagulated blood samples are implemented using the QIAgen DNA Blood Mini Kit ( Qiagen, Hilden, Germany ) according to the manufacturer's instruction. All the DNA samples are dissolved in 1X TE-Buffer and stored at 80 degrees.

**Procedure:**

At first, 200ul QIAgen protease mixed with 500ul blood and poured into the sterile 15 ml falcon tube. Then 2.4 ml buffer AL was added and vortex thoroughly for homogenization and incubate at 70°C( for few enzymes it will be 50°C) for 10 minutes in a water bath. After that, flipping was followed by adding 2 ml absolute ethanol to all samples. Half of the solution was then transferred in the QIAamp Midi column placed in a 1.5 ml centrifuged tube. Then centrifuge the sample at 3000 x g for 5 minutes and repeat this process if the solution had not completely passed through the membrane. The QIAamp Midi column was then removed and the filtrate of the column is discarded and repeat this step for the rest of the sample. Without moistening the rim, 2ml buffer AW2 was added to the column and centrifuge again at 3000 x g for 30 minutes. The uncapped column was incubated at 70°C( for few enzymes it will be 50°C) for 10 minutes, to evaporate all the ethanol it contains. The column was removed and wiped with a tissue and placed in a clean 1.5ml centrifuge tube. The old filtrate was removed. 300ul of buffer AE was added and pipetted directly into the membrane of the column and Incubate at room temperature for 5 minutes and then centrifuge it at 3000 x g for 10 minutes repeat this step. All the filtrate was transferred to a label, autoclave 1.5ml Eppendorf tube and 40ul autoclaved 3M sodium acetate (pH=6.5) and 800ul absolute ethanol were added to the filtrate and mixed through flipping and incubate at -70°C. After 30 minutes, centrifuge the sample at 13000 x g for 15 minutes. Discard the supernatant and 1 ml 70% ethanol and mixed it and centrifuge at 13000 x g for 15 minutes. Then discard the supernatant and dry the pellet for 20-30 minutes in a laminar hood. Then dissolve the pellet at NF (nuclease free) water and quantify it at Nanodrop and stored at -80°C.

### 2.2.3. SNP selection and primer design:

A. **N363S:** N363S originally listed as rs6195, currently listed in dbSNP as (rs56149945) is results in an asparagine (N) to serine (S) modification in the amino acid in codon 363, the molecular mechanism through which the N363S exerts its effects is unknown. N363S has been associated with a clinical phenotype consistent with increased glucocorticoid sensitivity.
B. **ER22/23EK:** The two linked polymorphisms in codons 22 and 23 of the GR gene, GGA.GAG to GAA.AAG lead to one change in amino acid (glutamic acid

(E).arginine(R) to glutamic acid (E).Lysine (K)). The ER22/23EK (rs6189 + rs6190) polymorphism (sometimes referred to as R23K) affects GC sensitivity in a remarkable way. It has previously been shown that both AUG-1 and AUG-27 (and other AUG-codons further downstream) in the GR mRNA are used as translation start sites and that the GR protein resulting from translation starting at AUG-27 (termed GR-B) has greater transactivating capacity than that starting from AUG-1 (termed GR-A). The ER22/23EK polymorphism induces changes in the secondary structure of the GR mRNA that lead to a shift in the balance between usages of AUG-1 an AUG-27 in favor of the less active GR-A (starting at AUG-1). Testing of the transactivating capacity, in both transfection experiments and in peripheral blood mononuclear lymphocytes of carriers of this polymorphism yielded results consistent with a reduced transactivating capacity, both in vitro and ex vivo. Transrepression, studied with a bioassay, measuring corticosteroid induced repression of interleukin-2 gene expression, seems to be unchanged. Alternatively or additionally, as for the N363S polymorphism this variant is also located in the N-terminal domain of the receptor protein and the amino acid change resulting from the ER22/23EK polymorphism may alter the interaction of the GR with other transcription factors. ER22/23EK has been associated with a clinical phenotype consistent with reduced glucocorticoid sensitivity.

C. **TthIII-1:** The Tth111I polymorphism (rs10052957), is located in 6305 bp upstream from the first initiation codon, in an intronic region between two of the alternative exons 1, which causes C/T change.

D. **BclI:** The BclI polymorphism (rs41423247) located in intron 2, 646 bp downstream of the end of exon 2, causes C/G change. However, the molecular function of this polymorphism is still unknown. BclI has been related to a clinical phenotype consistent with increased glucocorticoid sensitivity.

E. **GR-9beta:** GR-9b (rs6198) is an A to G nucleotide replacement positioned in the 30-UTR of exon 9b, the terminal exon of the mRNA of the b isoform (nucleotide 3669 in X03348; rs6198). The A to G nucleotide replacement is located in an ‘ATTTA’ motif (changing it to GTTTA). This ‘ATTTA’ motif is known to destabilize mRNA and decrease receptor protein expression in vitro. The GRb splice variant has been reported to have a dominant negative effect on GRb action. In vitro data show that the GR-9b polymorphism leads to a more stable GRb mRNA and possibly to a relative GC resistance. Ex vivo, GC-induced up-regulation of GILZ mRNA via transactivation did not significantly differ in GR-9b homozygotes, while the downregulation of IL-2

expression via transrepression was decreased. Polymorphism in GR-9b has been associated with a clinical phenotype consistent with effects of reduced glucocorticoid sensitivity on the immune system.

**Table 2.2.3.1. SNP selection:**

| SNP name | Accession id | Codon position | Amino acid position | Allele | dbSNP Allele | Protein residue | Functional Consequence |
|---|---|---|---|---|---|---|---|
| TthIIII | rs10052957 | | | [A/G] | | | Intron variant |
| ER22/23EK | rs6189 | 3 | 22 | [A/G/T] | T | Asp(R) | Pathogenic |
| | | | | | A | Glu(E) | |
| | | | | | G | Glu(E) | |
| | rs6190 | 2 | 23 | [A/C/G] | A | Lys (K) | |
| | | | | | C | Thr (T) | |
| | | | | | G | Arg(R) | |
| N363S | rs6195 | | | [A/G/T] | | | Likely benign |
| | | | | | | | |
| | | | | | | | |
| Bcl I | rs41423247 | | | [C/G] | | | Intron variant |
| GR-9beta | rs6198 | | | [A/G] | | | Likely benign |

**Table 2.2.3.2. Primer sequence:**

| Primers Name | Sequence (5'----3') | Total Size |
|---|---|---|
| ***TthIIII*** | For: TGAGGATCTTGAGGGTTATCTG | 22 bp |
| | Rev: TCCAGTGTGCCAGAAAGGA | 19 bp |
| **ER22/23EK** | For: CTCTTACTAATCGGATCAGG | 20 bp |
| | Rev: CTCTTAGGGTTTTATAGAAGTCC | 23 bp |
| **N363S** | For: TTAATGTCATTCCACCAGTTCCC | 23 bp |
| | Rev: CACTGATCTTACCTTGAATAGCC | 23 bp |
| ***BclI*** | For: TCAGGAGAGTATAATGTCTC | 20 bp |
| | Rev: CAGTTATCTACTTGAGAACTTG | 22 bp |
| **GR-9beta** | For1: GGAAATGTTTAACTTTTATTTTTGCA | 26 bp |
| | For2: GGAAATGTTTAACTTTTATTTTTGCG | 26 bp |
| | Rev: AAATCAGATTGGACAATCGGAA | 22 bp |

### 2.2.4. PCR-RFLP & Allele-specific PCR :

#### 2.2.4.1. PCR (polymerase chain reaction):

The polymerase chain response (PCR) is a laboratory method for DNA replication that permits an "objective" DNA arrangement to be specifically enhanced. PCR can utilize the slightest

example of the DNA to be cloned and open up it to a large number of duplicates in only a couple of hours. Found in 1985 by Kerry Mullis, PCR has turned out to be both basic and routine apparatus in most natural research facilities. However, it includes the groundwork interceded enzymatic enhancement of DNA. PCR depends on utilizing the capacity of DNA polymerase to incorporate new strand of DNA reciprocal to the offered format strand. Preliminary is required in light of the fact that DNA polymerase can include a nucleotide just onto a previous 3′-OH gathering to include the primary nucleotide. DNA polymerase at that point lengthens its 3' end by adding more nucleotides to produce an expanded locale of twofold stranded DNA.

**2.2.4.1.1. PCR procedure:**

- **Amplification:**

1. Before the preparation of master-mix, all the reagents except Taq polymerase have been spinned.

2. Then all the materials are mixed and spin again.

3. After that, the master-mix has been poured to the PCR tubes and mix it with the desired samples.

4. Reaction step started right after the denaturation of the template, during the initial denaturation process.

5. It will cool down during annealing temperature and primers will find their complementary regions to bind.

6. During, Extension process dNTP will find the sequence and extend it by adding nucleotide.

**• VALIDATION OF THE PCR:**

Once PCR reaction is processed to run, it will either succeed or fail. However, It will be properly evaluated during gel electrophoresis process. As a result, optimization plays a superior role during the PCR procedure.

- **CONTROL:**

Two types of controls are used during PCR. One is positive and another negative, for ensuring the PCR condition.

**2.2.4.1.2. Steps:**

**a. Denaturation:**

The DNA template is warmed to 95° C. This breaks the frail hydrogen bonds that hold DNA strands together in a helix, enabling the strands to isolate making single-stranded DNA.

**b. Annealing:**

The mixture is cooled to somewhere in the range of 50-70° C. This enables the primers to tie (temper) to their complementary sequence in the layout DNA.

**c. Extension:**

The response is then warmed to 72° C, the ideal temperature for DNA polymerase to act. DNA polymerase expands the primers, including nucleotides onto the preliminary in the following way, utilizing the objective DNA as a layout.

With one cycle, a solitary fragment of double-stranded DNA layout is enhanced into two separate bits of double-strand DNA. These two pieces are then accessible for amplification in the following cycle. As the cycles are repeated, an ever increasing number of duplicates are created and the quantity of duplicates of the format is expanded exponentially.

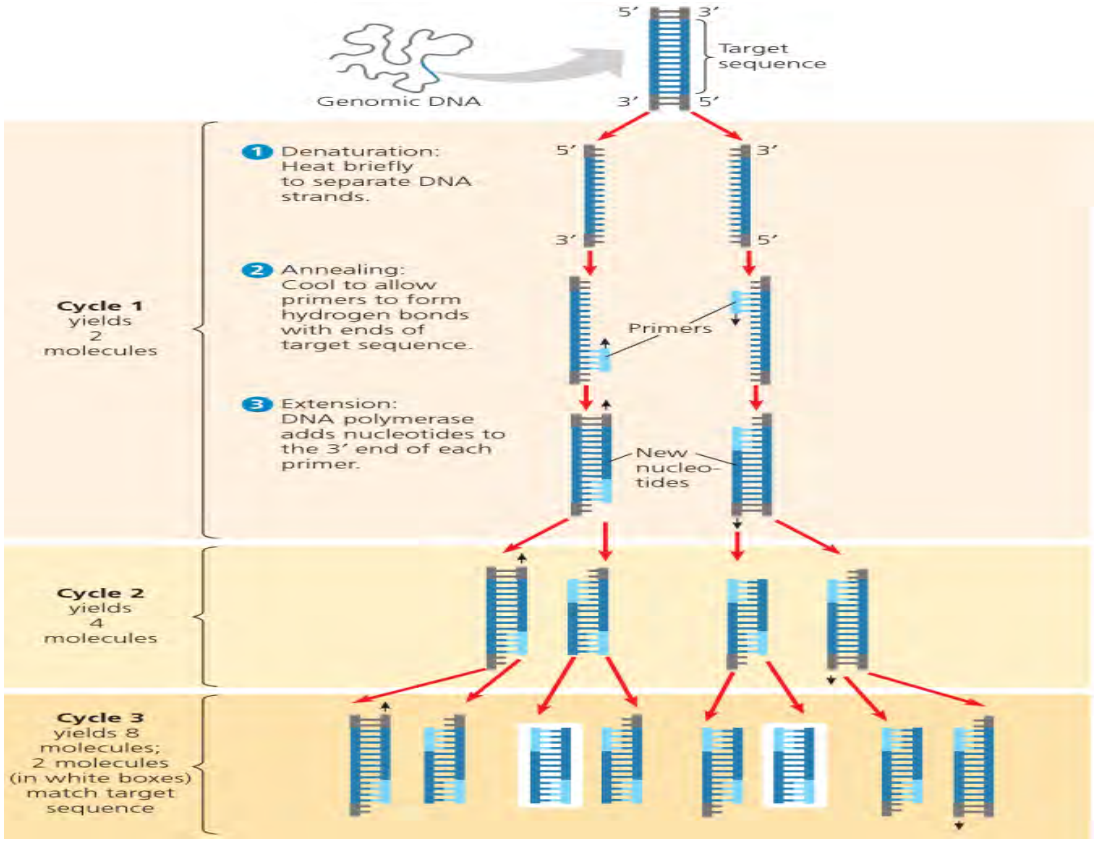



**Figure 2.1. Exponential amplification of polymerase chain reaction (PCR).**The illustration shows three basic steps along with cycle repetition.

### 2.2.4.2. PCR PROGRAM:

Five different PCR program has been set for TthIIII, ER22/23EK, N363S, BclI, GR-9beta. All of them are done using BIO-RAD, DNA Engine (Thermal Cycler) and Gradient cycler.

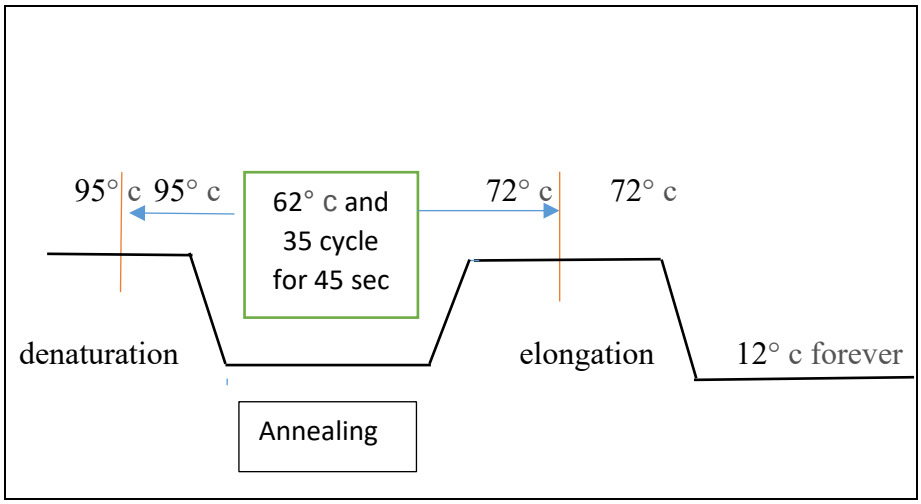
For above-mentioned types, the master mix for each of them is optimized differently along with the thermal programme amplification. These are given below :

1. **TTH111l:**

- Master mix preparation :

| **Master mix composition** | **Volume (µl)** |
|---|---|
| **Filtrated Deionized Water** | 10.5µl |
| **5X BUFFER** | 5.0 µl |
| **25mM Mgcl2** | 5.0 µl |
| **10mM dNTPs** | 1.0 µl |
| **Forward-primer** | 0.15 µl |
| **Reverse-primer** | 0.15 µl |
| **Taq polymerase** | 0.2 µl |
| **Template DNA** | 3.0 µl |
| **Total volume** | 25 µl |

- Thermal program :

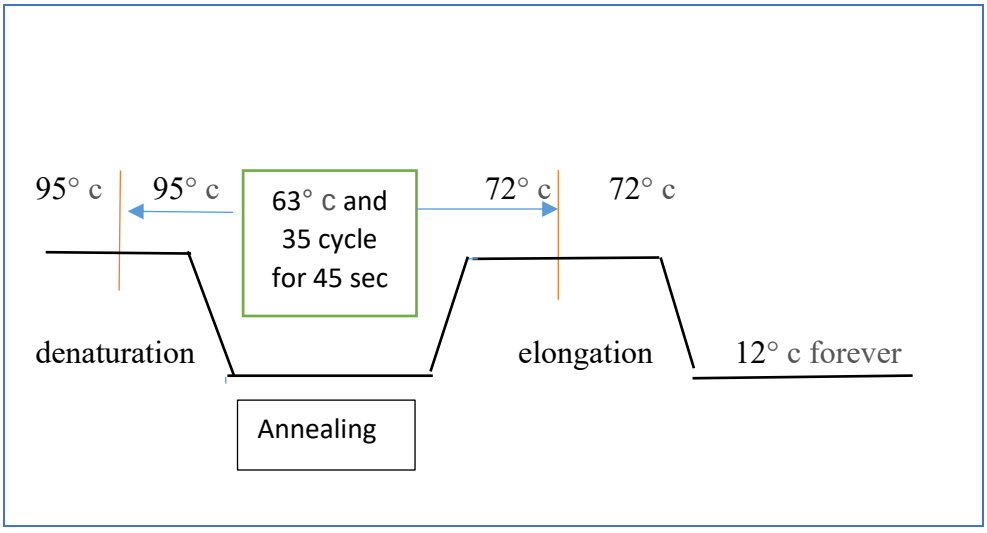


2. **N363S:**

- Master mix preparation :

| **Master mix composition** | **Volume (µl)** |
|---|---|
| **Filtrated Deionized Water** | 10.5µl |
| **5X BUFFER** | 5.0 µl |
| **25mM Mgcl2** | 5.0 µl |
| **10mM dNTPs** | 1.0 µl |
| **Forward-primer** | 0.15 µl |
| **Reverse-primer** | 0.15 µl |
| **Taq polymerase** | 0.2 µl |
| **Template DNA** | 3.0 µl |
| **Total volume** | 25 µl |

- **Thermal program :**

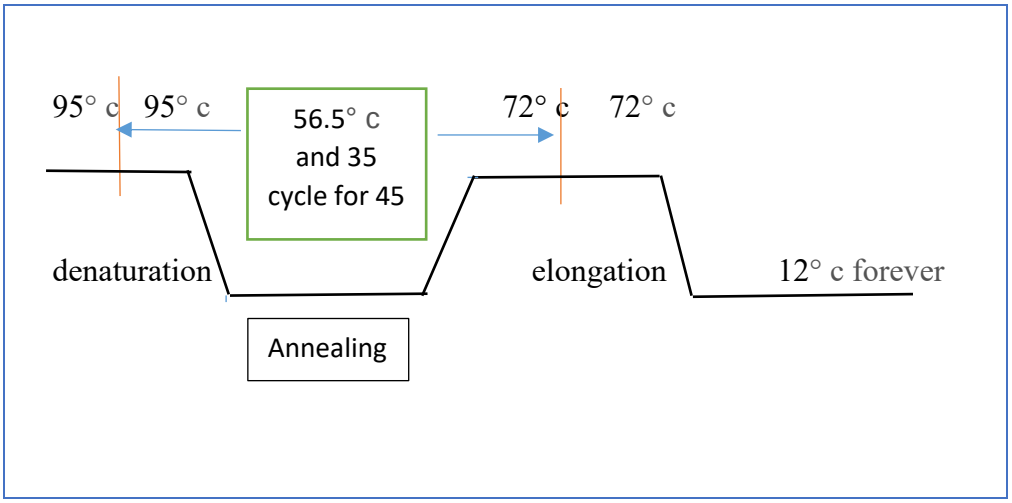


### 3. BCL1:

- Master mix preparation :

| Master mix composition | Volume (µl) |
|---|---|
| **Filtrated Deionized Water** | 10.5µl |
| **5X BUFFER** | 5.0 µl |
| **25mM Mgcl2** | 5.0 µl |
| **10mM dNTPs** | 1.0 µl |
| **Forward-primer** | 0.15 µl |
| **Reverse-primer** | 0.15 µl |
| **Taq polymerase** | 0.2 µl |
| **Template DNA** | 3.0 µl |
| **Total volume** | 25 µl |

- **Thermal program :**

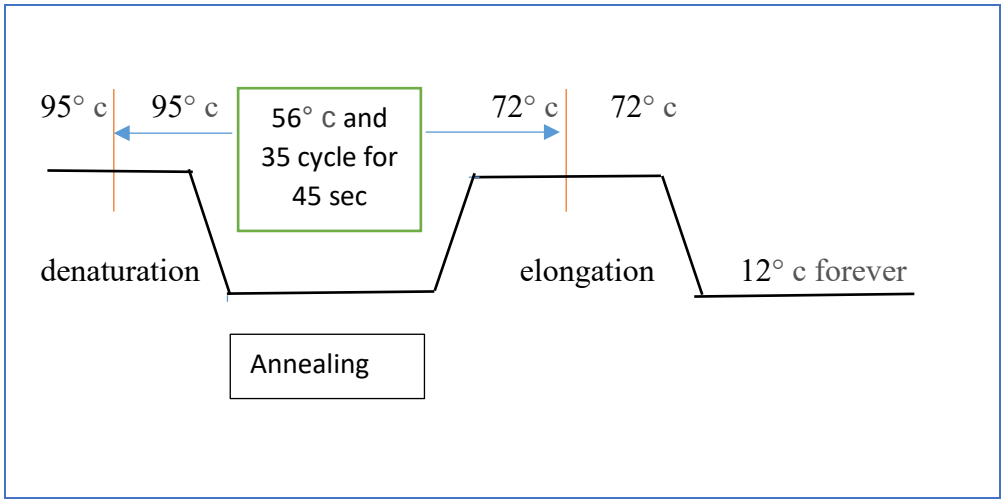


4. **ER22/23EK:**

- Master mix preparation :

| **Master mix composition** | **Volume (µl)** |
|---|---|
| **Filtrated Deionized Water** | 10.5µl |
| **5X BUFFER** | 5.0 µl |
| **25mM Mgcl2** | 5.0 µl |
| **10mM dNTPs** | 1.0 µl |
| **Forward-primer** | 0.15 µl |
| **Reverse-primer** | 0.15 µl |
| **Taq polymerase** | 0.2 µl |
| **Template DNA** | 3.0 µl |
| **Total volume** | 25 µl |

- **Thermal program :**

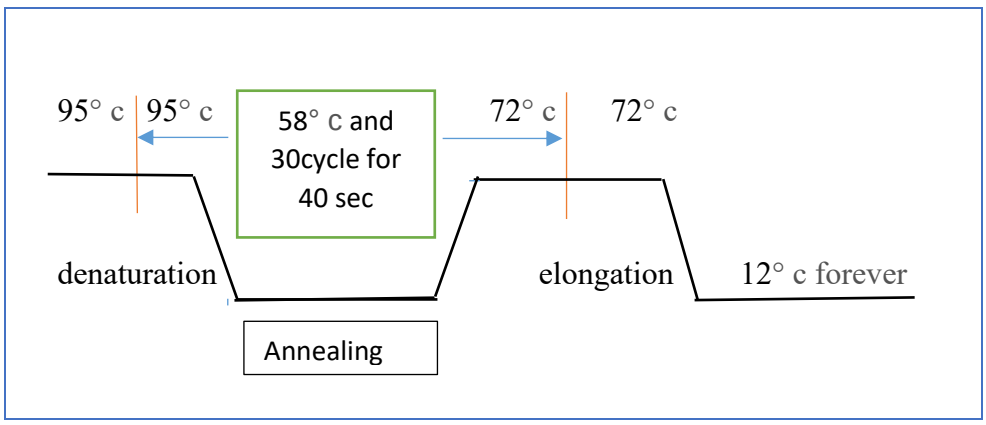


**5. GR-9BETA:**

- Master mix preparation :

| Master mix composition | Volume (µl) |
|---|---|
| **Filtrated Deionized Water** | 10.5µl |
| **5X BUFFER** | 5.0 µl |
| **25mM Mgcl2** | 5.0 µl |
| **10mM dNTPs** | 1.0 µl |
| **Forward-primer** | 0.15 µl |
| **Reverse-primer** | 0.15 µl |
| **Taq polymerase** | 0.2 µl |
| **Template DNA** | 3.0 µl |
| **Total volume** | 25 µl |

- **Thermal program:**



**2.2.4.1.3. RFLP:**

Restriction Fragment Length Polymorphism (RFLP) is a strategy in which living beings might be separated by investigation of examples got from cleavage of their DNA. In the event that two living beings contrast out there between locales of cleavage of a specific restriction endonuclease, the length of the pieces delivered will contrast when the DNA is processed with a restriction enzyme. The familiarity of the examples produced can be utilized to separate species (and even strains) from each other. However, the fragments can be separated by gel electrophoresis.

**The procedure of completing RFLP:**

**a)** At first, we will take sterile Eppendorf (1.5 ml) to prepare master-mix which includes a buffer, NFW, an enzyme. All the reagents must keep on ice.

**b)** Secondly, for each Eppendorf, we will dispense13 ul of mastermix to mix with 7ul of PCR product. All of them must pipette properly for a homogeneous mixture.

**c)** Once all this process is done, all the samples will be incubated at 37 degrees for two hours in a water bath.

**d)** After incubation, the result will be examined by using 2% agarose gel at 80V. In this process, we will not add a buffer as it is already mixed with PCR product.

**e)** Since the restriction fragments have a negative charge, so it can be separated by gel electrophoresis. However, it will separate the pieces of DNA based on their size.

**f)** When the samples will migrate a sufficient distance through the gel, the machine will be turned off and gel will be observed under UV light for taking a gel image.

**Table 2.2.4.1.4. RESTRICTION ENZYME SELECTION:**

| SNP NAME | RE | Cutting sites | Catalog | Size(UNITS) | Genotypes: Band Size (bp) |
|---|---|---|---|---|---|
| TthIIII | ***TthIIII*** | 5'-GACNNNGTC-3'<br>3'-CTGNNNCAG-5' | R0185S | 400 | CC : 482bp<br>CT:482,271,211bp<br>TT: 271 and 211bp |
| ER22/23EK | ***MnlI*** | 5'-CCTC(N)7-3'<br>3'-GGAG(N)6-5' | R0163S | 500 | **A**A**A**: 197bp<br>**G**A**G** & **A**A**A**: 197, 152,45bp<br>**G**A**G**: 152, 45bp |
| N363S | ***MluCI*** | 5'-AATT-3'<br>3'-TTAA-5' | R0538S | 1000 | GG:145bp<br>AG :145bp, 110bp, 35bp<br>AA :110bp ,35bp |
| BclI | ***BclI*** | 5'-TGATCA-3'<br>3'-ACTAGT-5' | R0160S | 3000 | CC:431bp<br>CG:431bp,338bp,93bp<br>GG:338bp, 93bp |

### 2.2.4.2. ALLELE-SPECIFIC PCR:

Allele-specific PCR is a type of reaction that allows direct detection of a point mutation in DNA by using common reverse primer and two forward primers with different tails amplify two allele-specific –PCR products of same or different length, which are further separated by agarose gel electrophoresis. Due to the increasing need for large-scale genotyping for SNP in typical or atypical organism's requirements, the development of the simple and inexpensive yet effective method is allele-specific PCR.

### 2.2.4.3.GEL ELECTROPHORESIS:

In molecular biology, it is the process of separating gel and identifying molecules according to its size. For this technique, things which are required are given below :

- TBE/TAE buffer as the electrophoresis buffer
- Agarose powder for making gel
- Ethidium bromide for staining
- Deionized water as a destaining gel
- Sample combs for making wells on the gel
- Gel casing tray for pouring gel and solidifying gel. Usually, it is UV-transparent plastic.
- Transilluminator (an ultraviolet lightbox )
- Gel running machine

- **Preparation of gel**
  - **a)** First of all, the gel tray must be rinsed well and dried before it is ready to use (75 % ethanol ).
  - **b)** Secondly, the tray must be taped at sides of the trays to avoid leakage. However, sample combs must be set on the level surface.
  - **c)** The gel powder must weigh in the measurement machine according to the percentage of gel and mix with 100 ml TBE/TAE buffer and melt it for 1.30 mins. However, It has to mix completely with the buffer, so that no particles are left. For checking PCR product 1.5% and for RFLP 2% gel will be used.
  - **d)** After melting the powder with buffer, it must be poured on the gel tray and set it for cool down about 15-20 minutes.
  - **e)** When the gel is solidified, the comb and tap both are ready to be removed and the gel is ready to merge in the buffer solution.
- **Loading and running gel**
  - **a)** No loading dye will be used as it is already mixed with the sample.
  - **b)** All the samples must spin and pipette properly before they are loaded on the wells.
  - **c)** The well must be closed to the negative (black) electrode as DNA is negatively charged. During, electrophoresis, it will move from negative to positive charge.

**d)** The buffer must be poured on the chamber in a manner where the gel will be covered submerged into it.

**e)** After loading samples in wells carefully avoiding bubbles, it should be run by plugging into the power supply and adjusting the volt at 120.

**f)** The gel must run till the bromophenol blue shows up through the gel and after that, it can be turned off for further procedures.

**g)** After switching off the power supply, the machine must be carefully unplugged and the gel tray must be lifted from the chamber.

- **Staining and photographing gel**

**a)** For, staining solution, ethidium bromide is mixed with autoclaved water and tilted carefully for mixing.

**b)** Once it is ready the gel has been carefully placed in the solution from the tray. During this time, the tray must avoid touching the solution as ethidium bromide is carcinogenic.

**c)** Furthermore, the gel must keep in staining for 20 minutes.

**d)** After that, it is ready for destaining for 5-10 minutes.

**e)** At, the end, when the destaining will be completed, the gel is ready for capturing an image under UV light. The entire process will be processed by using the Gel-doc machine.

**f)** When the process is finished, the gel will be discarded to bio-hazard box.

**g)** In addition, the entire steps will be done wearing gloves and lab coat to avoid all terms of hazard.

**h)** If staining or destaining solutions are required to change, it must be discarded to EtBr-DISCARD and destaining -DISCARD bottle.

### 2.3. Detection of polymorphism in the glucocorticoid receptor gene:

The detection or observation has been completed by performing the methods accordingly and observing them after PCR-RFLP and ASO-PCR by gel electrophoresis and comparing the band sizes in the confirmation of the premises.

**2.4.Statistical analysis :**

All the analysis has been performed with the help of Logistic regression analysis in R statistics. The comparison of every association with significant categories is observed by using OR, 95% CI, and P value. In the end, haplotype analysis and pair-wise Linkage disequilibrium have been performed based on D'-Value and P-value as well. All of these analysis includes 71 population of Bangladesh which comprises with a patient with GBS and healthy control.

### 3.1.PCR-RFLP :

The result includes five SNPs of the GR gene in 71 population of Bangladesh which is initially studied by using ASO primer and PCR-RFLP method. Four SNPs named N363S, ER 22/23Ek, BCIl, TthIIIl are studied by PCR –RFLP method where GR-9beta (Figure 3.8) are studied using ASO primer followed by gel electrophoresis which determines the difference between wild-type and variant type by diverse band sizes along with allele alteration. Following this step, for N363S the PCR product size is 145bp (Figure3.1.) which gets separated into 110 bp and 35 bp(AA) after RFLP(Figure 3.2). However, for ER22/23EK the product size is 197 bp (Figure 3.3.) which divided into 152, 45 bp (GAG) (Figure 3.4.) and for BclI the PCR product is 431bp(Figure 3.3.) that has been divided into three groups which are 431bp(CC); 431 bp,338 bp,93 bp (CG); 338 bp and 93 bp (GG) (Figure 3.5). Subsequently, TthlllI is also separated with the similar method from 482 bp PCR product (Figure 3.6) to three groups after RFLP and these are 482bp(CC);482bp,271bp,211bp(CT); 271bp,211bp(TT) (Figure 3.7.).All the data has been interpreted by performing Logistic regression analysis, haplotype frequencies and pair-wise Linkage disequilibrium between a patient with GBS and healthy control of 71 Bangladeshi population.

### 3.1.N363S:

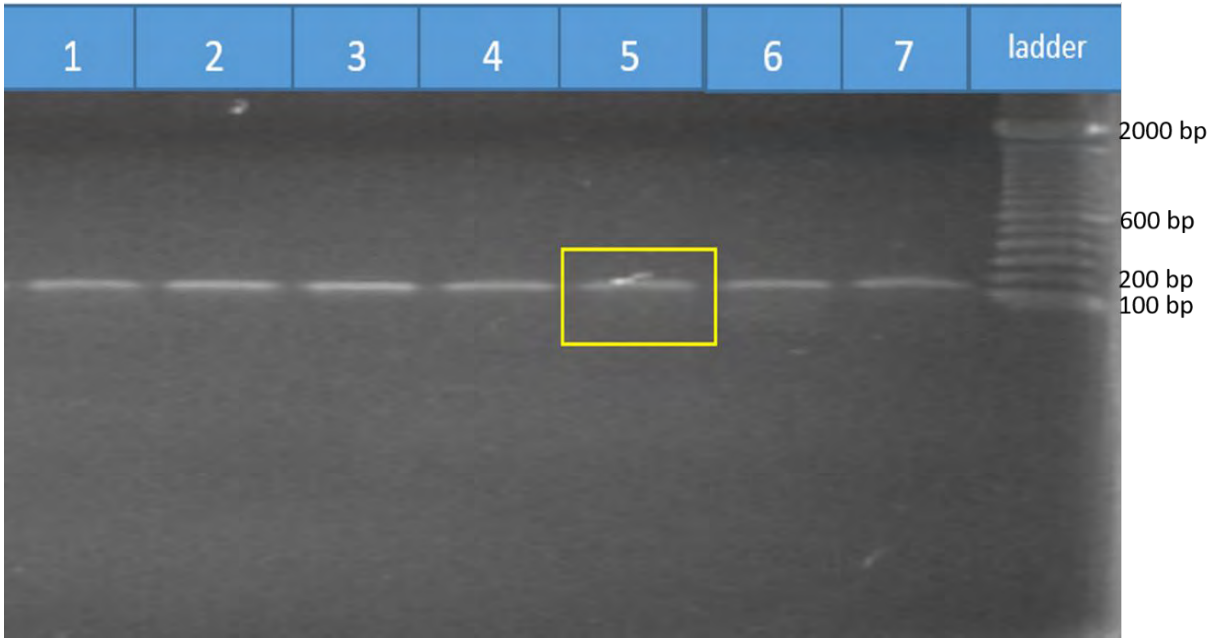



**Figure 3.1.N363S Agarose gel electrophoresis (1.5%) PCR image.**The image shows 145bp bp of DNA (PCR Product) marked yellow in color and the ladder starts from 100bp which is marked in orange color.

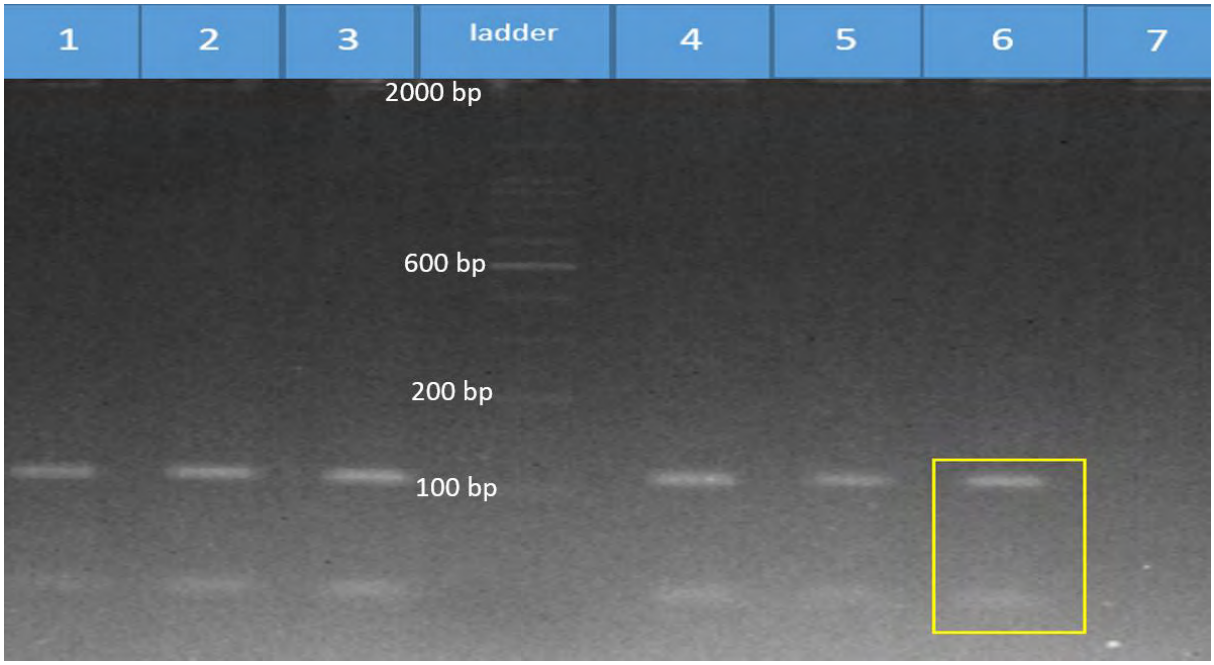



**Figure 3.2.N363S RFLP Agarose gel electrophoresis (2.5%) image .**This image shows RFLP product of 145bp bp of DNA band that has been cut into 110 bp and 35 bp (AA), marked as yellow in color. The RFLP product resembles no polymorphism in the result. In addition, the ladder starts from 100bp where number 7 represents negative control in the gel image.

**3.2.BclI & ER22/23EK :**

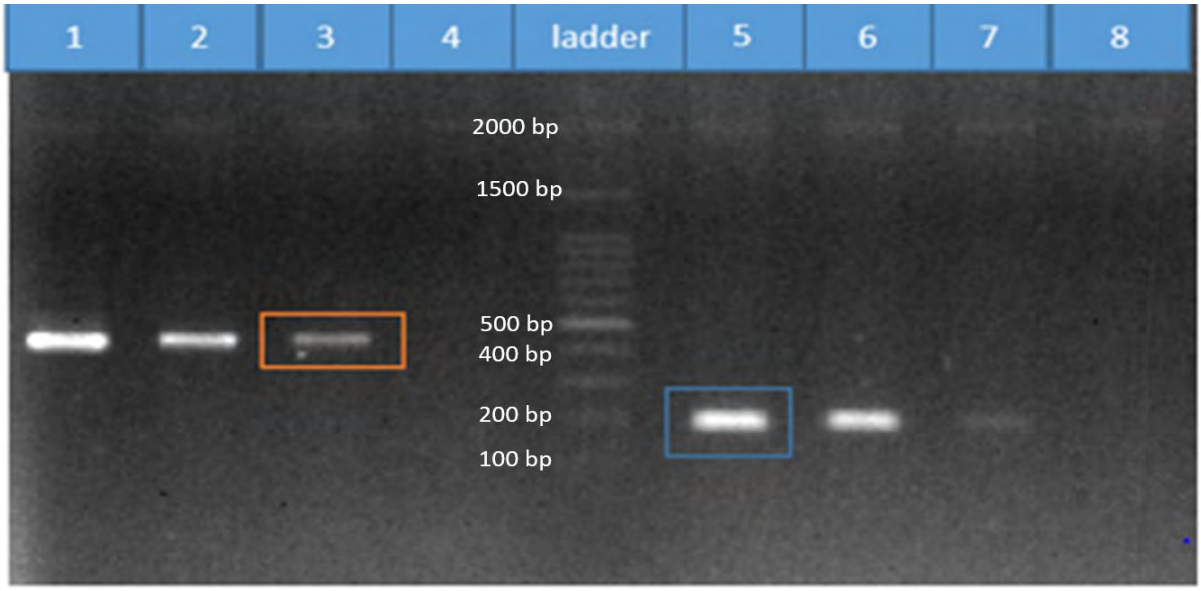



**Figure 3.3.BclI and ER22/23EK PCR Agarose gel electrophoresis (1.5%) image.** Two different sizes of bands (PCR product )are captured and marked in orange (BclI=431bp) and blue (ER22/23EK=197bp) color. In addition, Number 4 and 8 shows negative control with no band. Here ladder starts from 100bp.

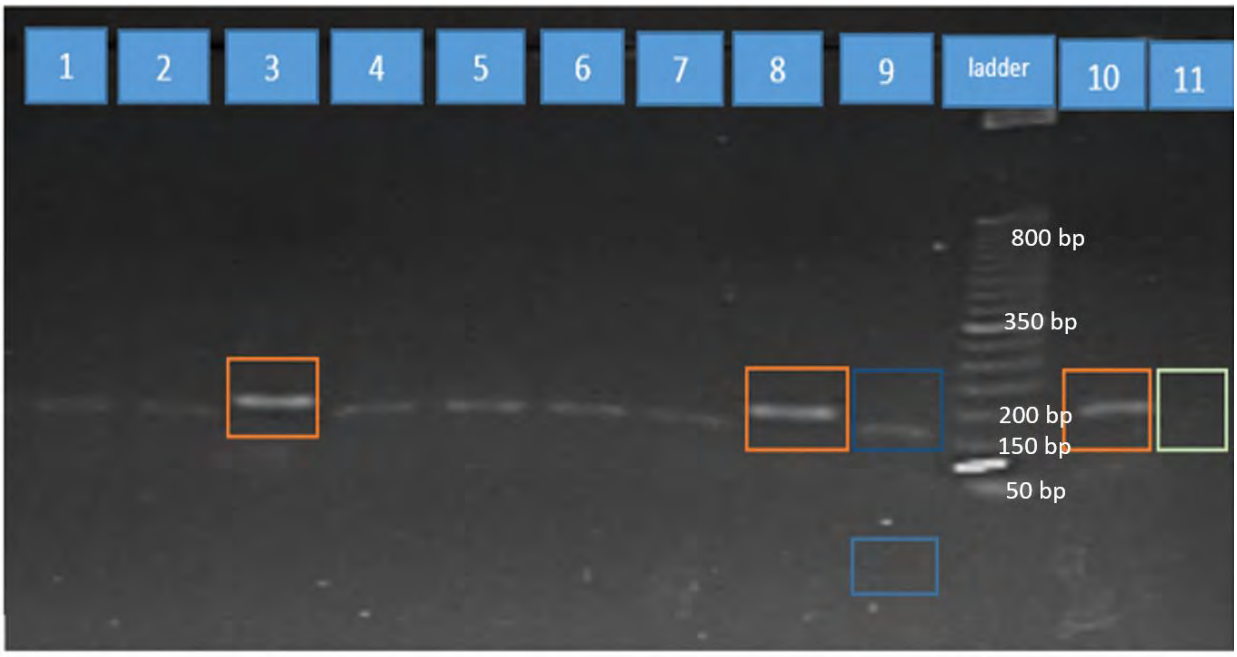



**Figure 3.4. ER22/23EK Agarose gel electrophoresis (2.5%) RFLP image**.It shows 197 bp of DNA band where GAG (152 and 45 bp) is marked as orange in color and Negative control is green in color. In addition, the ladder starts from 50bp.

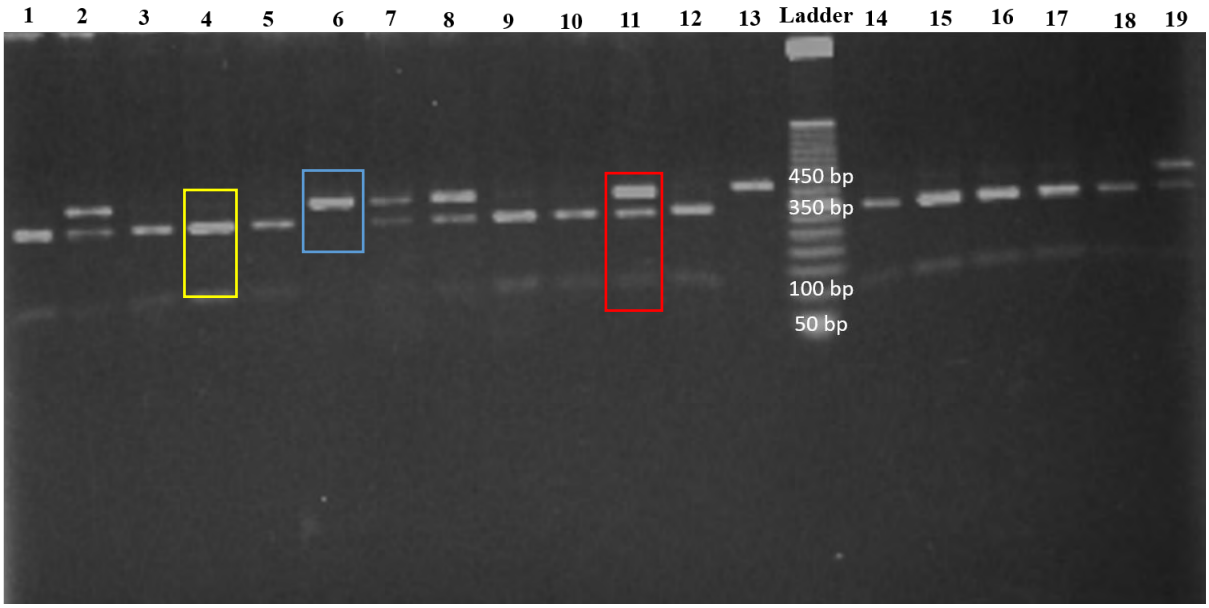



**Figure 3.5. BclI Agarose gel electrophoresis(2.5%) RFLP image.**The Image shows 431 bp of DNA band where GG(338 and 93 bp) is yellow in color and CG (431, 338 and 93 bp) is red in color and CC (431 bp) is blue in color. In addition, the ladder starts from 50bp.

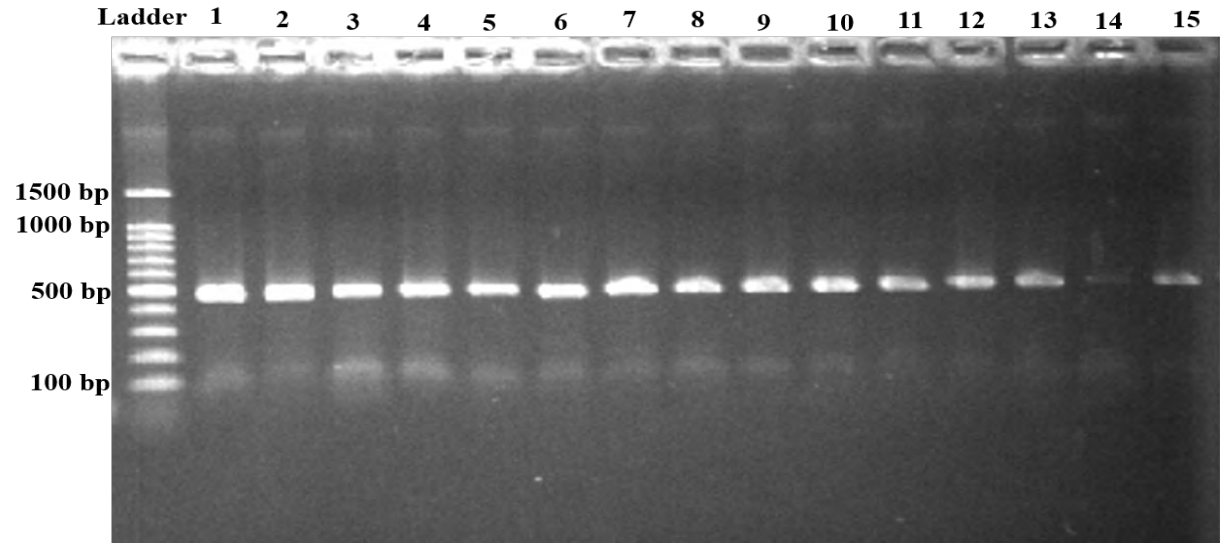



**Figure 3.6. TthIIII Agarose gel electrophoresis PCR (1.5%) image.** It shows 482 bp of DNA band where it is marked as red in color and ladder is marked as yellow in color which starts from 150bp.

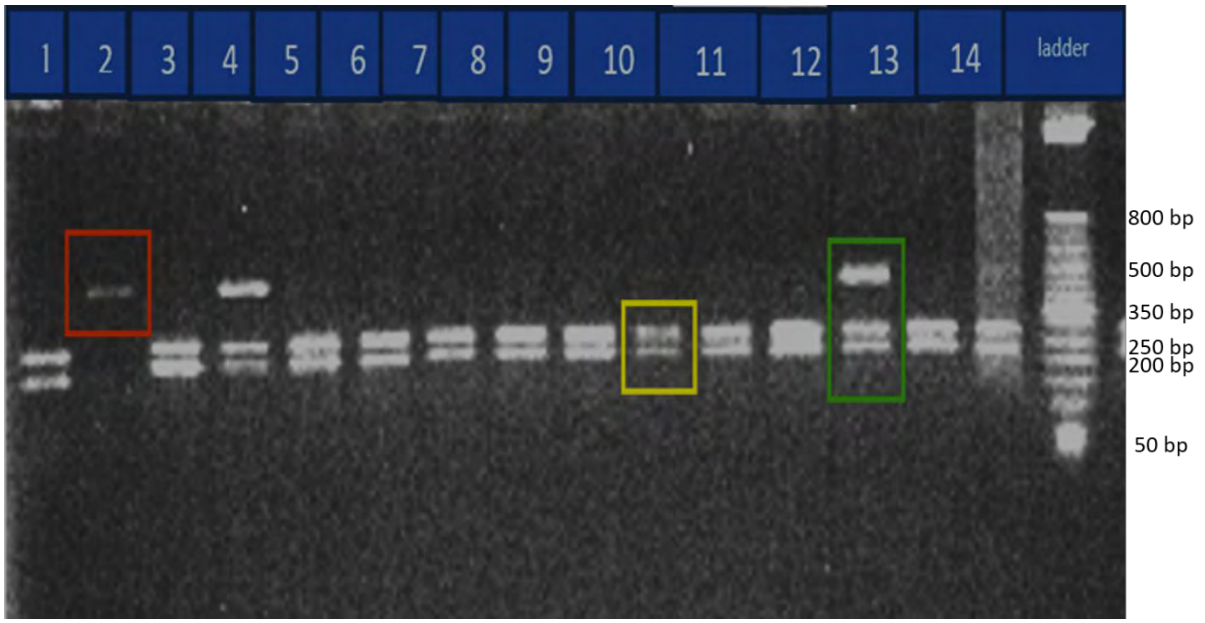



**Figure 3.7. TthIIII Agarose gel electrophoresis RFLP (2.5%) image.** Which shows 482 bp of DNA band where TT(271 and 211 bp) is yellow in color and CT (482,271 and 211 bp) is green in color and CC (482 bp) is orange in color. In addition, the ladder starts from 50bp

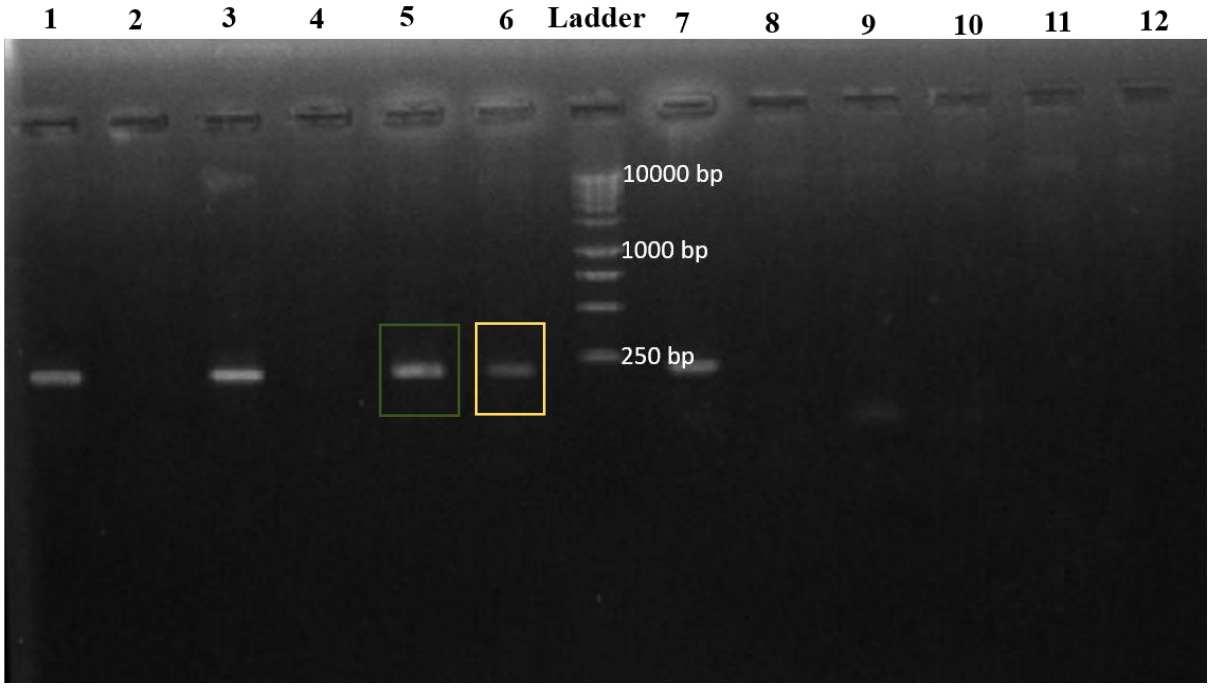



**Figure 3.8. GR-9 beta Agarose gel electrophoresis(2.5%) PCR image.**The image shows 208bp of DNA band where forward 1 is green in color and forwards 2 is yellow in color.

### 3.2.Individual association with GBS :

In the current study, at first individual association with GBS has been observed (Table 3.1.). However, the CI level does not show significant value because it has ranged more than 1 to less than 1. The study shows no association with GBS (Table3.1.) because the P-values are not significant for any SNPs. Additionally, ER22/23EK and N363S have shown no result due to one type of genotype presence for 71 population.

**Table 3.1.Individual association with GBS:**

| Gene name | genotype | OR | 95% CI | P value |
|---|---|---|---|---|
| GR 9-Beta | A/A | 0.51 | 0.15-1.74 | 0.281 |
| | A/G | 1.97 | 0.57-6.75 | 0.281 |
| | G/G | - | - | - |
| Bcll | C/C | 2.25 | 0.2-26 | 0.516 |

| | | | | |
|---|---|---|---|---|
| | C/G | 0.61 | 0.22-1.64 | 0.328 |
| | G/G | 1.56 | 0.6-4.05 | 0.363 |
| TthIIIl | C/C | 1.809689e+07 | 0.0-Inf | 0.992 |
| | C/T | 1.29 | 0.48-3.5 | 0.617 |
| | T/T | 0.66 | 0.26-1.81 | 0.446 |
| ER22/23Ek | G/A | - | - | - |
| | G/G | - | - | - |
| | A/A | - | - | |
| N363S | A/A | - | - | - |
| | G/G | - | - | - |
| | A/G | - | - | - |

### 3.3. Haplotype analysis:

All the five SNP then studied for their haplotype structure. The frequencies of haplotype alleles are performed by categorizing 18 numerous patterns among 243 patterns in the 71 population study (Figure 3.9). Thus, 6 possible haplotypes are observed to be most common with 18 different patterns in this study. These 6 haplotypes (Figure 3.10) includes 5 SNPs of GR gene where 31% is haplotype 1, 22.53% is haplotype 2, 15.49% is haplotype 3, 7.04% is haplotype 4, 5.63% is haplotype 4 and 5.63% is haplotype 5. The haplotype 1 is characterized by the presence of T/T allele of the TthIIIl and G/G of the BclI where the haplotype 2 has a combination of T/T allele of the TthIIIl, C/G of the BclI and haplotype 3 with the combination of C/T allele of the TthIIIl, C/G of the BclI. In the haplotype 4, the arrangements include C/T allele of the TthIIIl, G/G of the BclI, and A/G of GR-9 beta. On the other hand, both haplotype 5 and 6 are 5.63 % frequent in the population but haplotype 5 happened due to the presence of C/T allele of the TthIIIl and C/G of the BclI, haplotype 6 with the combination of T/T allele of the TthIIIl, G/G of the BclI and A/G of GR-9 beta.

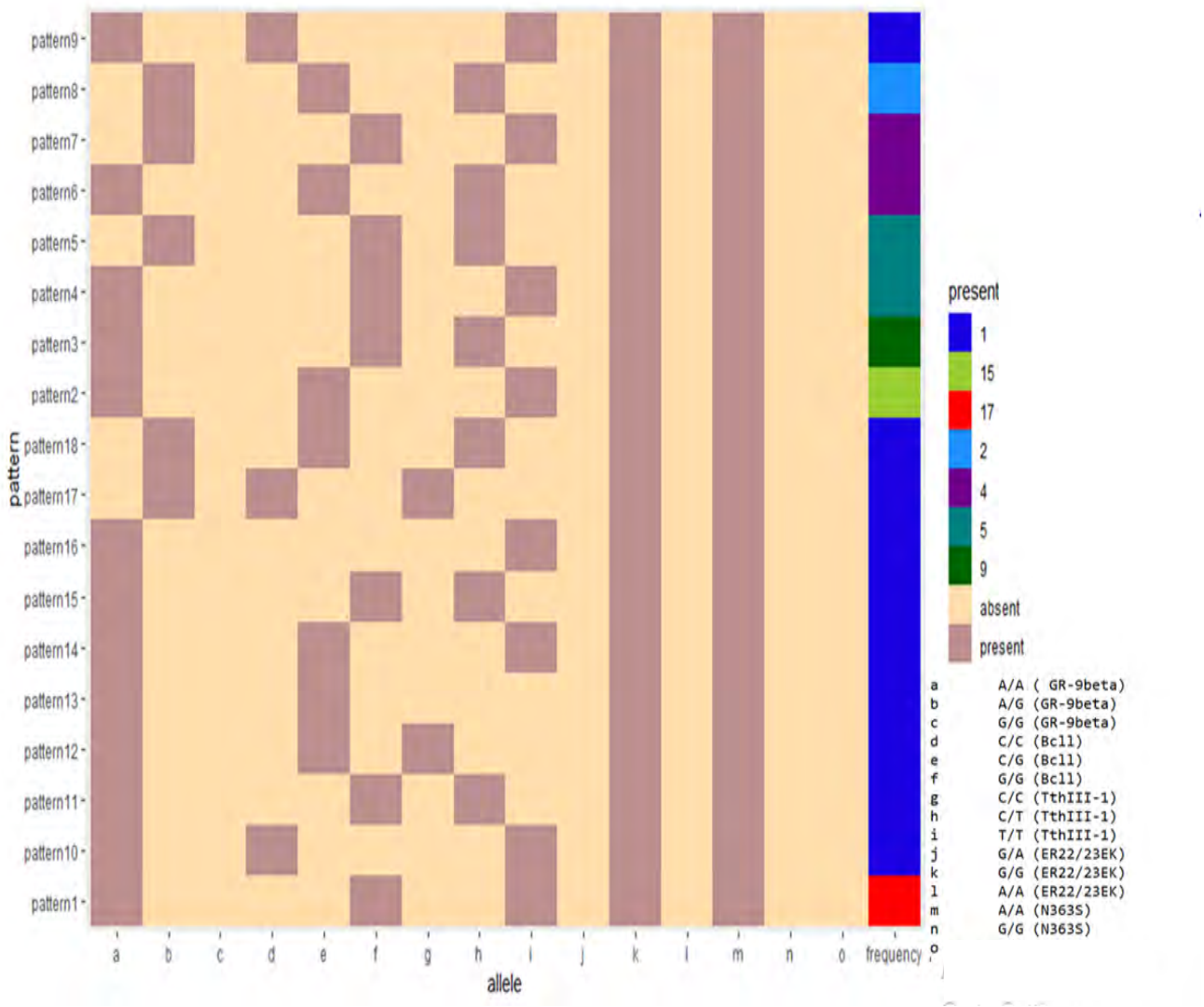



**Figure 3.9: 18 different output find in 71 population for 5 different polymorphism in the GR gene.** In the left corner, it shows the frequencies of presence and absence of a particular pattern with different genotype in our population.


**Figure 3.10. : Haplotype frequencies of the glucocorticoid receptor gene**. The analysis includes 6 possible haplotypes using 5 SNPs in 71 population with a total frequency of 77 % where haplotype 1 is most frequent by 31 %, haplotype 4 and 5 are least frequent with 5.63 %.

### 3.4. Linkage disequilibrium (LD):

Pair-wise linkage disequilibrium is performed by comparing with the level of D' value. As a result, only N363S with BclI and GR-9beta and ER22/23EK with TthIIIl shows strong association to be present together in a population because according to D' value the more it's nearer to 1 the more significant it demonstrates (Figure 3.11).On the other hand, other pairs colored as green or orange, are not strongly associated because of their very low D'value.In contrast, the pairs which illustrated strong association are not significant in terms of P-value (Figure 3.11).

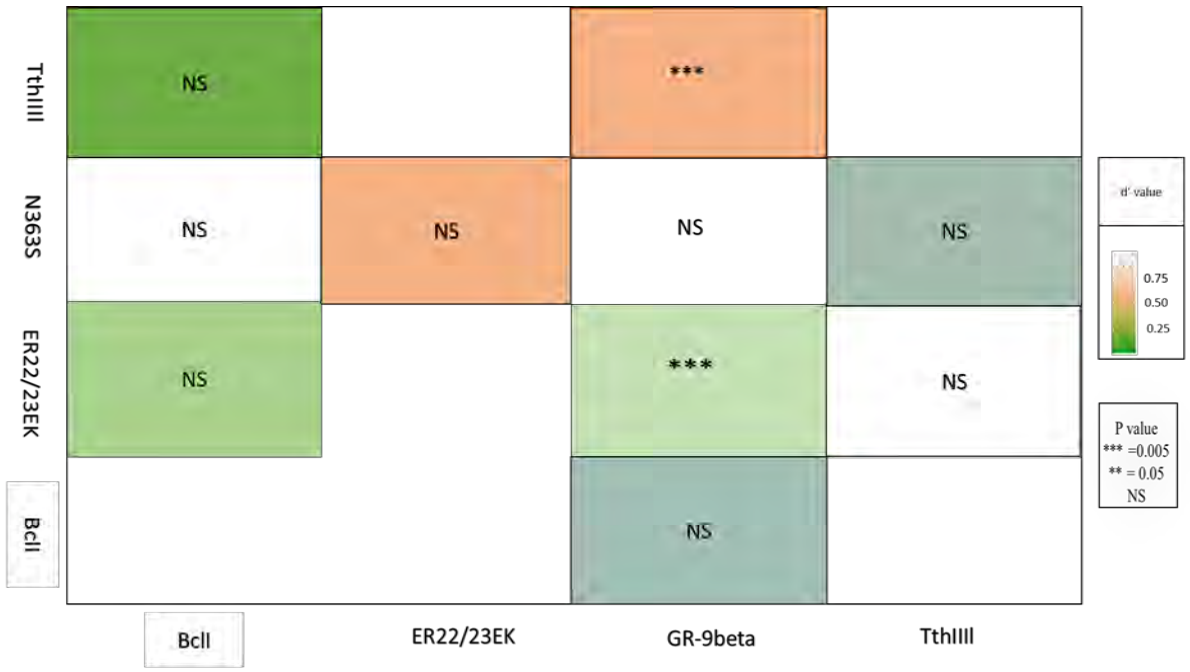



**Figure 3.11. Pair-wise Linkage disequilibrium illustration.** According to D'-value, only three pairs comprising N363S & BclI, N363S & GR-9beta, and ER22/23EK & TthIIIl show a strong association, even though their P-values are not significant. Others labeled as orange and green do not have a strong association with each other.

### 3.5.Individual associations of alleles with GBS :

After haplotype analysis (Figure 3.10) the individual association of haplotype with GBS has shown no association with GBS (Table3.2.) because the CI level does not show significant value as it has ranged between more than 1 to less than 1. However, the P-values are not significant as well. Additionally, ER22/23EK and N363S have shown no result due to one type of genotype presence for 71 population.

### 3.2. Table: Hap vs GBS:

| Hap vs GBS | OR | 95% CI | P value |
|---|---|---|---|
| **Hap_1 vs GBS** | 1.130 | 0.413-3.093 | 0.811 |
| **Hap_2 vs GBS** | 0.578 | 0.184-1.812 | 0.374 |
| **Hap_3 vs GBS** | 1.371 | 0.377-4.985 | 0.631 |
| **Hap_4 vs GBS** | 1.693 | 0.265 -10.806 | 0.577 |

| | | | |
|---|---|---|---|
| **Hap_5 vs GBS** | 0.343 | 0.033-3.471 | 0.365 |
| **Hap_6 vs GBS** | 1.093 | 0.145-8.226 | 0.931 |

### 3.6. Individual associations of alleles with autoantibody:

All the associations have been compared with OR (odd-ratio), 95% CI (95% Confidence Interval) and P-value (≤ 0.05). For the association of Allele with autoantibody in table 3.3 and table 3.4, only BclI (C/G) with autoantibody GD1a shows a positive association (p=0.024, OR, 6.94; 95% CI, 1.28-37.58) (Table 3.4). However, for ER22/23EK and N363S, the result cannot be deduced, caused by a similar outcome for individuals in 71 population ( Table3.3, 3.4).

### 3.3. Allele association with autoantibody:

| Gene name | Genotype vs GM1 | OR | 95% CI | P value |
|---|---|---|---|---|
| **GR 9 Beta** | A/A | 0.87 | 0.21-3.66 | 0.849 |
| | A/G | 1.15 | 0.27-4.84 | 0.849 |
| | G/G | - | - | - |
| **BclI** | C/C | 2.257525e-07 | 3 | 0.991 |
| | C/G | 0.61 | 0.17-2.15 | 0.438 |
| | G/G | 2.21 | 0.63-7.82 | 0.215 |
| **TthIII-1** | C/C | 2.300119e-07 | 0.0-inf | 0.993 |
| | C/T | 1.06 | 0.314-33.543 | 0.90 |
| | T/T | 1.2 | 0.36-3.99 | 0.766 |
| **ER 22/23EK** | G/A | - | - | - |
| | G/G | - | - | - |
| | A/A | - | - | |
| **N363S** | A/A | - | - | - |
| | G/G | - | - | - |
| | A/G | - | - | - |

### 3.4. Allele association with autoantibody:

| Gene name | Genotype vs GD1a | OR | 95% CI | P value |
|---|---|---|---|---|
| **GR 9 Beta** | A/A | 0.63 | 0.112-3.57 | 0.6059 |
| | A/G | 1.57 | 0.28-8.86 | 0.606 |
| | G/G | - | - | - |
| **BclI** | C/C | 1.762847e-07 | 0.0-inf | 0.995 |
| | C/G | 6.94 | 1.28-37.583 | 0.0244 * |
| | G/G | 0.1916667 | 0.04- 1.03 | 0.054 |
| **TthIII-1** | C/C | 4.871781e-07 | 0.0-inf | 0.993 |
| | C/T | 0.67 | 0.123-3.59 | 0.637 |
| | T/T | 1.84 | 0.34-9.89 | 0.474 |
| **ER22/23EK** | G/A | - | - | - |
| | G/G | - | - | - |
| | A/A | - | - | - |
| **N363S** | A/A | - | - | - |
| | G/G | - | - | - |
| | A/G | - | - | - |

**'*'Significant code of P–value (≤ 0.05).**

**Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

### 3.7. Table: Hap vs subtype, outcome, *c.jejuni*, *c.jejuni*+AMAN:

#### 3.7.1. Hap vs type:

The following table shows no association with subtypes of GBS. Here, as a subtype, only AMAN has been identified. Since the CI are varying between more than 1 to less than 1, even though the OR shows positive association among Hap1 vs type, Hap4 vs type Hap6 vs type. However, none of them shows significant P value.

**3.5.Table Hap vs subtype:**

| Hap vs type | OR | 95% CI | P value |
|---|---|---|---|
| **Hap1 vs type** | 1.904 | 0.432-8.387 | 0.394 |
| **Hap2 vs type** | 0.309 | 0.031-3.012 | 0.3122 |
| **Hap3 vs type** | 0.900 | 0.139- 5.811 | 0.911 |
| **Hap4 vs type** | 4.199 | 0.339- 51.972 | 0.263 |
| **Hap5 vs type** | 2.457476e-08 | 0-Inf | 0.997 |
| **Hap6 vs type** | 1.909 | 0.108-33.537 | 0.658 |

#### 3.7.2. Hap vs outcome:

The following study only shows a significant association between haplotype 3 with the outcome (p=0.008, OR, 0.06; 95% CI, 0.007-0.478), otherwise, no significant association has been observed for additional Haplotype and Outcome.

**3.6.Table Hap vs outcome:**

| Hap vs outcome | OR | 95% CI | P value |
|---|---|---|---|
| **Hap1 vs outcome** | 1.25 | 0.201 -7.750 | 0.811 |
| **Hap2 vs outcome** | 3.304251e+07 | 0-Inf | 0.994 |
| **Hap3 vs outcome** | 0.060 | 0.007- 0.478 | 0.00794 ** |

| | | | |
|---|---|---|---|
| **Hap4 vs outcome** | 3.929575e+07 | 0-Inf | 0.997 |
| **Hap5 vs outcome** | 1.209100e+07 | 0-Inf | 0.997 |
| **Hap6 _outcome** | 0.230 | 0.012-4.238 | 0.323 |

**Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

### 3.7.3.Hap vs c.jejuni:

No individual association has been observed between haplotype and *c.jejuni* following OR, CI and P value.

### 3.7. Table Hap vs *c.jejuni*:

| **Hap vs. *c.jejuni*** | **OR** | **95% CI** | **P value** |
|---|---|---|---|
| **Hap1 vs *c.jejuni*** | 2.909 | 0.612 -13.827 | 0.179 |
| **Hap2 vs *c.jejuni*** | 0.323 | 0.050-2.076 | 0.2342 |
| **Hap3 vs *c.jejuni*** | 0.323 | 0.05041234 - 2.076303 | 0.2342 |
| **Hap4 vs *c.jejuni*** | 0.361 | 0.029 -4.4175 | 0.425 |
| **Hap5 vs *c.jejuni*** | 4.747419e+07 | 0-Inf | 0.997 |
| **Hap6 vs *c.jejuni*** | 1.005336e+08 | 0-Inf | 0.997 |

### 3.7.4. Hap vs *c.jejuni* with AMAN:

A positive association between haplotype 1 with *c.jejuni* positive and AMAN (p=0.048, OR, 5.55; 95% CI, 1.02-30.33) has been found. ), otherwise, no significant association has been observed for additional Haplotype and *c.jejuni* with AMAN.

**3.8.Table Hap vs *c.jejuni* with AMAN:**

| **Hap Vs *cjejuni*+AMAN** | **OR** | **95% CI** | **P value** |
|---|---|---|---|
| **Hap1 vs *cjejuni*+AMAN** | 5.555 | 1.017-30.331 | 0.04769 * |
| **Hap2 vs *cjejuni*+AMAN** | 0.600 | 0.059 -6.051 | 0.665 |
| **Hap3 vs *cjejuni*+AMAN** | 2.88229e-08 | 0.0000000 - Inf | 0.993 |
| **Hap4 vs *cjejuni*+AMAN** | 1.714 | 0.134-21.819 | 0.677 |
| **Hap5 vs *cjejuni*+AMAN** | 2.925566e-08 | 0-Inf | 0.997 |
| **Hap6 vs *cjejuni*+AMAN** | 3.571 | 0.197-64.632 | 0.389 |

**Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '**

**4.Discussion:**

The overall aim of this study was to investigate the association between polymorphisms in the GR gene and GBS. As a first step, The SNPs prevalent in autoimmune diseases have been identified to study in another autoimmune disease named GBS. Following this step, five SNPs have been sequestered for this investigation, which yields towards the process of haplotype analysis and Linkage disequilibrium in the population of Bangladesh. As a matter of fact, individual genotype and haplotype associated with GBS have been measured through logistics regression analysis in the patient with GBS and healthy control including association study with autoantibody presence, disease outcome, *c.jejuni* presence and *c.jejuni* with AMAN type GBS. The study includes 5 SNPs in 71 population of Bangladesh with 18 different patterns observed during haplotype analysis and establishes 6 possible haplotypes.

The reason for enrolling *c.jejuni* positive GBS patients is because of being a dominant infectious agent in Bangladesh. However, *c.jejuni* is also responsible for molecular mimicry and cross-reactivity with the presence of a cst-II gene(Figure1.2.) that is essential for biosynthesis in ganglioside like LOSs(Yuki, 2007). Additionally, GD1a, as well as GM1, is an autoantigen for IgG antibodies in patients with AMAN succeeded by *C. jejuni*.

This study shows that polymorphisms of glucocorticoid receptor gene are not associated with developing GBS. However, the analysis performed in Bangladesh may differ from patients with other geographical areas with respect to genetic or environmental aspects. Also, a Large population study in Bangladesh may diverge the interpretation of the study too.

In this study, only BclI(C/G) with autoantibody GD1a showed a positive association (p=0.024, OR, 6.94; 95% CI, 1.28-37.58). Besides, all the haplotypes have been compared with GBS, autoantibody presence (GM1, GD1a), disease outcome, the presence of *c.jejuni* and *c.jejuni* with AMAN. The previous study says,*c. jejuni* positive patients are more likely to develop AMAN or AMSAN in Bangladesh(Zhang *et al.*, 2010). As a result, a positive association between haplotype 1 with *c.jejuni* positive and AMAN (p=0.048, OR, 5.55; 95% CI, 1.02-30.33) has been found. The haplotype 1 which is more frequent in 71 population of Bangladesh is similar to the haplotype 2 in Netherlands study (Dekker et al., 2009). The variance found between these two populations are the frequency rate and the number of the population because the Netherland study has been accomplished in 318 Dutch patients, where haplotype 2 is only 15.5 %. Nevertheless, this haplotype 2 for Dutch patients have found an association with a

severe subform of GBS based on an MRC sum score ≤40 at study entry, preceding diarrhea, and the presence of serum anti-GM1/GD1a antibodies.

On the other side, haplotype 3 with the outcome (p=0.008, OR, 0.06; 95% CI, 0.007-0.478), shows 94 % slower progression of recovery. Which means in the presence of Haplotype 3 the disease recovery rate will get slower compared to the absence of haplotype 3. Additionally, the study performed in Bangladesh includes 71 total population accompanied by patients with GBS and healthy control, where 6 haplotype shows a total of 77% frequency rate. However, the Netherland examination for a similar objective has been accompanied by 100 % frequency rate of 6 haplotypes and 318 population. Most previous studies with SNPs of glucocorticoid receptor gene found that BclI polymorphism in GR has a consequence in GR sensitivity (Stevens *et al.*, 2004). Haplotypes of GR gene can modify the action of the link (Rautanen *et al.*, 2006) which has been found in our study, where all three associations have a mutual occurrence of BclI variants in the haplotypes. Additionally, for haplotype 5 and 6, the quantity of population is probably insufficient to conclude the associations with clinical aspects and GBS. In pair-wise Linkage disequilibrium, the study shows an association between N363S with BclI, N363S with GR-9beta and TthIII-1 with ER22/23EK as well based on D'-value and P-value.

Though GBS is an immune-mediated disease, the genetic contribution to GBS is still indistinguishable. Not to forget, Host factors play an important role in developing GBS. Human leukocyte antigen (HLA) genes encoded within major histocompatibility complex (MHC), plays a significant role in the immune system as a genetic regulator. Thus, genome-wide association study (GWAS) has been performed to analyze the association of SNPs as a susceptible factor for developing GBS(Blum et al., 2018). The investigation shows 25% of susceptibility to develop GBS by common genetic variants(Blum *et al.*, 2018), which still needs a large variant of study to conclude.

In conclusion, the study performed in a Bangladeshi population with an amount of 71 patients with GBS and healthy control, could not find any association with polymorphisms of the glucocorticoid receptor gene and GBS. Even though there has been a small association found between BclI with GD1a autoantibody and among haplotype 1 with *c.jejuni* positive AMAN and haplotype 3 with the outcome, future study is required to comprehend the correlation between GR sensitivity with GBS and its clinical aspects. Overall, Colossal population study is required to understand the significance and associations with GBS.

1. Asbury, A. K. (1990) ‘Guillain-Barrea syndrome: Historical aspects’, Annals of Neurology. Wiley-Blackwell, 27(S1), pp. S2–S6. doi: 10.1002/ana.410270703.
2. Asbury, A. K. and Cornblath, D. R. (no date) ‘Assessment of Current Diagnostic Criteria for Guillain-Barr6 Syndrome’. Available at: https://pdfs.semanticscholar.org/b806/abb929620fa46544770cc4a0f583a17dda10.pdf (Accessed: 25 May 2018).
3. Burns, T. (2008) ‘Guillain-Barré Syndrome’, Seminars in Neurology, 28(2), pp. 152–167. doi: 10.1055/s-2008-1062261.
4. van Doorn, P. A., Ruts, L. and Jacobs, B. C. (2008) ‘Clinical features, pathogenesis, and treatment of Guillain-Barré syndrome’, The Lancet Neurology. doi: 10.1016/S1474-4422(08)70215-1.
5. Geleijns, C. P. W. (2005) Immunogenetic polymorphisms in Guillain-Barré syndrome, Department of Immunology.
6. Hughes, R. A. et al. (2016) ‘Corticosteroids for Guillain-Barré syndrome’, Cochrane Database of Systematic Reviews, 10(8), p. CD001446. doi: 10.1002/14651858.CD001446.pub5.
7. Hughes, R. A. C. and Cornblath, D. R. (2005) ‘Guillain-Barré syndrome.’, Lancet (London, England). Elsevier, 366(9497), pp. 1653–66. doi: 10.1016/S0140-6736(05)67665-9.
8. Hughes, R. A. C., Cornblath, D. R. and Willison, H. J. (2016) ‘Guillain-Barré syndrome in the 100 years since its description by Guillain, Barré and Strohl’, Brain. doi: 10.1093/brain/aww247.
9. Hughes, R. A. and Cornblath, D. R. (2005) ‘Guillain-Barré syndrome’, The Lancet, 366(9497), pp. 1653–1666. doi: 10.1016/S0140-6736(05)67665-9.
10. Islam, Z. et al. (2011) ‘High incidence of Guillain-Barre syndrome in children, Bangladesh.’, Emerging infectious diseases. Centers for Disease Control and Prevention, 17(7), pp. 1317–8. doi: 10.3201/eid1707.102031.
11. Jacobs, B. (1997) Antecedent infections and anti-ganglioside antibodies in Guillain-Barré syndrome: their role in pathogenesis and heterogeneity.
12. Joshi, K. et al. (2016) ‘Guillain–Barre syndrome: a case report’, International Journal of Medical Science and Public Health, 5. doi: 10.5455/ijmsph.2016.05052015157.
13. Martyn, C. N. and Hughes, R. A. C. (1997) ‘Epidemiology of peripheral neuropathy’, Journal of Neurology Neurosurgery and Psychiatry, 62(4), pp. 310–318. doi: 10.1136/jnnp.62.4.310.

14. Mcgrogan, A. et al. (2009) 'The Epidemiology of Guillain-Barré Syndrome Worldwide A Systematic Literature Review', Neuroepidemiology, 32, pp. 150–163. doi: 10.1159/000184748.
15. Oakley, R. H. and Cidlowski, J. A. (2011) 'Cellular processing of the glucocorticoid receptor gene and protein: New mechanisms for generating tissue-specific actions of glucocorticoids', Journal of Biological Chemistry, 286(5), pp. 3177–3184. doi: 10.1074/jbc.R110.179325.
16. Paralysis, A. (1859) 'The History of Guillain-Barré Syndrome', (1), pp. 1–47.
17. Pithadia, A. B. and Kakadia, N. (2010) 'Clinical Profile Guillain-Barré syndrome ( GBS )', Polish Academy of Sciences, pp. 220–229.
18. Schaaf, M. J. M. and Cidlowski, J. A. (2003) 'Molecular mechanisms of glucocorticoid action and resistance', Journal of Steroid Biochemistry & Molecular Biology, 83, pp. 37–48. doi: 10.1016/S0960-0760(02)00263-7.
19. Seneviratne, U. (no date) 'Guillain-Barré syndrome'. doi: 10.1136/pgmj.76.902.774.
20. Zhang, H.-L. et al. (2010) 'Axonal Variant of Guillain-Barre Syndrome Associated With Campylobacter Infection in Bangladesh', Neurology, 75(2), pp. 194–195. doi: 10.1212/WNL.0b013e3181e7920c.
21. Blum, S., Ji, Y., Pennisi, D., Li, Z., Leo, P., McCombe, P., & Brown, M. A. (2018). Genome-wide association study in Guillain-Barré syndrome. *Journal of Neuroimmunology*, *323*, 109–114. https://doi.org/10.1016/j.jneuroim.2018.07.016
22. Burns, T. (2008). Guillain-Barré Syndrome. *Seminars in Neurology*, *28*(2), 152–167. https://doi.org/10.1055/s-2008-1062261
23. Hughes, R. A., Brassington, R., Gunn, A. A., & van Doorn, P. A. (2016). Corticosteroids for Guillain-Barré syndrome. *Cochrane Database of Systematic Reviews*, *10*(2), CD001446. https://doi.org/10.1002/14651858.CD001446.pub5
24. Islam, Z., Gilbert, M., Mohammad, Q. D., Klaij, K., Li, J., van Rijs, W., … Jacobs, B. C. (2012). Guillain-Barré Syndrome-Related Campylobacter jejuni in Bangladesh: Ganglioside Mimicry and Cross-Reactive Antibodies. *PLoS ONE*, *7*(8), e43976. https://doi.org/10.1371/journal.pone.0043976
25. Louwen, R., Heikema, A., Van Belkum, A., Ott, A., Gilbert, M., Ang, W., … Nieuwenhuis, E. E. (2008). The sialylated lipooligosaccharide outer core in Campylobacter jejuni is an important determinant for epithelial cell invasion. *Infection and Immunity*, *76*(10), 4431–4438. https://doi.org/10.1128/IAI.00321-08
26. Mcgrogan, A., Madle, G. C., Seaman, H. E., & De Vries, C. S. (2009). The Epidemiology

of Guillain-Barré Syndrome Worldwide A Systematic Literature Review. *Neuroepidemiology*, *32*, 150–163. https://doi.org/10.1159/000184748

27. Rac, H. (n.d.). Plasma exchange for Guillain-Barré syndrome (Review). Retrieved from http://www.thecochranelibrary.com
28. Schaaf, M. J. M., & Cidlowski, J. A. (2003). Molecular mechanisms of glucocorticoid action and resistance. *Journal of Steroid Biochemistry & Molecular Biology*, *83*, 37–48. https://doi.org/10.1016/S0960-0760(02)00263-7
29. Seneviratne, U. (n.d.). Guillain-Barré syndrome. https://doi.org/10.1136/pgmj.76.902.774
30. Van Der Velden, V. H. J. (1998). Glucocorticoids: Mechanisms of action and anti-inflammatory potential in asthma. *Mediators of Inflammation*, *7*(4), 229–237. https://doi.org/10.1080/09629359890910
31. van Rossum, E. F. C., Lamberts, S. W. J., Regression, L., Burns, T., Therapeutics, P., Panek, M., … Szemraj, J. (2004). Guillain-Barre Syndrome. *Seminars in Neurology*, *5*(2), 572–580. https://doi.org/10.1210/rp.59.1.333
32. Webster, J. I., & Sternberg, E. M. (2004). Role of the hypothalamic-pituitary-adrenal axis, glucocorticoids and glucocorticoid receptors in toxic sequelae of exposure to bacterial and viral products. *Journal of Endocrinology*, *181*(2), 207–221. https://doi.org/10.1677/joe.0.1810207
33. Wilder, R. L., Cupps, T. R., Balow, J. E., Endocrinol-, P., Branch, D. E., Develop-, H., & Receptors, G. (1993). Glucocorticoid Therapy for Immune-mediated Diseases: Basic and Clinical Correlates, *119*(12), 1198–1208. https://doi.org/10.1059/0003-4819-119-12-199312150-00007
34. Winer, J. B., Hughes, R. A. A. C., Cornblath, D. R., & Willison, H. J. (2005). Guillain-Barré syndrome in the 100 years since its description by Guillain, Barré and Strohl. *J Clin Pathol: Mol Pathol*, *139*(11), 1653–1666. https://doi.org/10.1093/brain/aww247
35. Yuki, N. (2007). Campylobacter sialyltransferase gene polymorphism directs clinical features of Guillain-Barré syndrome. *Journal of Neurochemistry*, *103 Suppl*, 150–158. https://doi.org/10.1111/j.1471-4159.2007.04707.x
36. Asbury, A. K. (1990) 'Guillain-Barrea syndrome: Historical aspects', Annals of Neurology. Wiley-Blackwell, 27(S1), pp. S2–S6. doi: 10.1002/ana.410270703.
37. Asbury, A. K. and Cornblath, D. R. (no date) 'Assessment of Current Diagnostic Criteria for Guillain-Barr6 Syndrome'. Available at: https://pdfs.semanticscholar.org/b806/abb929620fa46544770cc4a0f583a17dda10.pdf (Accessed: 25 May 2018).

38. Burns, T. (2008) 'Guillain-Barré Syndrome', Seminars in Neurology, 28(2), pp. 152–167. doi: 10.1055/s-2008-1062261.
39. van Doorn, P. A., Ruts, L. and Jacobs, B. C. (2008) 'Clinical features, pathogenesis, and treatment of Guillain-Barré syndrome', The Lancet Neurology. doi: 10.1016/S1474-4422(08)70215-1.
40. Geleijns, C. P. W. (2005) Immunogenetic polymorphisms in Guillain-Barré syndrome, Department of Immunology.
41. Hughes, R. A. et al. (2016) 'Corticosteroids for Guillain-Barré syndrome', Cochrane Database of Systematic Reviews, 10(8), p. CD001446. doi: 10.1002/14651858.CD001446.pub5.
42. Hughes, R. A. C. and Cornblath, D. R. (2005) 'Guillain-Barré syndrome.', Lancet (London, England). Elsevier, 366(9497), pp. 1653–66. doi: 10.1016/S0140-6736(05)67665-9.
43. Hughes, R. A. C., Cornblath, D. R. and Willison, H. J. (2016) 'Guillain-Barré syndrome in the 100 years since its description by Guillain, Barré and Strohl', Brain. doi: 10.1093/brain/aww247.
44. Hughes, R. A. and Cornblath, D. R. (2005) 'Guillain-Barré syndrome', The Lancet, 366(9497), pp. 1653–1666. doi: 10.1016/S0140-6736(05)67665-9.
45. Islam, Z. et al. (2011) 'High incidence of Guillain-Barre syndrome in children, Bangladesh.', Emerging infectious diseases. Centers for Disease Control and Prevention, 17(7), pp. 1317–8. doi: 10.3201/eid1707.102031.
46. Jacobs, B. (1997) Antecedent infections and anti-ganglioside antibodies in Guillain-Barré syndrome: their role in pathogenesis and heterogeneity.
47. Joshi, K. et al. (2016) 'Guillain–Barre syndrome: a case report', International Journal of Medical Science and Public Health, 5. doi: 10.5455/ijmsph.2016.05052015157.
48. Martyn, C. N. and Hughes, R. A. C. (1997) 'Epidemiology of peripheral neuropathy', Journal of Neurology Neurosurgery and Psychiatry, 62(4), pp. 310–318. doi: 10.1136/jnnp.62.4.310.
49. Mcgrogan, A. et al. (2009) 'The Epidemiology of Guillain-Barré Syndrome Worldwide A Systematic Literature Review', Neuroepidemiology, 32, pp. 150–163. doi: 10.1159/000184748.
50. Oakley, R. H. and Cidlowski, J. A. (2011) 'Cellular processing of the glucocorticoid receptor gene and protein: New mechanisms for generating tissue-specific actions of glucocorticoids', Journal of Biological Chemistry, 286(5), pp. 3177–3184. doi: 10.1074/jbc.R110.179325.

51. Paralysis, A. (1859) ‘The History of Guillain-Barré Syndrome’, (1), pp. 1–47.
52. Pithadia, A. B. and Kakadia, N. (2010) ‘Clinical Profile Guillain-Barré syndrome ( GBS )’, Polish Academy of Sciences, pp. 220–229.
53. Schaaf, M. J. M. and Cidlowski, J. A. (2003) ‘Molecular mechanisms of glucocorticoid action and resistance’, Journal of Steroid Biochemistry & Molecular Biology, 83, pp. 37–48. doi: 10.1016/S0960-0760(02)00263-7.
54. Seneviratne, U. (no date) ‘Guillain-Barré syndrome’. doi: 10.1136/pgmj.76.902.774.
55. Zhang, H.-L. et al. (2010) ‘Axonal Variant of Guillain-Barre Syndrome Associated With Campylobacter Infection in Bangladesh’, Neurology, 75(2), pp. 194–195. doi: 10.1212/WNL.0b013e3181e7920c.