PREVALENCE OF ZIKA VIRUS INFECTION IN PATIENTS WITH GUILLAIN-BARRE SYNDROME IN BANGLADESH

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A thesis submitted to the Department of Mathematics and Natural Science
in partial fulfilment of the requirements for the degree of
Bachelor of Science in Microbiology

Department of Mathematics and Natural Science
BRAC University
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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

The thesis titled "Prevalence of Zika Virus infection in patients with Guillain-Barre Syndrome in Bangladesh" submitted by Tabassum Binte Matiur (15126015) of Spring, 2015 has been accepted as satisfactory in partial fulfilment of the requirement for the degree of Bachelor of Science in Biotechnology on 3rd October 2019.

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Ethics Statement

This study has been conducted with samples from the blood serum study performed by International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and the consent was taken in agreement to use the samples for thesis purpose.

Abstract/ Executive Summary

Guillain–Barre syndrome (GBS) is an autoimmune disease where the immune system of the body attacks the peripheral nervous system causing demyelination of the axon. This is the leading cause of acute flaccid paralysis (AFP) in Bangladesh. Previous studies have established an association between Zika virus (ZV) infection and GBS in South America and Oceania. In Asia, recently, ZV is known to circulate widely, but the association with Guillain–Barre syndrome is unclear. This prospective study aimed to describe the clinical profile and the frequency of ZIKA virus infected pathophysiology of GBS in Bangladesh. Detailed information of the clinical presentation, electrophysiology, diagnosis, disease severity, and clinical course were obtained from 98 patients with GBS prospectively and followed up for 6 months using a standard questionnaire. In this study, ZV specific IgG and IgM antibody were detected by Elisa to distinguish GBS patients with prolonging and recent ZV infection respectively. Moreover, RT-PCR was performed to confirm if patients had recent ZV infection. Among the 98 patients 18 (18%) GBS patients had ZV infection for a long time. Among the 18 Zika IgG positive patients, 15 patients with GBS (83%) were male and 3 of them were female (17%). The majority of ZV IgG positive patients with GBS were adults with a mean age of 40 years, almost 67% (n=66) had reported with preceding event and more than 40% (n=8) had gastrointestinal diarrhea. Around 45% of the ZV-IgG positive patients with GBS develop axonal variants of GBS and 38% had demyelinating variants of GBS. 56% had cranial nerve involvement and only 6% had sensory deficits. The MRC score of weeks 13 and week 26 were compared to observe the rate of recovery between Zika positive and Zika negative patients, however, there was no significant difference (week 13, p-value=0.31326 and week 26, p-value=0.430478). In conclusion, we can say that ZV can be considered a causative agent of Guillain-Barre Syndrome and male population are more vulnerable. However, further studies are warranted to see why ZV infection is predominant in male GBS patients comparing to their female counterparts.

Dedication

All the animals in the world

Acknowledgment

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Tabassum Binte Matiur

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Table of Contents

List of Tables

List of Figures

List of Acronyms

AFP	Acute Flaccid Paralysis
AIDP	Acute Inflammatory Demyelinating Polyradiculoneuropathy
AMAN	Acute Motor Axonal Neuropathy
AMSAN	Acute Motor and Sensory Axonal Neuropathy
MFS	Miller -Fisher Syndrome
C. jejuni	Campylobacter jejuni
ZV	Zika Virus
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
et al.	'et alia' meaning 'and others'
GBS	Guillain-Barre Syndrome
Elisa	Enzyme-linked immune sorbent assay
RT-PCR	Reverse transcriptase-polymerase chain reaction.
MRC	Medical Research Council
OR	Odd ratio
OD	Optical density
TBE	Tris-Borate EDTA
p-value	Estimated probability

-Introduction-

1.1 Guillain-Barré syndrome

Guillain-Barré syndrome is an immune-mediated polyradiculoneuropathy, a disease of the peripheral nervous system with a wide clinical spectrum and particular pathological and electrophysiological features (Hahn, 1998). At present, it is the world's most prevalent cause of acute paralysis after the extinction of poliomyelitis (van Doorn et al., 2008). Since 2000, no virally and clinically validated polio instances had been observed in Bangladesh; however, non-polio acute flaccid paralysis (AFP) is frequently diagnosed. Most of the non-polio acute flaccid paralysis is thought to be GBS in Bangladesh (Rasul et al., 2002). In the last two decades, a great deal of development has been made grasping the pathophysiology and epidemiology of GBS in the developed world. Record is scarce on GBS from the developing world which includes Bangladesh.

1.2 History of GBS

The first correct description of GBS was made in 1859 by Jean Baptiste Octave Landry de Thezillat (Skalski et al., 2018). By 1876, the phrase 'Landry's ascending paralysis' grew to be the preferred identity for the disease. The syndrome used to be named after G. Guillain and J. A. Barre, two French military neurologists, who is 1916 described, along with A. Strohl, the common findings in the cerebrospinal fluid (Afifi, 1994). They reported the combination of increased protein concentration with a normal cell count in the cerebrospinal fluid (CSF), or albuminocytological dissociation, which differentiated the circumstance from poliomyelitis (Afifi, 1994). In 1927, H. Draganesco and J. Claudian coined the phrase '**Guillain-Barre syndrome**' and regretfully failed to acknowledge Strohl's contribution to the research. Until now, GBS is a descriptive disease entity defined through a set of clinical, laboratory and electrodiagnostic standards for which there are no specific diagnostic tests `(van Doorn et al., 2008). To date, GBS is no longer viewed as one single sickness entity, but a non-stop spectrum of distinct overlapping variants, ranging from a pure motor to a combination of motor and sensory nervous system disturbance except cranial nerve involvement.

1.3 Epidemiology

In developed countries like, Europe and North America, AIDP is the most frequent form of GBS (accounting for around 90% of cases) and thus the term GBS in general is synonymous with AIDP in Western countries. The axonal forms account for only 3-5% of cases in Western countries but are

much more frequent (30%-50% of GBS cases) in Asia and Latin America (Van Doorn,2009). The annual US incidence of GBS is 1.2-3 per 100,000 inhabitants, making GBS the most common cause of acute flaccid paralysis in the United States (Seneviratne, 2000). However, if we look into the scenarios of developing countries, we can see that, according to WHO, in India overall incidence of GBS is 0.4 to 4.0 people per 100 000 per year. Therefore, we can see that there is no specific pattern in the prevalence of GBS in developing and developed countries.

1.4 Guillain-Barré syndrome in Bangladesh

Guillain-Barre Syndrome is the most frequent cause of acute flaccid paralysis in Bangladesh since polio myelitis has been eradicated. Here, majority of the GBS patients were infected with *C. jejuni* infection. An incidence rate of 3.25 cases of GBS patients per 100,000 children under 15 years of age were reported. The majority of the patients were men, less than 30 years old, and suffering from a severe, pure motor and axonal variant of GBS and 60% of them had diarrhoea as an antecedent infection (Islam et al., 2010). In 2006 and 2007, 608 (37%), and 855 (46%) cases of children under 15 years of age fulfilled the GBS symptoms criteria. Whereas, 1619 and 1844 cases were enrolled as AFP (Acute flaccid paralysis) patients. It is believed that, 1.5 to 2.5 cases per 100,000 population per year among all division of Bangladesh, are children less than 15years of age, who have developed GBS. Perhaps seasonal variants may affect the percentage of Guillain-Barre Syndrome. The incidence of GBS seems to be highest between January and March especially the AMAN subgroup in Bangladesh. Whereas, the majority of the GBS cases are pure motor variants (92%) with predominant axonal degeneration (67%) (Islam et al., 2010).

1.5 Clinical Features of GBS

1.5.1 Diagnosis of GBS

The purpose of creating diagnostic criteria for GBS was associated with its growing incidence during the swine flu vaccination two periods in 1976 and 1977. The standards have been first published in 1978 at the request of the National Institute of Neurological and Communicative Disorders and Stroke (NINDS) two (Ress et al.,1995). The standards were reaffirmed in 1990, and the electrodiagnostic standards have been expanded. These diagnostic standards were extensively conventional and appeared suitable to clinically outline GBS (Asbury & Cornblath, 1990).
Diagnostic standards for regular GBS are shown in **Table 1.1**.

Table 1.1: Diagnostic criteria for Guillain Barre Syndrome

Features required for diagnosis
❖ **Progressive motor weakness for more than one limb** ❖ **Low or absent reflexes** ❖ **No other identifiable cause** ❖ **Features that strongly support the diagnosis**
Clinical
❖ **Progression of signs over days to 4 weeks** ❖ **Relative symmetry of symptoms** ❖ **Mild sensory symptoms or signs** ❖ **Cranial nerve involvement, especially bilateral weakness of facial muscles** ❖ **The onset of recovery 2-4 weeks after development stops** ❖ **Autonomic dysfunction** ❖ **Pain (often present)** ❖ **Cerebrospinal Fluid (CSF)** ❖ **A high concentration of Cerebrospinal Fluid (CSF) protein after the first week** ❖ **Less than 50 mononuclear leukocytes per µl CSF** ❖ **Electrodiagnosis** ❖ **Conduction slowing or block**
Feature casting doubt on the diagnosis
❖ **Bladder or bowel dysfunction at the onset** ❖ **Sharp sensory level** ❖ **Marked persistent asymmetry of weakness** ❖ **Persistent bladder or bowel dysfunction** ❖ **More than 50 mononuclear leukocytes per µl CSF** ❖ **Presence of polymorphonuclear leukocytes in CSF**

1.6 Disease course and prognosis

The clinical severity, path, and effect of GBS are quite variable (van Doorn et al., 2008). The maximum level of weakness is reached within 4 weeks after onset of signs accompanied by

spontaneous recovery. It appears to be a typical belief that most patients suffering from a slight clinical course of ailment may additionally exhibit a neurological good recovery. However, patients with a mild course of the disease may additionally go through from residual morbidity (R Van Koningsveld et al., 2002). Many case sequence file that advanced age is indicative of worse prognosis (Hughes et al., 2003).

In children, the recuperation is more rapid and more likely to be completed and death is exceptional (Donofrio, 2017). Respiratory failure takes place in 25% of patients and may need mechanical ventilation for an extended period. Prognosis degrees from complete neurological recuperation with or without fatigue (van Doorn et al., 1999), to wheelchair dependency or death. Approximately 20% of patients have an extreme functional deficit, and continue to be unable to walk or bedridden, and they even may need continuous artificial ventilation (Ropper, 1991; Koningsveld et al., 2007). The mortality normally ranges from 2 to 13% (Winer, 2009) and is mostly the result of respiratory failure, cardiovascular and autonomic disturbance (R.A.C. Hughes et al., 2003).

1.7 Antecedent infection

GBS is considered a post-infectious disease because of the commentary enrollment of two-third of the patients suffering from GBS (Jacobs, 1997). These infections are thought to affect the immune reaction against the peripheral nerve antigens in the human body, between the intermission of 1-4 weeks between the precursor of the infections and onset of weakness (Jacobs, 1997). However, thorough clinical research shows that the root of GBS is involved with a range of infectious agents. Among the GBS patients, *Campylobacter jejuni* infection was most common (Islam et al.,2010). However, cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumonia*, and *Haemophilus influenza* are also responsible for causing GBS(van Doorn et al., 2008). Also, cytomegalovirus is reported to be the second most common infectious pathogen to cause GBS (Seneviratne, 2000). It has also been reported to be associated with human immunodeficiency virus (HIV) (Burns, 2008). Hepatitis A, B, C and D, typhoid and falciparum malaria are retrained to anecdotal case reports (Seneviratne, 2000). Furthermore, a study in Japan shows that most of the antecedent events of GBS and related disorders are Fever (52%), cough (48%), sore throat (39%), nasal discharge (30%) and diarrhea (27%) (van Doorn et al., 2008).

1.8 Zika Virus

Zika virus (ZV) is an arthropod-borne virus that was first found in 1947 in a forest named Zika in Uganda. Since then, more than one outbreaks of ZV have been suggested in different countries. It is transmitted generally through Aedes mosquitoes, and the signs of fever, joint pain, red eyes, headache, and maculopapular rash closely resemble chikungunya and dengue. The most extreme issues of ZV infection encompasses the danger of microcephaly and other congenital brain anomalies in contaminated pregnant women. It is a condition that can be effortlessly averted with ideal education.

However, ZV can infect several other parts of our body and cause inflammation. ZV infects ocular tissues, including the cornea, neurosensory retina, and optic nerve (mice), as well as the aqueous humor of the anterior chamber (humans). ZV also targets cells of the reproductive tract, including spermatogonia, Sertoli cells, and Leydig cells (in the testis of mice), sperm (samples from mice and humans), and the vaginal epithelium (mice) and uterine fibroblasts (in vitro infection of human samples). The extensive tropism results in ZV detection in multiple body fluids, including conjunctival fluid or tears (mice and humans), saliva (non-human primates and humans), semen (mice, non-human primates, and humans), cervical mucus (humans), vaginal washings (mice and human), and urine (non-human primates and humans) (Miner., 2004).

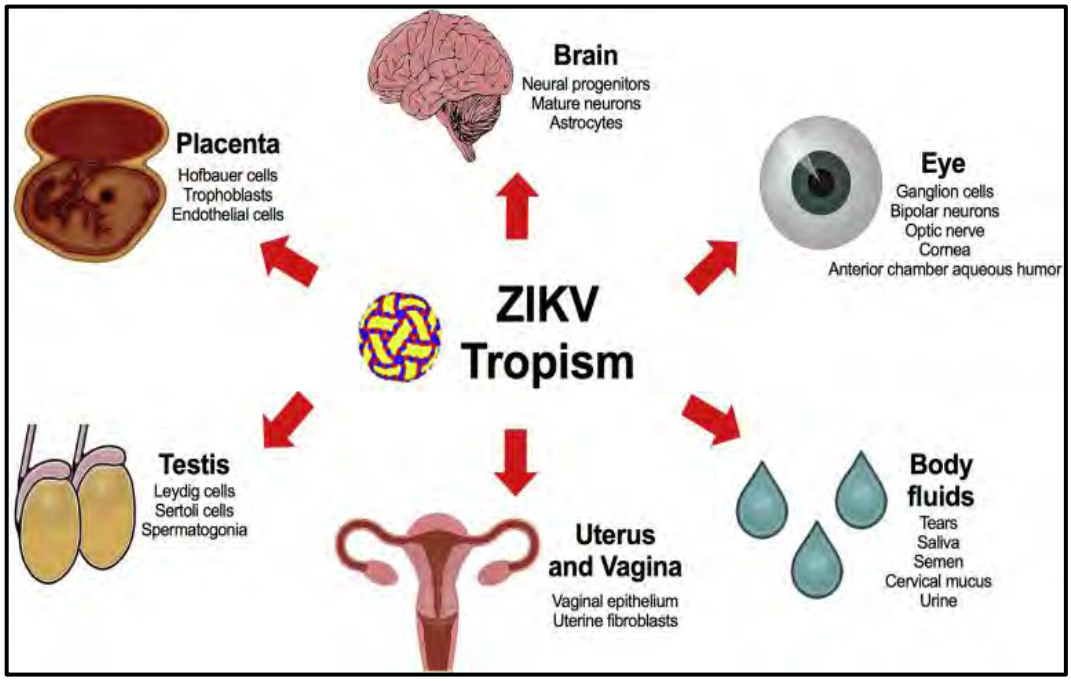


Fig 1.1 ZV Tissue and Cell Tropism

1.8.1 History and outbreak of Zika Virus

Zika virus (ZV) is an arbovirus that was first isolated from a rhesus sentinel monkey in Kampala, Uganda, in 1947 (Dick, 1952). The first case of infection in humans was described in a 10-year-old girl from Nigeria in 1954 (MacNamara, 1954). In 2007, the first major outbreak of Zika fever took place in the Western Pacific Island of Yap in the Federated States of Micronesia (Duffy et al., 2009). Since then, more than one outbreaks of ZV have been suggested worldwide. Two larger epidemics affecting over 30,000 people took place in 2013 and 2014 in French Polynesia (Cao-Lormeau, 2014). Smaller outbreaks also cropped up in different Pacific Islands, such as New Caledonia, the Easter Island, and the Cook Islands, along with the Solomon Islands, Samoa, and Vanuatu. The condition slowly crawled its way to the Americas on March 2015, and at the end of January 2016, more than 20 countries reported outbreaks of the ZV(Zanluca et al., 2015).

The primary complication of Zika virus infection is the danger of microcephaly and other congenital brain anomalies in infected pregnant women. The World Health Organization (WHO) raised the alarm for this condition, as most instances of microcephaly and defects in newborns from Zika virus disease were noted in economically disadvantaged countries. The employer emphasized that it is vital to properly educate the public on preventive measures to avoid a similar occurrence in the future.

1.8.2 Transmission of Zika Virus

Zika virus has one-of-a-kind modes of transmission. The Aedes mosquito bite is the most frequent one, and it involves exclusive families of Aedes: A. aegypti, A. albopictus, A. polynesiensis, A. vittatus, A. unilineatus, and A. hensilli amongst many other before stated sub-types (Li et al.,2012). Vertical transmission from a contaminated mother through the placenta to the fetus has additionally been until now reported. Furthermore, Zika virus can be transmitted via blood transfusions and via sexual encounters, whether oral, vaginal, or anal through an infected individual (Foy et al., 2011). In 2016, the first male-to-male transmission was once additionally registered in Texas, US. However, the foremost and most serious danger of transmission is mosquito bites (Chan et al., 2016).

1.8.3 Pathogenesis of Zika virus

Zika virus most usually follows the sylvatic transmission cycle. It is categorized as an arbovirus that is transmitted via one vertebrate to some other through a mosquito bite. The virions exist as immature (non-infectious), mature (infectious), and fusogenic (host membrane binding) states. Humans are incidental hosts in the lifecycle of the virions. Monkeys and apes can also serve as host to the virions, while some researchers have determined that sheep, elephants, and goats had antibodies against Zika, suggesting viable host states as well (Haddow et al., 2012). The cycle begins when an Aedes mosquito ingests blood containing the Zika virus after biting a contaminated person. The virus begins replicating in the epithelial cells of midgut and goes to the salivary glands of the mosquito. After an incubation period of 10 days, the saliva becomes infected, making the mosquito a vector for infecting a human. Upon entry into the human skin, the virus infects the dermal fibroblasts that serve as receptors for attachment of Zika virus (Hamel et al., 2015).

1.8.4 Symptoms of Zika virus disease

Misdiagnosis of Zika virus disease is very common because signs and symptoms in former healthy patients resemble chikungunya and dengue. After incubation of three to 12 days, Zika fever appears (Plourde & Bloch, 2016). The most frequently reported symptoms to encompass fever, joint pain, red eyes, headache, and a maculopapular rash (Musso et al., 2014). The symptoms generally last less than a week and no mortality has yet been reported from the initial infection phase.

The complications of Zika virus in pregnant ladies include microcephaly and other brain malformations (Rasmussen et al., 2016). Infections in earlier healthy adults have been related to Guillain-Barre syndrome (GBS) as well (Petersen et al., 2016).

1.8.5 Diagnosis and cross reaction of Zika virus disease

Diagnosis of ZV infection can be confusing due to cross-reactivity since ZV shares a considerable degree of genetic identity and structural homology with other flaviviruses, including dengue virus (DENV). In particular, the surface glycoprotein envelope (E), which is involved in viral fusion and entry and is therefore a chief target for neutralizing antibody responses, contains regions that are highly conserved between the two viruses. This results in immunological cross-reactivity, which in the context of prior DENV exposure, may have significant implications for the generation of immune responses to ZV and affect disease outcomes.

ZV is positive sense, single-stranded RNA genome is contained within a nucleocapsid core that is surrounded by an outer envelope made up of two structural proteins: envelope (E) and pre-membrane (prM). The cryo-EM structure of ZV reveals that the virus has a nearly identical organizational structure to DENV, including the characteristic herringbone arrangement of E protein head-to-tail homodimers on the virus surface. In addition to structural similarities between the viral particles, the main targets for antibody responses in dengue infections, namely E, prM and the non-structural protein NS1, share substantial amino-acid sequence identity between ZV and DENV. The considerable structural and genetic relatedness between ZV and DENV has been hypothesized to cause immunological cross-reactivity between these closely related viruses, which may make diagnosing patients challenging as well as potentially impact protective/pathologic immune responses to these infections (Priyamvada L., 2017).

1.9 ZIKA virus and autoimmune disease

The mechanisms underlying ZV-induced neuropathogenesis are still poorly understood. However, studies in mice and guinea pigs showed that ZV can replicate and affect CNS cells (Dick, 1952; Bell et al., 1971; Kumar et al., 2017). Also, recent studies have used *in vitro* technologies to elucidate mechanisms that contribute to development of autoimmune neurological alterations after Zika infection (Figure 1.2). Some studies have described the mechanisms by which ZV avoids the host IFN signalling of STAT2. During viral infection IFN-I pathways are activated, allowing the expression of hundreds of IFN-stimulated response elements. ZV protein NS5 binds and destroys STAT2 via proteasomal degradation, conferring viral resistance to IFN in cell cultures (Grant et al., 2016; Kumar et al., 2016).

1.10 ZV triggering GBS

GBS was already associated with ZV infection during the outbreak in French Polynesia. Indeed, the rise and fall of Zika cases were followed by a similar trend in the onset of GBS (Broutet et al., 2016). However, the mechanisms by which ZV infection causes GBS has not yet been established; it has been suggested that the virus could exacerbate the immune response triggering an immunopathogenic process that determines, in turn, the onset of GBS (Blázquez & Saiz, 2016). Some patients “developed neurological symptoms during or immediately after the [ZV] infection, suggesting a parainfectious rather than postinfectious pattern that is typically seen in GBS”, as noted

in a recent review (Uncini, Shahrizaila, & Kuwabara, 2017). These symptoms include muscle weakness, inability to walk, facial palsy, and respiratory distress. In 2015, 1708 cases of GBS were reported in Brazil representing a 19% increase in comparison to the previous year (Araujo et al., 2016).

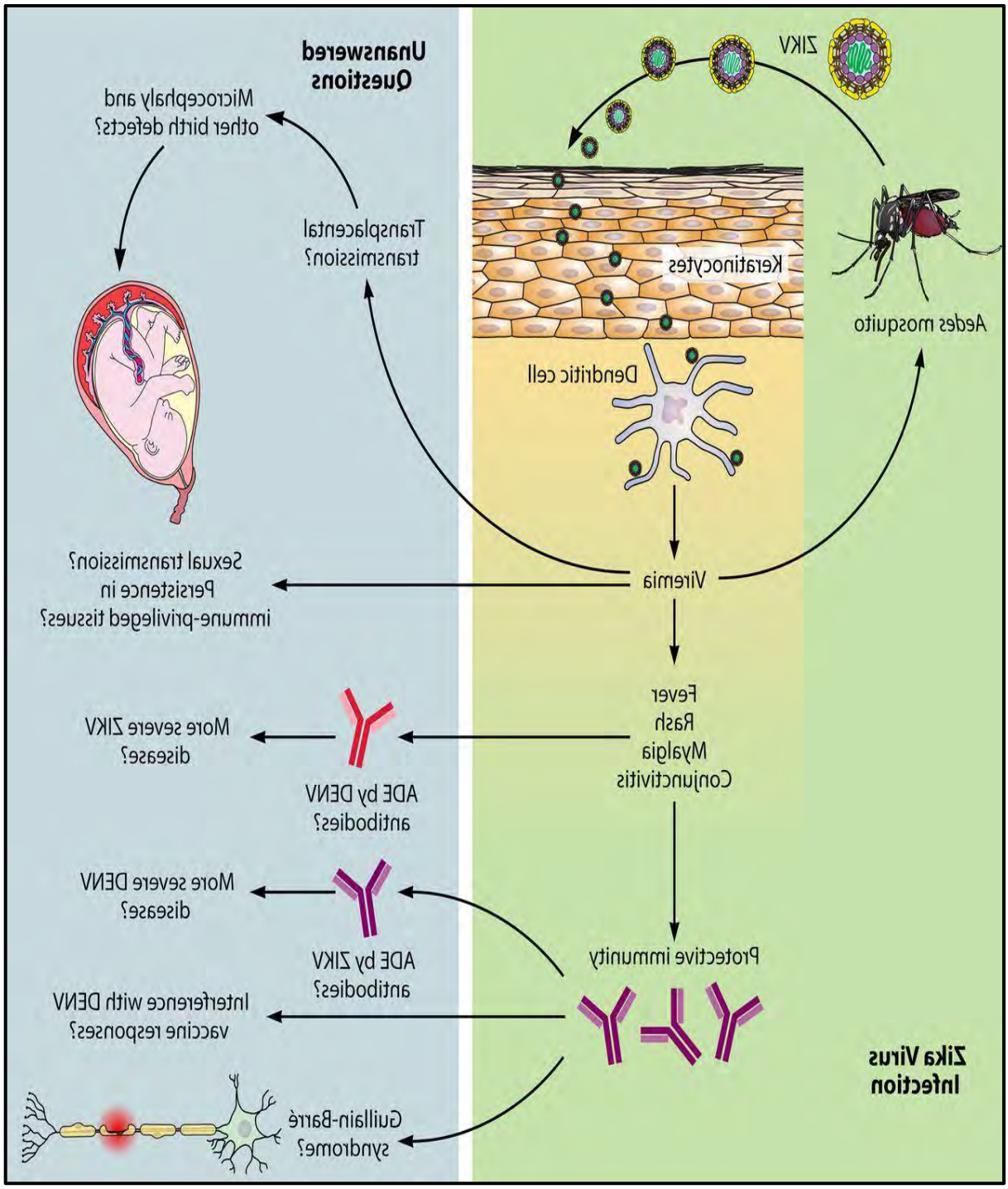


Fig 1.2: Figure illustrating ZV infection triggering Guillain Barre Syndrome.

1.11 Aims and objectives

The major aim to find the prevalence of Zika virus infection among patients with GBS and correlate with the clinical presentation and of GBS across different age and sex groups.

Primary objectives

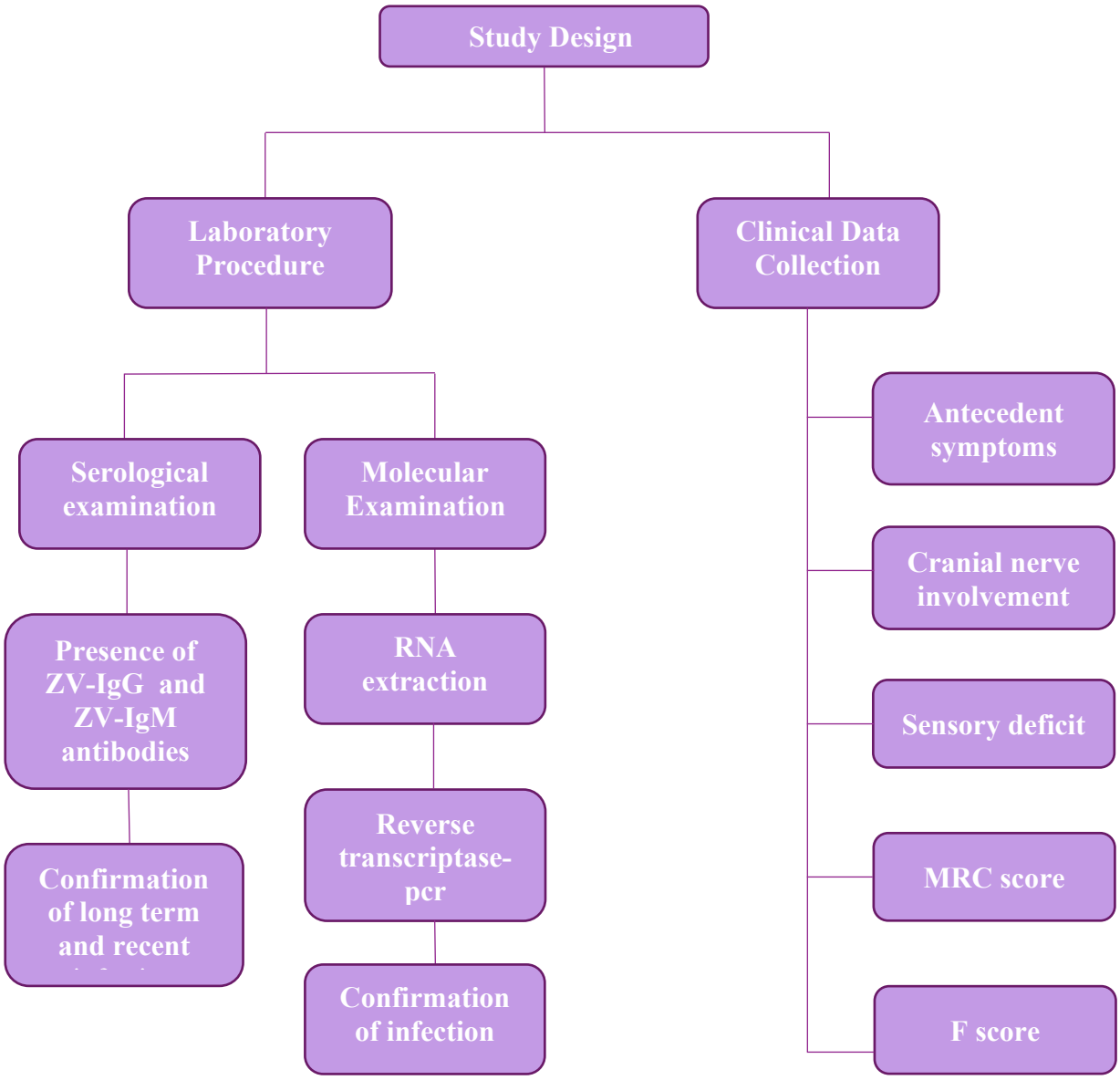
- Determine the ZV-IgG and IgM among patients with GBS and confirm the recent ZV infection.
- Determine the correlation of ZV infection with clinical manifestation among patients with GBS

Materials and Methods

2.1 Study Design

A prospective study was conducted in National Institute of Neuroscience (NINS) from 2016 to 2019. A pre-defined protocol was used to select patients, data was collected regarding baseline characteristics, clinical presentation and course and electrophysiology. Among all the patients, 98 patients were selected randomly. Detailed, standardized information on demographic and clinical data were collected, including age, sex, place of residence (district of Bangladesh); clinical symptoms of preceding infections or other events; time and degree of maximum weakness; cranial, sensory, and autonomic nerve involvement; respiratory failure; requirement for mechanical ventilation; GBS disability score (F score) and Medical Research Council (MRC) score at standard points (Entry, 2 weeks, 4 weeks, 3 months, 6 months). The severity of the disease was evaluated using GBS disability score (Hughes et al., 1978) and the MRC score. GBS disability score is a widely accepted scoring system used to assess functional status of patients with GBS, ranging from 0 (normal) to 6 (death) (Jacobs BC, 2007). The collected samples were then taken to the laboratory for Serological and Molecular examinations. Anti-Zika IgG and IgM Elisa was done to detect long term and recent infection respectively. As for molecular examination, reverse transcriptase-pcr was done to detect the presence of ZV RNA and therefore, to confirm whether the infection was recent or long term.

2.1.1 Study Design Flowchart



2.2. Laboratory experiments

Serological and Molecular examinations were done on the samples to detect the presence of long term and recent infections by immunological assays, such as the Elisa technique. Anti-Zika IgG and IgM Elisa was done to detect the presence of long term and recent infections respectively. Reverse transcriptase-PCR was done to detect the presence of ZV RNA in the patient samples

2.2.1 Immunological assays

Presence of ZV-reactive IgM and IgG antibodies was assessed by the NS1 ELISA assay (Euroimmun™, Lübeck, Germany) for all GBS patient following manufacturers' instructions. The total number of samples were 98 for this experiment.

The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant non-structural proteins (NS1) of Zika virus. In the first reaction step, diluted patient samples are incubated in the wells. In case of positive samples, specific IgG (or IgM) antibodies will bind to antigens. To detect bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalyzing a color reaction (**Figure 2.1**).

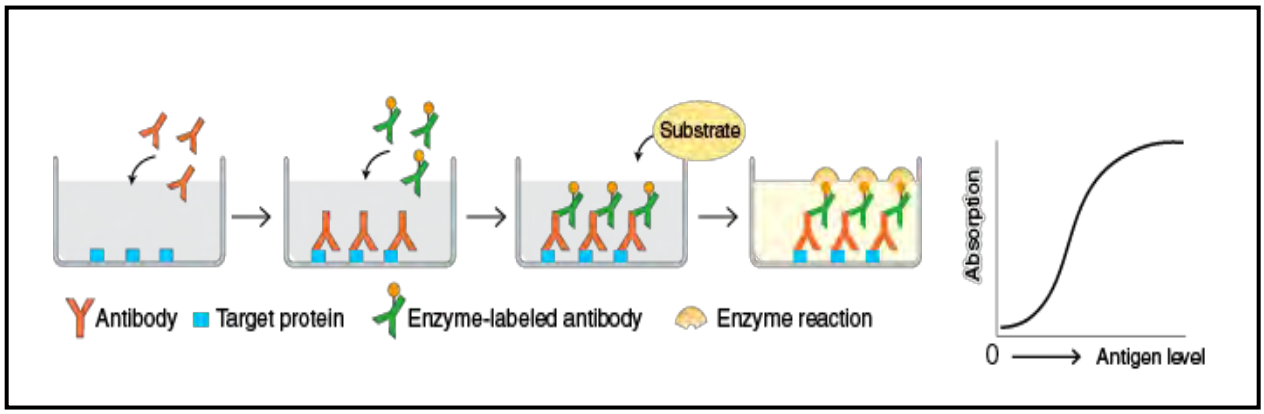


Fig 2.1. The basic setup of an ELISA assay.

2.2.2 Detection of anti-Zika IgG

2.2.2.1 Materials

- 10 µl, 200 µl & 1000 µl pipette
- 300 µl multichannel pipette
- Sterile blue, yellow and nano pipette tips
- Autoclaved distilled water

- 1.5ml eppendorf tube
- Parafilm
- Aluminum foil

2.2.2.2 Preparation of the reagents

All reagents and microplate wells of the kit except wash buffer are ready for use. All reagents were brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. The thermostat adjusted ELISA incubator was set at +37°C ± 1°C

Wash buffer

The wash buffer was a 10X concentrate. Due to the formation of crystals in the concentrated buffer, it was warmed at +37°C and mixed well before diluting. The quantity required was removed from the bottle using a clean pipette and diluted with autoclaved distilled water (one-part reagent with 9 parts distilled water). For one microplate strip, 5 ml concentrate wash buffer was added in 45 ml of distilled water.

Preparation of the patient samples

10 µl of sample was diluted in 1.0 ml sample buffer and mixed well by the vortex (1:101).

2.2.2.3 Procedure

For semiquantitative analysis, Calibrator 2 was incubated along with the positive and negative controls and patient samples. For quantitative analysis Calibrator 1, 2 and 3 were incubated along with the positive and negative controls and patients' samples.

- **Sample incubation (1st step)**

  100µl of the calibrator, positive and negative controls or diluted patient samples were transferred into the individual microplate wells according to the pipetting protocol. Since microplate wells were processed manually, the finished test plate was covered with the protective foil. The plate was incubated for 60 minutes at +37°C ± 1°C.

- **Washing**

  The protective foil was removed, the wells were emptied and subsequently washed three times using 300 µl of working-strength wash buffer for each wash. The wash buffer was left in each well for 30-60 seconds per washing cycle, then emptied the wells. After washing, all the liquid was thoroughly disposed of from the microplate by tapping it on absorbent paper with the openings downwards to remove all residual wash buffer. Insufficient washing (e.g. less than three wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction readings, thus the plates were washed thoroughly. Free positions on the microplate strips were filled with blank wells of the same plate format as that of the parameter to be investigated.

- **Conjugate incubation (2nd step)**

  100 µl of enzyme conjugate (peroxidase labeled anti-human IgG) was pipetted into each of the microplate wells. The plate was incubated 30 minutes at room temperature (+18°C to +25°C).

- **Washing**

  The wells were emptied and then washed following the protocol mentioned above.

- **Substrate incubation**

  100µl of chromogen substrate solution into each of the microplate wells. The microplate was incubated for 15 minutes at room temperature (+18°C to +25°C) and was protected from direct sunlight by using aluminum foil.

- **Stopping**

  100µl of stop solution was pipetted into each of the microplate wells in the same order and at the same speed as the chromogen or substrate solution was introduced.

- **Measurement**

  Elisa plate thermo Elisa reader was used to measure the absorbance of the color intensity of the plate. Ascend software was used to calculate the measurements of the concentration and OD. Photometric measurement of the color intensity was made at a wavelength of 450 nm and a reference wavelength between 620 nm within 30 minutes of adding the stop solution. Before measuring, the microplate was slightly shaken to ensure a homogeneous distribution of the solution

  The method used here to detect the presence of anti-Zika IgG antibody was a semi-quantitative method, meaning the results can be interpreted both quantitatively and qualitatively. Here. For the semiquantitative detection of IgG antibody the following ratio was used:

$$\frac{Extinction\ of\ control\ X\ patient\ sample}{Extinction\ of\ calibrator\ 2} = Ratio$$

Here, the extinction of the control or patient sample over the extinction of the calibrator 2 ratio was used. The results were interpreted using the following standards:

Table 2.1 Elisa OD interpretation ratio

Ratio	Interpretation
<0.8	Negative
≥0.8 to <1.1	Borderline
≥1.1	Positive

The standard curve from which the concentration of antibodies is measured in the patient sample was obtained by a point to point plotting method. In which, two or more points are found on the x and y-axis. Point to point plotting of the extinction readings measured for the 3 calibration sera against the corresponding units (linear/linear) was obtained here. 'point to point' plotting was used for calculation of the standard curve. The following curve is an example of a typical calibration curve.

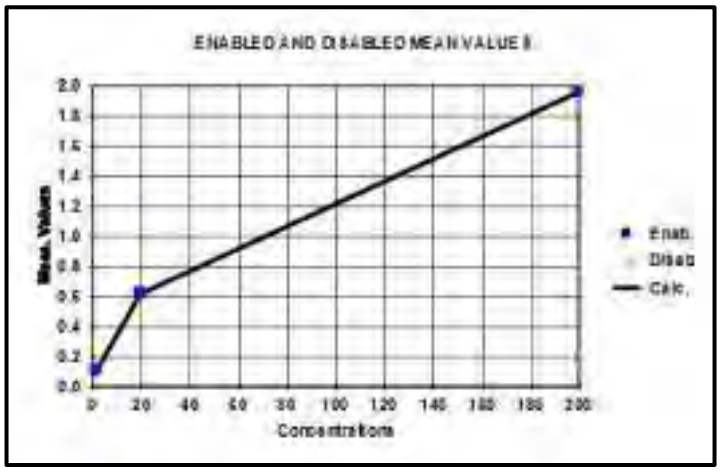


Fig 2.2 Standard curve of the concentration of antibodies in patient sample obtained by point to point

2.2.3 Detection of IgM antibodies

2.2.3.1 Materials

- 10 µl, 200 µl & 1000 µl pipette
- 300 µl multichannel pipette
- Steriled blue, yellow and nano pipette tips
- Autoclaved distilled water
- 1.5ml eppendorf tube
- Parafilm
- Aluminum foil
- ELISA incubator

2.2.3.2 Preparation of the reagents

All reagents and microplate wells of the kit except wash buffer are ready for use. All reagents were brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. The thermostat adjusted ELISA incubator was set at +37°C ± 1°C

Wash buffer

The wash buffer was a 10X concentrate. Due to the formation of crystals in the concentrated buffer, it was warmed at +37°C and mixed well before diluting. The quantity required was removed from the bottle using a clean pipette and diluted with autoclaved distilled water (one-part reagent with 9 parts distilled water). For one microplate strip, 5 ml concentrate wash buffer was added in 45 ml of distilled water.

Preparation of the patient samples

Before the determination of specific antibodies of class IgM, antibodies of class IgG should be removed from the patient sample. The sample buffer contains IgG/RF absorbent. 10 µl of sample was diluted in 1.0 ml sample buffer and mixed well by the vortex (1:101). The mixture was incubated for 10 minutes at room temperature.

2.2.3.3 Procedure

For semiquantitative analysis, Calibrator 2 was incubated along with the positive and negative controls and patient samples. For quantitative analysis Calibrator 1, 2 and 3 were incubated along with the positive and negative controls and patients' samples.

- **Sample incubation (1st step)**

  100µl of the calibrator, positive and negative controls or diluted patient samples were transferred into the individual microplate wells according to the pipetting protocol. Since microplate wells were processed manually, the finished test plate was covered with the protective foil. The plate was incubated for 60 minutes at +37°C ± 1°C.

- **Washing**

  The protective foil was removed, the wells were emptied and subsequently washed three times using 300 µl of working-strength wash buffer for each wash. The wash buffer was left in each well for 30-60 seconds per washing cycle, then emptied the wells. After washing, all the liquid was thoroughly disposed of from the microplate by tapping it on absorbent paper with the openings downwards to remove all residual wash buffer. Insufficient washing (e.g. less than three wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction readings, thus the plates were washed thoroughly. Free positions on the microplate strips were filled with blank wells of the same plate format as that of the parameter to be investigated.

- **Conjugate incubation (2nd step)**

  100 µl of enzyme conjugate (peroxidase labeled anti-human IgG) was pipetted into each of the microplate wells. The plate was incubated 30 minutes at room temperature (+18°C to +25°C).

- **Washing**

  The wells were emptied and then washed following the protocol mentioned above.

- **Substrate incubation (3rd step)**

  100µl of chromogen substrate solution into each of the microplate wells. The microplate was incubated for 15 minutes at room temperature (+18°C to +25°C) and was protected from direct sunlight by using aluminum foil.

- **Stopping**

  100µl of stop solution was pipetted into each of the microplate wells in the same order and at the same speed as the chromogen or substrate solution was introduced.

- **Measurement**

  Elisa plate thermo Elisa reader was used to measure the absorbance of the color intensity of the plate. Ascend software was used to calculate the measurements of the concentration and OD. Photometric measurement of the color intensity was made at a wavelength of 450 nm and a reference wavelength between 620 nm within 30 minutes of adding the stop solution. Before measuring, the microplate was slightly shaken to ensure a homogeneous distribution of the solution

  The method used here to detect the presence of anti-Zika IgM antibody was a semiquantitative method, meaning the results can be interpreted both quantitatively and qualitatively. Here. For the semiquantitative detection of IgM antibody the following ratio was used:

$$\frac{Extinction\ of\ control\ X\ patient\ sample}{Extinction\ of\ calibrator\ 2} = Ratio$$

Here, the extinction of the control or patient sample over the extinction of the calibrator 2 ratio was used. The results were interpreted using the following standards:

Table 2.2 Elisa OD interpretation ratio

Ratio	Interpretation
<0.8	Negative
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The standard curve from which the concentration of antibodies is measured in the patient sample was obtained by a point to point plotting method. In which, two or more points are found on the x and y-axis. Point to point plotting of the extinction readings measured for the 3 calibration sera against the corresponding units (linear/linear) was obtained here. 'point to point' plotting was used for calculation of the standard curve. The following curve is an example of a typical calibration curve.

Fig 2.3 Standard curve of the concentration of antibodies in patient sample obtained by point to point

2.3 ZV quantitative real-time polymerase chain reaction

In molecular biology, **real-time polymerase chain reaction** also called *quantitative real-time polymerase chain reaction* (qPCR) or kinetic polymerase chain reaction, is a laboratory

technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted nucleic acid molecule. It enables both detection and quantification (as an absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in an nucleic acid sample. All samples were tested to detect the presence of Zika virus by Real Time PCR. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified nucleic acid is quantified as it accumulates in the reaction in *real-time* after each amplification cycle. For this experiment, fluorescent dyes that intercalate with double-stranded DNA were used.

An excel sheet designating particular PCR well location in 96 well PCR plate for Zika virus was prepared. The number of reactions (N) to setup per assay was determined. It is necessary to make an excess reaction cocktail for the control reactions and to account for pipetting error.

- If number of samples (n) including NTC + VTC controls = 1 to 48, then N = n + 2
- If number of samples (n) including NTC + VTC controls > 48, then N = n + 4

2.3.1 Procedure

QIAamp Viral RNA Mini Kits were used to isolate Zika virus RNA. The sample was first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions were then adjusted to provide optimum binding of the RNA to the membrane, and the sample was loaded onto the column. The RNA bound to the membrane, and contaminants were efficiently washed away in two steps using two different wash buffers. High-quality RNA was eluted in a special RNase-free buffer (provided with the kit). The purified RNA was free of protein, nucleases, and other contaminants and inhibitors **(Fig 2.4)**.

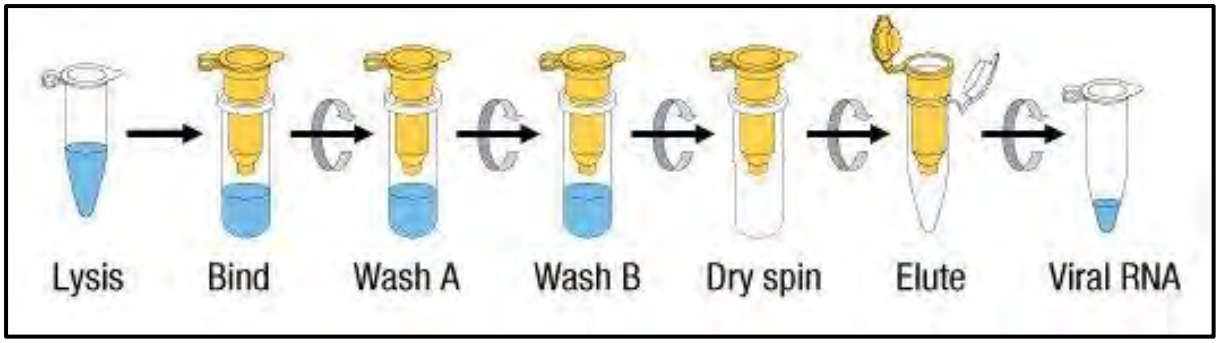

Fig 2.4: Process of viral RNA isolation. The viral RNA was isolated using four different types of buffers. The spin procedure was used.

- **Lysis**

  The sample was first lysed under the highly denaturing conditions provided by Buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA was added to Buffer AVL which improved the binding of viral RNA to the membrane and limited possible degradation of the viral RNA due to any residual RNase activity.

- **Binding**

  The buffering conditions of the lysate were adjusted to provide optimum binding conditions for the viral RNA before loading the sample onto the column. Viral RNA was adsorbed onto the silica membrane during two brief centrifugation steps. Salt and pH conditions in the lysate ensured that protein and other contaminants, which can inhibit downstream enzymatic reactions, were not retained on the membrane.

- **Wash**

  Viral RNA, bound to the membrane, was washed free of contaminants during two short centrifugation steps. The use of two different wash buffers, AW1 and AW2, significantly improved the purity of the eluted RNA. Optimized wash conditions ensured complete removal of any residual contaminants without affecting RNA binding.

- **Elution**

  Buffer AVE is RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases. Sodium azide affects spectrophotometric absorbance readings between 220 and 280 nm but does not affect downstream applications, such as RT-PCR.

- **Spin procedures**

  The purification procedure was carried out in three steps using the columns in a standard microcentrifuge. The procedures were designed to ensure that there was no sample-to-sample cross-contamination and allowed safe handling of potentially infectious samples.

2.3.2 Master Mix and Plate Set-Up

Reaction assay mixtures are made as a cocktail and dispensed into the 96-well reaction plate. Extracted nucleic acid, viral template controls, or water is then added to the appropriate test reactions and controls. Label 1.5 or 2ml microcentrifuge tube for Zika virus primer/probe set (assay) within the PCR work station.

Preparation of master mix for Zika virus assay was as follows:

Table 2.3 Composition of the master mix for the reverse transcriptase PCR

Components	Measurement
2X RT-PCR Buffer	N* 12.5 µl
25X Enzyme Mix	N * 1 µl
Forward primer (100 µM)	N * 0.25 µl
Reverse primer (100 µM)	N * 0.25 µl
Probe (25 µM)	N* 0.25 µl
Nuclease-free water	N * 5.75 µl
Total volume	**N * 20 µl**

**N= Number of Reaction*

The reaction mixture was mixed by pipetting gently up and down, which facilitated avoiding the formation of bubbles. 20 µl of each master mix into each designated well were added. Before moving the plate to the nucleic acid handling area, 5 µL of nuclease-free water into the no template control (NTC) reactions were pipetted and the well was capped immediately in the assay set-up area. The reaction plate was moved to the nucleic acid handling area. The tubes containing the samples were vortexed for 5 sec. followed by brief centrifugation for 5 sec. 05 µl of the template was added to the appropriate well. There is no need to mix or vortex the reaction following sample addition. The column was capped immediately after the sample addition. Finally, 05 µl of the positive viral template was added to control into all VTC wells and was capped immediately. The plate was centrifuged at 1200 rpm for 1 min to remove bubbles or settled down any droplets that may be present in the lid or body of the wells.

2.3.3 Cycling conditions

The following thermal cycling protocol was set in the RT-PCR machine for detection of Zika virus detection:

thermocycler was set as follows:

- 30 min at 50°C
- 10 min at 95°C
- 45 cycles of 15 sec. at 95°C and 1 min at 60°C

(Data Acquisition)

The run completed plate was kept into the PCR machine block until new plate incorporated, and

subsequently discarded the plate into a biohazard bag.

2.4 Statistical analysis

The demographic data were calculated and represented by bar diagrams and pie charts using microsoft excel. Muscle strength scores (MRC-score) were normally distributed throughout the follow up and thus paired T-test was done using SPSS software to compare between Zika positive and negative patients. Graphical representation of the disease severity score was represented using the SPSS software with 95% confidence interval (CI). The MRC score was based on two-tailed probability; a p-value < 0.05 was considered statistically significant.

-Results-

3.1 Demography

In the studied cohort (n=98), majority of the patients were young adults ranging from the age of 16-30 years (39%). The second major group comprised of people who were more than 45 years old (28%). Patients in this cohort was predominantly male (64%).

Table 3.1 Demography and preceding illness of GBS patients (n=98)

Characteristics		Number of patients (%)
Sex	Male	63 (64.29%)
	Female	35 (35.71%)
Mean age		33
Preceding illness		
	Diarrhea	48 (48.97%)
	Respiratory tract infection	10 (10.20%)
	Fever	9 (9.18%)
	Chicken Pox	1 (1.02%)
	Others	10 (10.20%)

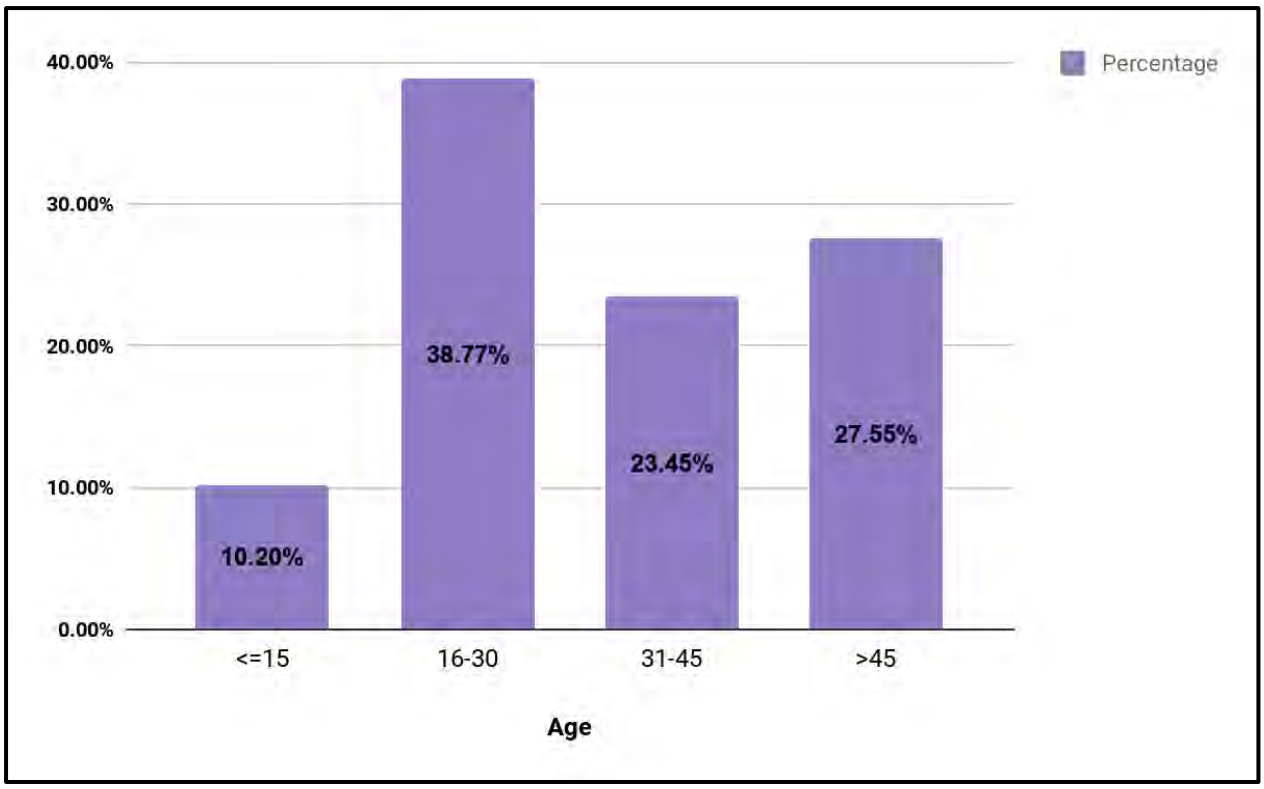

Fig 3.1: Age distribution of GBS patients in Bangladesh; most of the GBS patients in our country are within the age group of 16-30, making this the target age group for GBS.

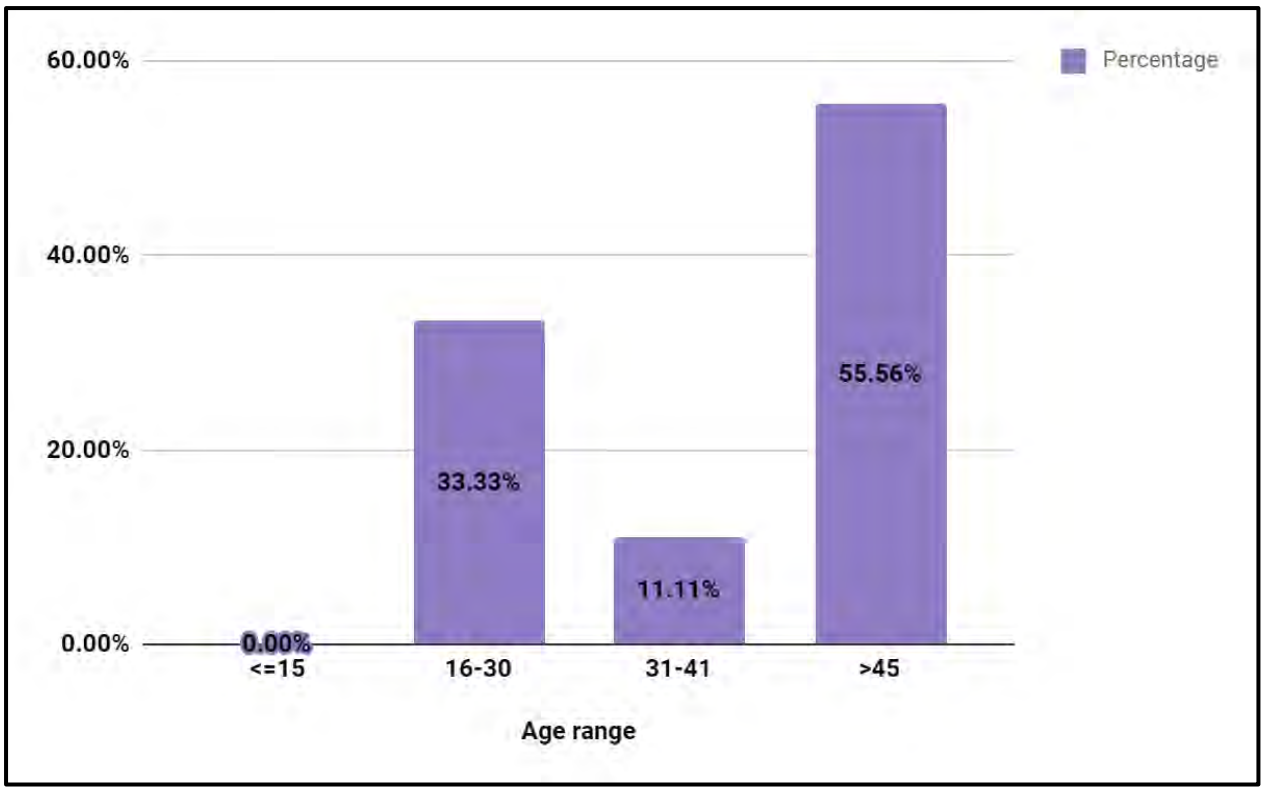

Fig 3.2: Age distribution of Zika positive GBS patients in Bangladesh; most of the Zika positive GBS patients in our country are over 45 years of age. It is also seen that, there was no Zika positive patient who were below 15 years of age.

3.2 Clinical features

Most of the patients had a pure motor variant of GBS in both groups. Among the Zika negative patient, 58% had cranial nerve impairment. 10% had sensory deficits, 60% had pain at entry. Whereas, 55% of Zika positive patient group had cranial nerve impairment, 5% had sensory deficit and 55% had pain at entry. Analysing the MRC score of both groups at entry it can be seen that, 67% of Zika positive patients had a very low MRC score indicating a critical condition at entry as opposed to 32% of Zika negative patients.

Table 3.2: Clinical and electrophysiological characteristics of GBS patients

Clinical Features	Zika Negative Patients	Zika Positive Patients
Cranial nerve impairment	57(58.16%)	10 (55.55%)
MRC sum score (at entry)		
60-51	5 (5.10%)	1 (55.55%)
50-41	4 (4.08%)	0 (0%)
40-31	11 (11.22%)	4 (22.22%)
30-21	5 (5.10%)	1 (5.55%)
20-0	32 (32.65%)	12 (66.67%)
Sensory deficits (at entry)		
None (pure motor variant)	88 (89.79%)	17 (94.44%)
Present	10 (10.20%)	1 (5.55%)
Pain (at entry)	59 (60.20%)	10 (55.55%)
GBS disability score (at entry)		
0 or 1 (healthy or minor symptoms)	1(1.02%)	5 (27.78%)
2 (able to walk independently)	4(4.08%)	2 (11.11%)
3 (unable to walk independently)	12(12.24%)	2 (11.11%)
4(chair bound or bedridden)	61(62.24%)	5 (27.78%)
5 (ventilation)	20(20.40%)	4 (22.22%)
Electrophysiology		
Classification (n=98)		
Axonal	40 (40.81%)	8 (44.44%)
AIDP	27 (27.55%)	7 (38.89%)

Unclassified	15 (15.31%)	2 (11.11%)

3.3 Prevalence of Zika virus infection among GBS patients in Bangladesh

According to the anti-Zika IgG and IgM Elisa result, it was known that 18 out of the 98 GBS patients had Zika virus infection (18.36%). 50% among them having cranial nerve impairment, 11% having sensory deficits, 5% having an axonal variant of GBS and 5% having AIDP variant, rest being unclassified.

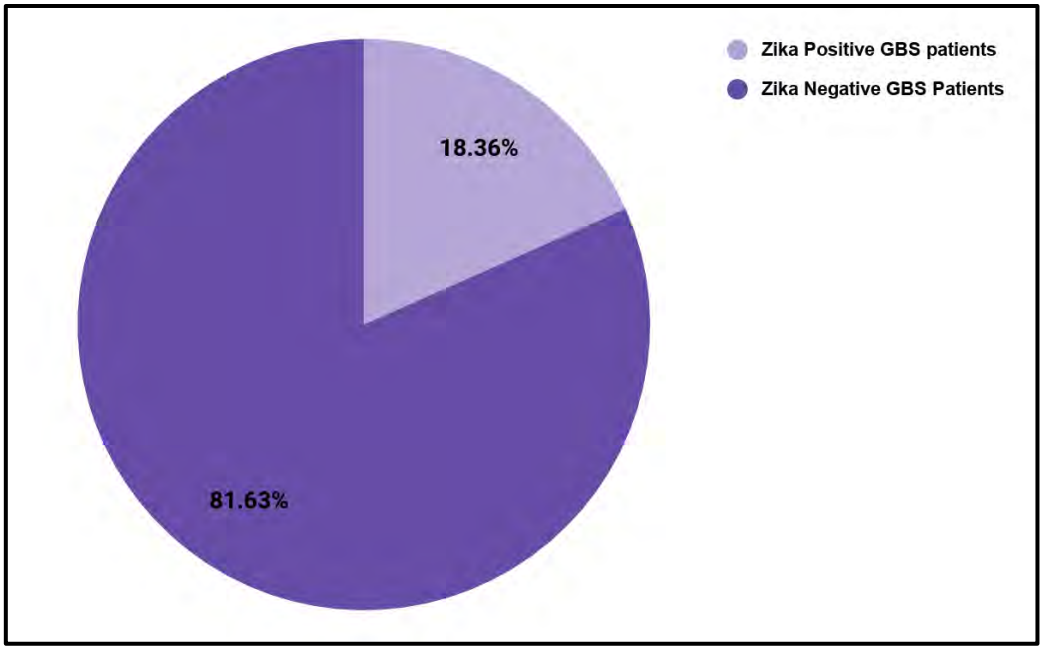

Fig 3.3 Prevalence of Zika virus infection among GBS patients in Bangladesh; among the total number of GBS patient samples, 18.36% had Zika virus infection.

3.4 Correlation of ZV infection with the GBS disease recovery

The MRC sum score determines the state of health of the patients. The MRC score is measured upon entry and every week onward. The MRC sum score on the 26 weeks follow-up was measured of the patients to determine their recovery rate. The higher the range of the MRC score is, the better the condition of the patient is. In a comparative study between Zika positive and negative patients, it was seen that most patients in both categories had the MRC sum score between 60-51 indicating that the patients' recovery was good till their 26 weeks follow-up. 44% for Zika positive patients and 40% for Zika negative patients fell within 60-51 score range indicating very good recovery condition**.** 5% of the Zika positive patients' MRC score ranges within 50-41 and 11% of the Zika negative patients fell within that range. 5% Zika positive patients and 11% of the Zika negative patients fell within 40-31 range. 11% of both Zika positive and Zika negative patients fell within 30-21 range. And lastly 33% of Zika positive and 19% of Zika negative patients fell within 20-0 range indicating quite a critical condition.

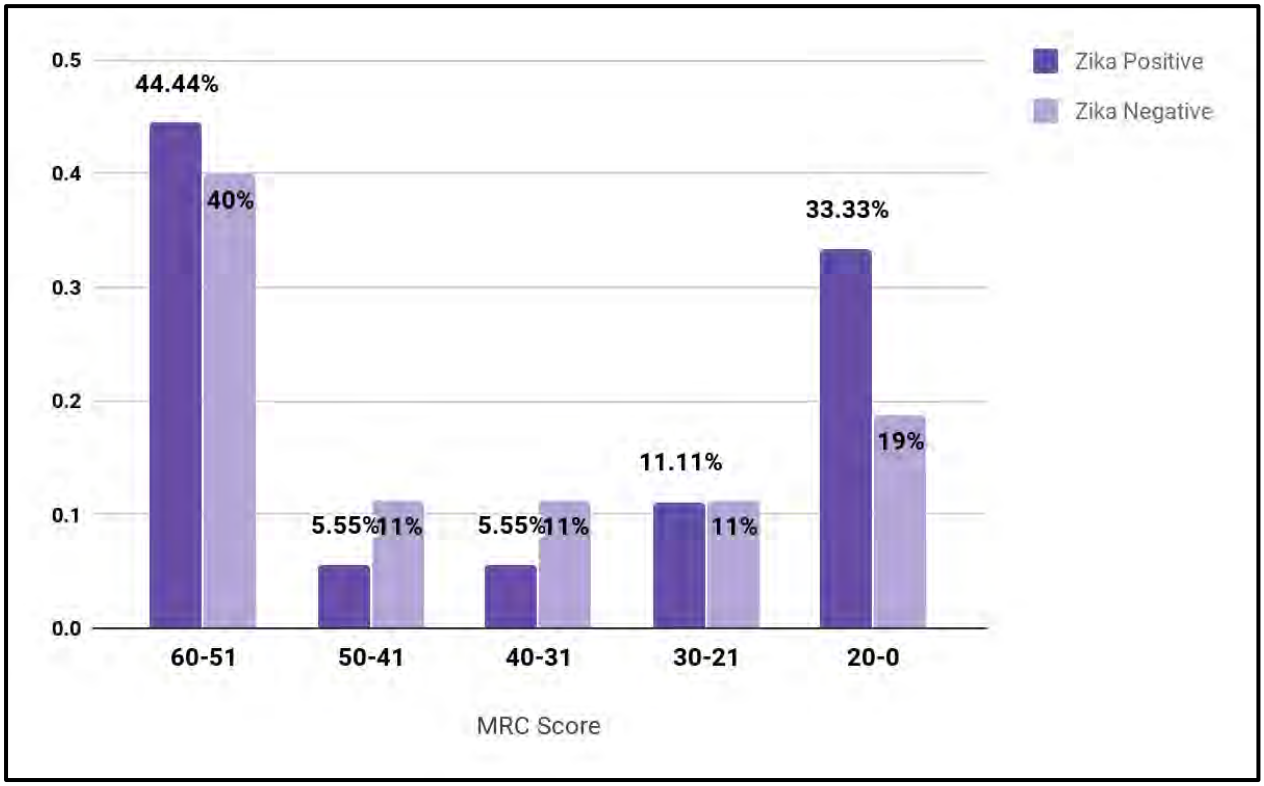

Fig 3.4 MRC sum scores between Zika positive and negative patients (week 26); the dark purple color stands for zika positive and light purple color stands for zika negative patients here. The MRC score increased gradually but with a similar frequency for both Zika positive and negative patients showing no significant correlation.

3.5 Correlation between GDS (GBS disability score) and GBS disease recovery

The GBS disability score (GDS) is also used to determine the physical state of the patients. The GDS was also measured upon entry and on each week onward. The GDS is measured by numbers from 0-6 where each number is assigned to a specific condition, which is, 0 or 1-healthy or minor symptoms, 2-able to walk independently,3-unable to walk independently, 4-chair bound or bedridden,5-ventilation.

In a comparative study between Zika positive and Zika negative patients, we found that most Zika positive patients had a GDS of 0 or 1 (38%) and most Zika negative patients had a GDS of 4 (77%). 16% of Zika positive patients and 27% of Zika negative patients had GDS of 2. 5% Zika positive and 7% Zika negative patients had GDS of 3. 27% Zika positive and 77% Zika negative patients had a GDS of 4 and finally 11% Zika positive and 1% Zika negative patients had GDS of 5. From this it is apparent that the recovery rate is higher among Zika positive patients than their Zika negative counterpart. However, since a GDS score of 5 indicates artificial ventilation required it is apparent from the graph that the percentage of Zika positive patients to require artificial ventilation than Zika negative counterpart (Fig 3.5)

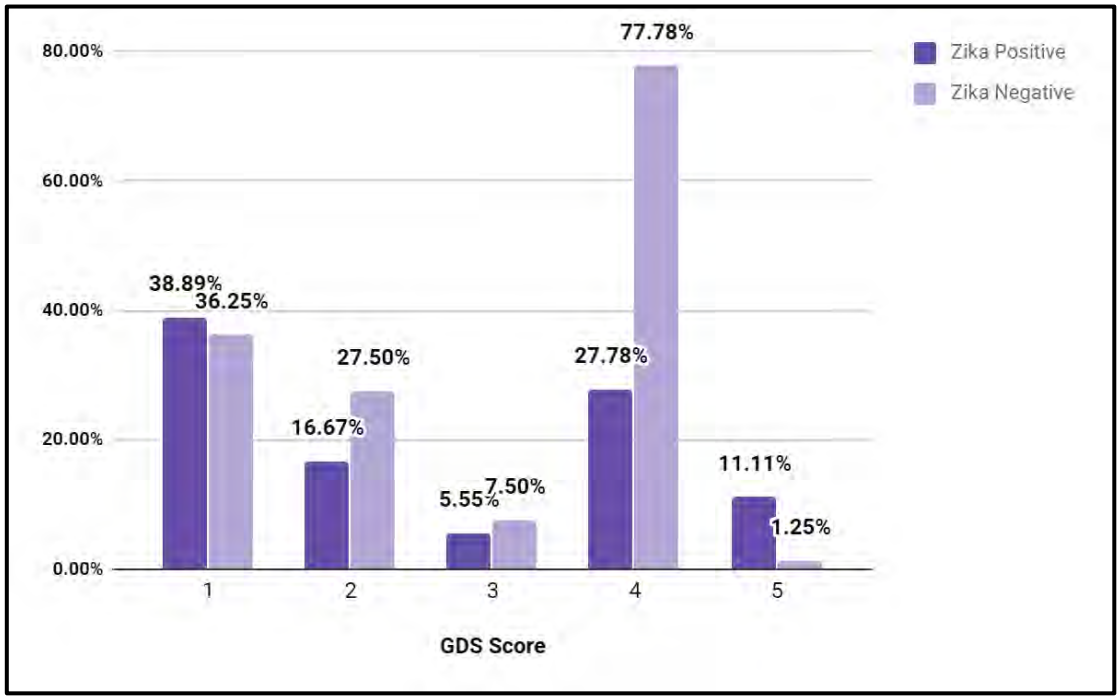

Fig 3.5 Correlation of GDS (GBS disability score) between Zika positive and Zika negative patients based on week 26 follow-up; the dark purple color stands for zika positive and light purple color stands for zika negative patients here. We can see that the GBS disability score is significantly low in Zika negative patients comparing to their Zika positive counterparts.

Table 3.3 Statistical analysis of MRC score of week 13 and week 26

Type		N	Mean	Std. Deviation	P-value
13 weeks follow up					
Baseline	Zika Positive	8	39.13	22.674	0.31326
	Zika Negative	48	35.63	18.064	
26 weeks follow up					
Baseline	Zika Positive	8	41.00	22.475	0.430478
	Zika Negative	48	42.02	20.457	

The data set was normally distributed here, therefore the distribution was symmetrical to the mean. Hence, T-test was done here to figure out the difference between the means of the two data sets. On 13 week follow up the mean value of Zika positive and negative patients are 39.13 and 35.63 with the standard deviation of 22.674 and 18.064 as follows. On 26 week follow up the mean value of Zika positive and negative patients are 41.00 and 42.02 with the standard deviation of 22.475 and 20.457 as follows. The p-values in both follow ups are larger than the cut-off value (>0.05) indicating very weak significance.

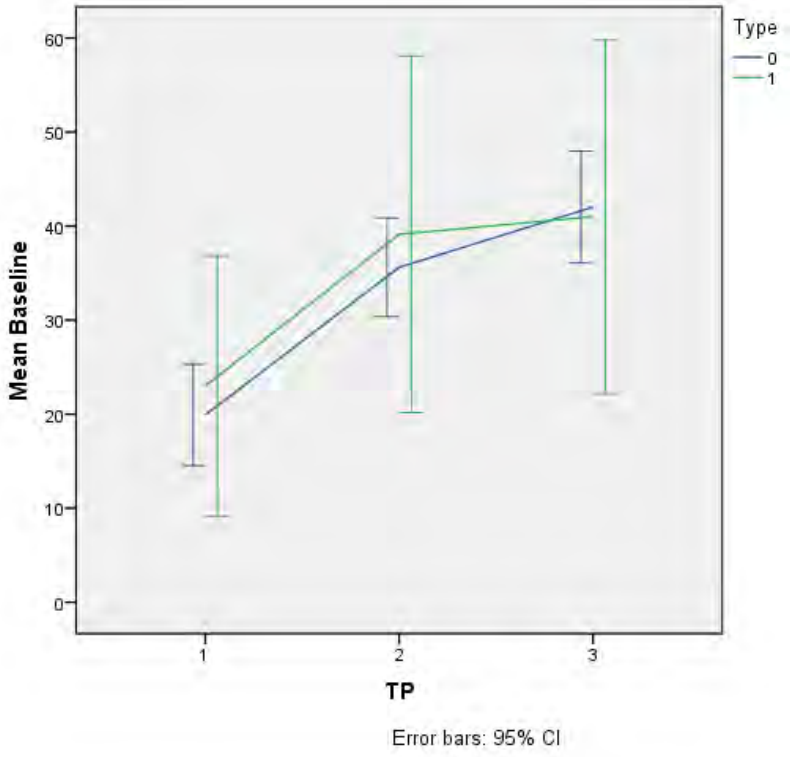

Fig 3.6 Analysis of MRC scores of both Zika positive and negative patients based on week 13 and week 26 follow up. Here, TP= time point; 1= Base line, 2= 13 week, 3= 26 week; 0 (blue) = negative, 1 (green) = positive. Both the green and blue lines are seen to be going up going up and the over lapping at one point, showing no significant correlation between the MRC scores.

-Discussion-

Discussion:

The overall aim of this study was to investigate the role of ZV infection on Guillain-Barre syndrome and to analyse the demography by which it affects the population. In a study previously done on Bangladeshi population on samples collected till 2015, no significant association was found between ZV infection and GBS (C.H. GeurtsvanKessel et al; 2018). However, ZV incidences were reported in Bangladesh later on as well. This led to conducting this study on the Bangladeshi population to assess the endemic Zika circulation for the formation of GBS.

From this study, it has been seen that 18% of the GBS patients previously had Zika virus infection. Up until now, majority of the studies related to Zika virus causing GBS focused on symptomatic Zika virus patients and their outbreak (Cao-Lormeau VM; 2014). Interestingly enough, Zika virus is symptomatic in only 20% of the cases. The first reported case of the association between ZV and GBS indicated that the axonal variant of GBS was exclusively associated with ZV infection, whereas recent reports from Brazil and Colombia show that AIDP is the subtype of GBS associated with ZV infection (Cao-Lormeau VM; 2014, Parra B; 2016). In our study, all included GBS patients, 44% had an axonal variant of GBS and 38% had AIDP variant, rest were being unclassified. The MRC score of most Zika positive patients ranged between 51-60 on their 26th week in the hospital. The MRC score was just as high for Zika negative GBS patients showing no significance.

In accordance with our earlier report from Bangladesh (Islam Z, Jacobs BC;2010) there was a considerable delay before the GBS patients reached the hospital (an average of 11 days after onset of weakness). Such delay can result in alteration of the mean interval between a possible antecedent infection and specimen collection. It is a plausible explanation for not detecting ZV genome by PCR in serum. Thus, anti-Zika IgM Elisa was done to detect the presence of IgM antibodies in the patient samples. The absence of IgM antibody shows that the ZV infection was not recent. The assessment of ZV in urine or whole blood would have been a valuable addition to the study protocol and would be considered in future studies (Lustig Y; 2017). Among the 98 patients with GBS 18 patients were Zika virus positive and had anti-Zika IgG antibodies. The IgG antibodies appear in a later phase of any infection and may last for the several months. This proves that those patients with GBS might have prolong ZV infection.

The most significant finding of this study was the fact that ZV induced GBS is predominant in male population (83%) and half of the patients were aged above 40 years. In a study previously done on Bangladeshi population also found that 88% of the ZV positive GBS

patients were male (C.H. GeurtsvanKessel et al; 2018). Generally, ZV infection itself is more common in women (Zika striking women in higher rates than men: U.S. study; 2016). However, since GBS is an infectious autoimmune disease, the predominant group differs from the aforementioned study. It is known that testosterone level in men plays a crucial role in inhibiting autoimmune disease (Vendramini AC, Soo C, Sullivan DA; 1991) by reducing the number of B cells, a type of lymphocyte that produces harmful antibodies (Doughman; 2018). However, study shows testosterone level starts reducing in men after they reach 40 years age (Testosterone, aging and the mind; 2008). This reduction in testosterone level is due to a dysfunction in the hypothalamic-pituitary-testicular axis (Feldman HA; 2002). Free testosterone is considered biologically the active form of the hormone. Aging is related with a reduction of the hormone as the sex hormone binding globulin (SHBG) increases, binding the free testosterone, which in turn reduces the total serum concentration of the hormone (Wu FC, Tajar A, Pye SR, et al.,2008). Another study states that, testosterone treatment induces a strong increase in the Treg cell population (Magdalena Walecki ; 2015) and Treg cells are a subpopulation of T-cells that maintain tolerance to self-cells and prevent autoimmune diseases. Thus, reduction of testosterone level decreases the activity of the Treg cells that can induce autoimmune diseases in male body, which in this case is GBS. In one large cross-sectional study of more than 3000 men ages 40 to 79, serum testosterone concentration fell 0.4% per year, free testosterone concentration 1.3% (FC Wu; 2008). These could be explanation behind the high percentage of male over the age of 40, found in our study, who developed ZV induced GBS.

This study had a few limitations, firstly, the sample size was too little to come down to a specific conclusion. Secondly, cross-reactivity with other mosquito-borne viral infections like DENV, Chikungunya virus, malaria virus was not checked which may lead to false to inaccurate results. Viral neutralization test needs to be done to confirm the Zika virus positivity. Several prospective studies can be conducted based on this study.

In conclusion, there may be some connection between ZV infection and Guillain Barre Syndrome and it is predominant in male population. However, the specific mechanism through which ZV causes GBS is still unknown, therefore, a study can be conducted to learn the specific pathogenesis of ZV causing GBS which can be a future study aspect.

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