Incidence of Norovirus Infection among Hospitalized Patients in Bangladesh: A Case-Control Study.

A Dissertation Submitted to East West University in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology

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Declaration

This is to certify that, thesis entitled **"Incidence of Norovirus Infection among Hospitalized Patients in Bangladesh: A Case-Control Study" by AnikSaha,** ID: 2016-1-77-054, to be submitted to the Department of Genetic Engineering and Biotechnology, East West University, Aftabnagar, Dhaka-1212, in partial fulfillment of the requirement for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology. This work was carried out under our supervision and the content of the thesis have been approved and recommended for the award of Bachelor degree.

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List of Abbreviations

^{0}C	Degree celcius
µl	Microliter
BLAST	Basic Local Allignment Search Test
bp	Base pair
CDC	Center for Disease Control and prevention
ddNTP	Dideoxynucleotide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
HBGA	Histo blood group sntigen
MEGA	Molecular Evolutionary Genetical Analysis
ml	mililiter
NCBI	National center for Biotechnology Information
mRNA	Messenger Ribonucleic acid
NoV	Norovirus
ORF	Open Reading frame
PCR	Polymerase Chain Reaction
VLP	Virus like protein
VP	Viral protein
RT-PCR	Real time Polymerase Chain Reaction
RMCH	Rajshahi Medical Collage Hospital
JIMCH	Jahurul Islam Medical Collage & Hospital
JRRMCH	Jalalabad Ragib-Rabeya Medical Collage & Hospital
JGH	Jessore General Hospital
SBMCH	Sher-e-Bangla Medical Collage Hospital
BGH	Bogra General Hospital
RpMCH	Rangpur Medical Collage Hospital
RGH	Rajbari General Hospital
CMCH	Chittagong Medical Collage Hospital

Abstract

Norovirus is the most common cause of both outbreaks and sporadic non-bacterial gastroenteritis worldwide and one of the most common causes of acute gastroenteritis among children in developing countries. In this study, norovirus infections were investigated in diarrheal (Case) and non- diarrheal (Control) patients who have attended in nine hospitals during October, 2019 in Bangladesh. A total of 140 fecal specimens from both children and adults were tested for the presence of norovirus using real-time RT-PCR. Among them, ~12% (n=16) of the specimens were found positive for norovirus RNA, among which only four were further genotyped by sequencing the partial capsid and polymerase gene. Norovirus GII was the most prevalent genogroup. Based on their clustering in the phylogenetic tree, two distinct genotypes were identified- GII.4 as the most prevalent genotype followed by one GII.3. A significant number of diarrhoeal patients hospitalized were found to be infected with norovirus; therefore, community surveillance is required to estimate the true burden of the virus in the country. Genomic analysis of the circulating norovirus strains would be very useful for understanding the pathogenesis and vaccine development. Moreover, the dynamic molecular epidemiology of norovirus requires routine strain surveillance to identify changes in prevailing strains.

Chapter I: Introduction

1.1 Background:

Gastroenteritis, mainly caused by virus, is the representation of the infection occurs in the gastrointestinal tract of human. It is also known as "Stomach flu" and the main symptoms are diarrhoea and vomiting. Despite significant decreases in recent decades, acute gastroenteritis causes the second greatest burden of all infectious diseases, estimated at 89·5 million disability-adjusted life-years (DALYs) and 1·45 million deaths worldwide every year (Medscape, 2019). Recent evaluations advocate the annual mortality is 2.5 million deaths every year in children in developing countries with quite 700 million of registered cases worldwide (Kosek et al,. 2003) (Wilhelmi et al,. 2003). Its associate in nursing acute illness that's usually self-limiting, however maybe devastating and grievous in elderly, very young and immunocompromised patients (Karst et al,. 2010).

Norovirus consider as the eminent causing agent of acute and sporadic diarrhoea among all other diarrhoea causing agents those infect individuals of all ages (Nahar et al,. 2013). Fecal-oral route of transmission along with high stability in high temperature, enormous genetic diversity due to lack of proof-reading activity and low infectious dose (10 to 100 virions) make this virus to be considered as prominent one (Nahar et al,. 2013) (Beuret et al,. 2002).

In recent time, low viral load of norovirus has been found in healthy individuals. This finding rises an urgency to determine the exact relationship between diarrhea and norovirus. Norovirus has been classified into eight genogroups, and among them genogroups GI, GII, and GIV are associated with human infection (Vinje 2015). Genogroup is further divided into multiple genotypes and variants based on either the VP1 amino acid or the nucleotide sequence divergence of RdRp. Along with point mutations, recombination events seem to contribute largely to the genetic variability of NoVs (Medici et al., 2015). Their inability to grow in tissue culture is a great obstacle of vaccine development.

In a recent study conducted in Bangladesh, six GI genotypes (GI.3–GI.8) and 18 GII genotypes (GII.1–GII.10, GII.12– GII.14, GII.17, GII.20–GII.22, and GII.25) have been found and among them the globally dominant GII.4 genotype was identified most frequently (~20% of sequences), including the GII.4-New Orleans (13%) and GII.4-Sydney (7%) variants (Nelson

et al., 2018). Although there is increase in reporting of norovirus diarrhoea throughout the world, involvement of NoV with diarrhoea is incompetently understood in Bangladesh and further studies are also required to establish the clinical relevance of norovirus genotypes causing diarrhoea.

1.2 Aims and Objectives:

1. To determine the incidence of human norviruses in hospitalized patients among nine hospitals around Bangladesh in October, 2019.
2. Phylogenetic analysis and Similarity analysis of norovirus strains circulating in Bangladesh in October with the global strain to reveal genetic diversity.

Chapter II: Literature Review

2.1 Historical Background

The first case of Norovirus gastroenteritis was described by Zahorsky in 1929 as 'winter vomiting disease'. But at that time the virus was not known by name. He termed the disease as "Winter Vomiting Sickness" due to its seasonal preference and the frequent predominance of patients with vomiting as a primary symptom. The first outbreak of this virus was reported in an elementary school in Norwalk, Ohio, USA, in 1968. The virus was identified in 1972 as a cause of acute gastroenteritis through an experiment where this virus passed from one sick volunteer to another. Small, round viruses of 27-31 nm in diameter were described by visualizing stool on immune electron microscopy. For the first time, viruses had been confirmed as a cause of gastroenteritis. This strain, known as the Norwalk agent or strain, became the prototype for what was then described as Norwalk-like viruses or Small Round-structured Viruses (SRSVs), now called noroviruses. Norovirus is known by many names that include Norwalk virus, Norwalk-like virus, Norwalk agent, small rounded structured viruses, winter vomiting bug, winter vomiting disease, Snow Mountain virus, acute non-bacterial gastro-enteritis, viral gastro-enteritis and stomach flu. The name `norovirus' was approved by the International Committee of Taxonomy of Viruses in 2002 (Kapikian et al., 1972).

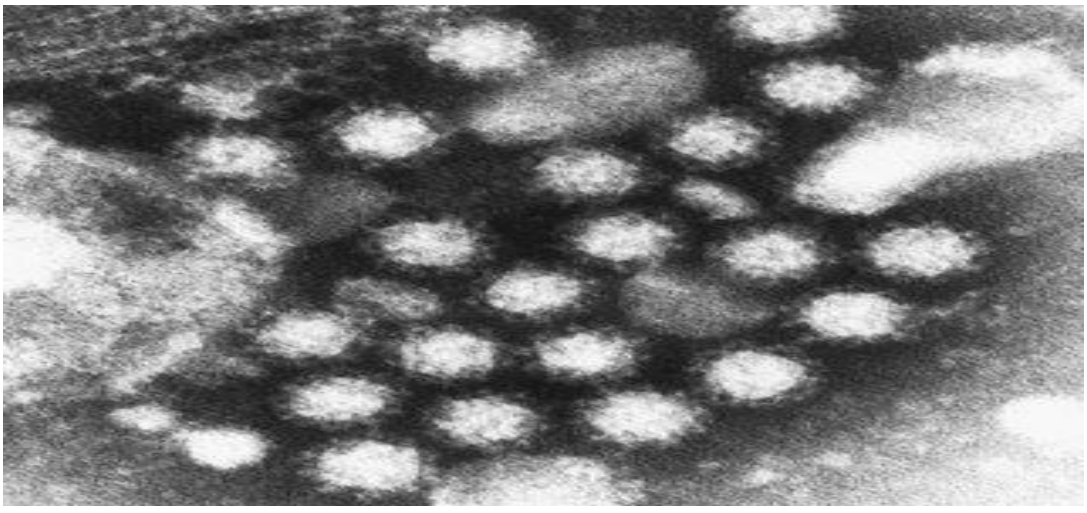


Figure 2.1 Norwalk virus (NV) particles in a stool filtrate visualized by immune electron microscopy (IEM) (Kapikian et al., 1972).

2.2 Classification of Norovirus

Norovirus belongs to the family Caliciviridae which is split into 5 genera: Vesivirus, Lagovirus, Nebovirus, Sapovirus and Norovirus. Norovirus and Sapovirus are the solely contributors which are capable to infect humans and motive gastroenteritis (Thorne and Goodfellow, 2014). As there is no culture method for Norovirus, genetic analysis is the main approach to classify norovirus. Norovirus is subdivided in eight genogroups (GI — GVIII) (Chan, Guan and Chan, 2016). Human NoVs are observed in GI, GII, and GIV, whereas animal NoVs are allotted among distinctive genogroups. Specifically, bovine NoVs are found in GIII, pigs NoVs in GII, murine NoVs in GV, and dogs NoVs in GVI.

NoVs are single-stranded RNA viruses, and they have highly mutation and recombination susceptible genome which leads to great genetic diversity and the emergence of new strains. The need to organize norovirus strains into different genetic groups or clusters was recognized in the mid-1990s when noroviruses were primarily divided into genogroups and genotypes based on partial RdRp sequences (P-type). When more sequences became available, classification shifted to designate genogroups and genotypes based on the complete VP1 amino acid sequence with 20% sequence difference used as a cut-off threshold for new genotypes, which was later adjusted to 15% (Chan, Guan and Chan, 2016).

Three (GI, GII, and GIV) genogroups are associated with human infection form which GI and GII are clinically important genogroups (Kageyama et al., 2003, Zheng et al., 2006). Currently, 9 genotypes in GI, 22 in GII, 2 in GIII, 2 in GIV, 1 in GV, 2 in GVI, and 1 in the tentative new GVII have been identified on the basis of complete VP1 amino acids (Vinje et al., 2000). However, for the last 20 years, GII.4 has been by far the most prevalent genotype worldwide, and has been responsible for global epidemics in persons of all ages (Bull et al., 2006, Tu et al., 2008).

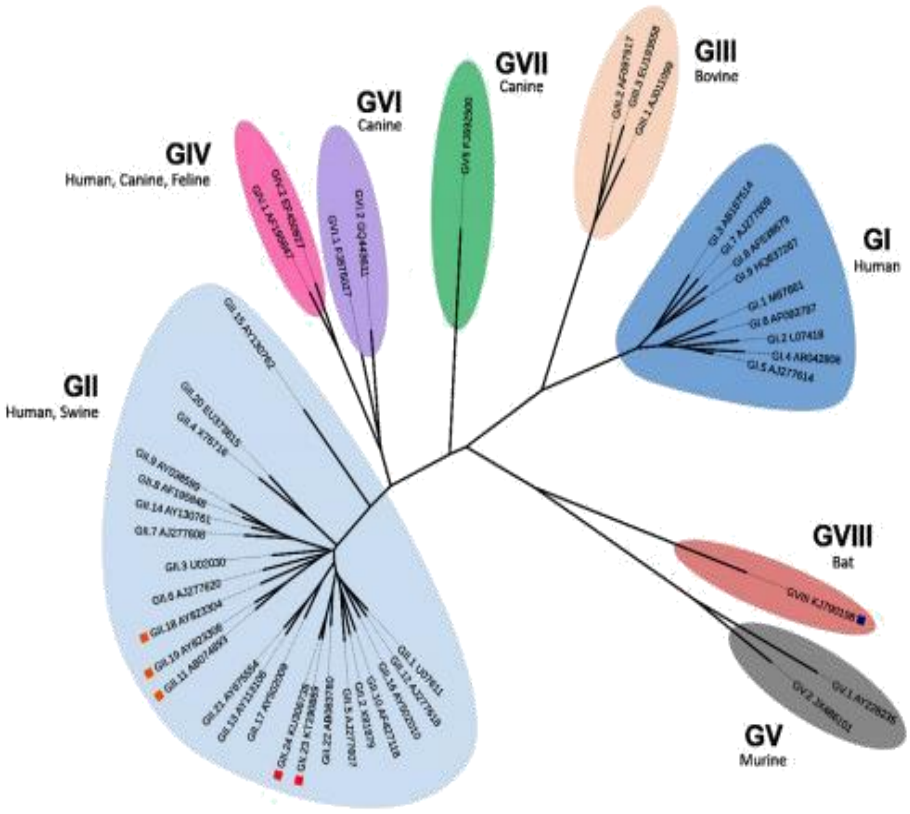


Figure 2.2: phylogenetic analysis of Norovirus. Classification of norovirus into eight genogroups (GI- GVIII) and 44 genotypes using neighbor-joining phylogenetic inference on complete amino acid sequences of VP1. One tentative new genogroup (GVIII from bats) and two tentative new genotypes (GII.23 and GII.24) not described in Vinje (2015) are indicated by blue (light gray in print versions) and magenta (dark gray in print versions) squares, respectively. The animal host of each genogroup is shown in gray text underneath genogroup designation. All genotypes within GII infect humans except GII.11, GII.18, and GII.19, which infect pigs (black squares) (Chan, Guan and Chan, 2016).

2.3 Norovirus Structure and Genome Organization

Norovirus is a small, round, structured non enveloped virus with various cup shaped depressions on its surface. Structural studies showed that the capsid is composed of 180 VP1 proteins which arranged as dimers and each of which divided into shell and protruding protein.

The protruding domain, P2, is highly variable region, and this region recognizes the histo-blood group antigens, which are considered as receptors and host-susceptibility factors for infection (Glass et al., 2009).

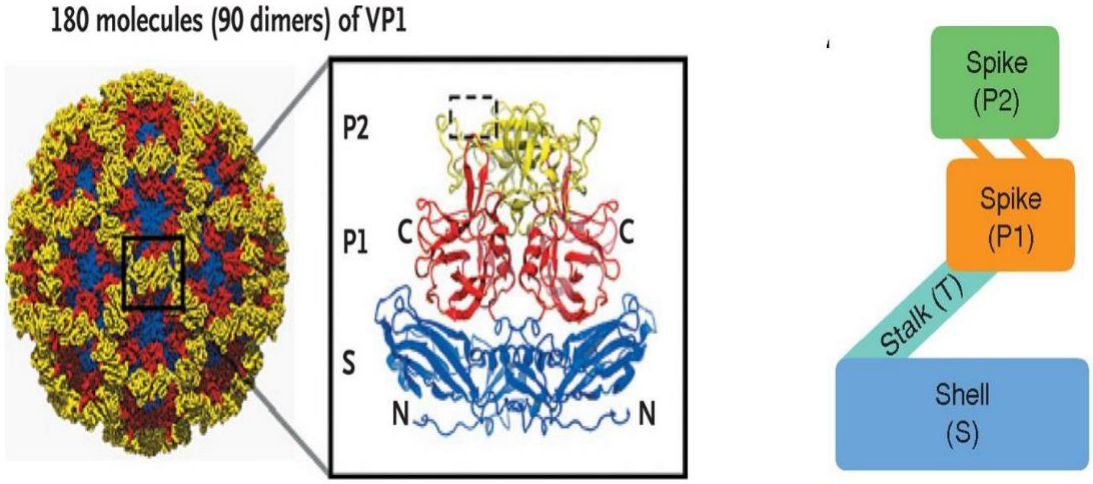


Figure 2.3: Structure of Norwalk Virus Capsid (Glass et al., 2009). A schematic diagram showing the modular organization of capsid subunits, consisting of trapezoid-shaped shell (S), long and flexible stalk (T) and protruding "spike" (P) domains. The P-domain consists of P1 subdomain emerging from the S-domain and P2, which is an insertion in P1 and positioned at the outermost surface of the virus.

The genome is of approximately 7.7 kb in length containing three open reading frames, ORF1, ORF2 and ORF3. There is a protein linked at the 5' end of the genome and polyadenylated at the 3' end. ORF 1 encodes the nonstructural proteins, p48, NTPase, p22, VPg, 3CLpro, and RNA dependent RNA polymerase (RdRp) that are processed co- and post-translationally by the viral 3C-like protease (3CLpro) (Thorne and Goodfellow, 2014). p48 is the N-terminal protein of ORF1. In this protein there is a significant amount of variation in length and in amino acid sequences between genogroupl and genogroupIl viruses. Sequence conservation increases toward the C terminus of the protein (Hardy, 2005). ORF2 and ORF3 are translated from a subgenomic RNA and encode the structural components of the virion, viral protein 1 (VP 1) and VP2, respectively. The subgenomic RNA is identical to the last 2.4 kb of the genome and is also attached covalently to VPg at the 5' end with a poly(A) tail at the 3' end (Hardy, 2005). The nomenclature and functions of the norovirus proteins are summarized in table 1.1 (Thorne & Goodfellow, 2014).

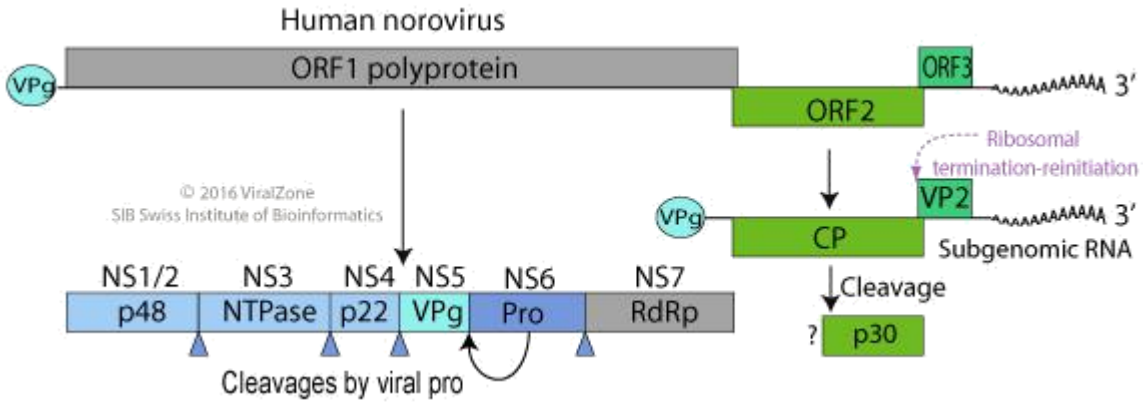


Figure 2.4: Human Norovirus Genome ("Norovirus ~ ViralZone page", 2019)

Table 2.1: Nomenclature for HuNoV proteins and their function

HuNoV (Human Norovirus)	Function	Reference
NS1	Unknown	
NS2/p48NS	Involved in replication complex formation	(Thorne and Goodfellow, 2014)
NS3/NTPase	RNA helicase/NTPase activity	(Natoni et al., 2006)
NS4/p22	Inhibits secretion of host proteins. Also involved in replication complex formation	(Karst, 2010)
NS5/VPg	Genome-linked protein involved in translation and replication	(Sosnovtsev et al., 2006)
NS6/Pro	Protease which mediates cleavage of ORF1 polyprotein	(Bok et al., 2009, Zeitler et al., 2006)
NS7/RdRp	RNA dependent RNA polymerase which replicates the viral genome	(Sosnovtsev et al., 2006)
VP1	Major capsid protein	(Radford et al., 2006)
VP2	Minor capsid protein, which increases expression of VP1 and stabilizes virus particles.	(Ko et al., 2006)

2.4 Norovirus Replication

Little is known about norovirus replication due to lack of an in vitro cell culture and small animal model systems. However, understanding of norovirus replications has come from animal calicivirus and the use of gnotobiotic pig as an animal model for the study of human norovirus. he binding of human norovirus VLPs (virus like particles) with cell surface receptor involves carbohydrate structures known as histo blood group antigens (HBGAs) (Thorne and Goodfellow, 2014). HBGAs are complex Glycans, which are found on the surfaces of red blood cells, expressed in the gut, and on respiratory epithelia also in many biological secretions of humans, such as saliva (Marionneau et al., 2001). Norwalk VLP binds to substrates modified by the A-out not B-enzymes, which results in binding to A blood type but not B. However once Norovirus RNA, which is linked with VPg at 5' end, recognized by cellular translation initiation factors and this mRNA is used as template for translation of viral RNA into protein by the cellular translational apparatus.

Calicivirus genomes involve VPg for translation of mRNA, that is not found in many other animal RNA viruses and this VPg also act as cap substitute. Viral proteins VP1 and VP2 are translated primarily from the subgenomic RNA. By using this strategy, Norovirus produce high amount of viral major capsid protein for virus assembly. And maybe that is why subgenomic RNA is more abundant in infected cells than the viral genomic RNA in infected cells (Thorne and Goodfellow, 2014). The Norovirus subgenomic RNA is polycistronic, so translation of VP2 occurs by a termination— re-initiation mechanism. After termination of ORF2 translation (VP1), ribosomes remain associated with the RNA and reinitiate the start of ORF3 (VP2). There are overlapping stop and start codons of ORF2 and ORF3, respectively, and this codon help in termination initiation. After translation of the ORF I polyprotein, co- and posttranslational processing takes place, which is done by the viral NS6 protease, and results in the release of the viral NS proteins ready for replication complex formation and their precursors, some of which are thought to be functionally active in replication (Belliot et al., 2005). Because of being a positive sense RNA virus, Norovirus use negative sense RNA intermediate for genome replication. The RNA dependent RNA polymerase (RdRp) catalyzes this replication. In Norovirus, NS7 is referred as RdRp, which is conserved structurally and functionally (Hogbom et al., 2009). After first round of translation of the parental viral RNA genome, the mRNA template then functions as a template for the formation of a double-stranded replicative form (RF). The RdRp has two mechanisms for initiation of replication one is: de novo and another is VPg dependent. The processes of norovirus assembly, encapsidation

and exit are not yet fully understood. The VP1 is capable to be self-assembled into virus like particles (VLPs) during virus replication which are indistinguishable morphologically and antigenically from native virions. Some cellular proteins may also be involved in assembly. VP2 is not required for VLP assembly, but it promotes the stability of VP1 and is essential for the production of infectious virions. VP1 and VP2 interact with each other and a conserved motif of VP2 was found in the shell domain of VP1 (Vongpunsawad et al., 2013). This place of VP2 in the interior of the capsid was thought to play role in encapsidation. After encapsidation, viruses have to exit from infected cell to complete their life cycle. A possible way of exit in case of norovirus is apoptosis, as accretion of apoptotic epithelial cells has been observed in infected patients. The mechanisms of subsequent assembly of the daughter virions, their maturation and release from the host cell are poorly understood (Green, 2007).

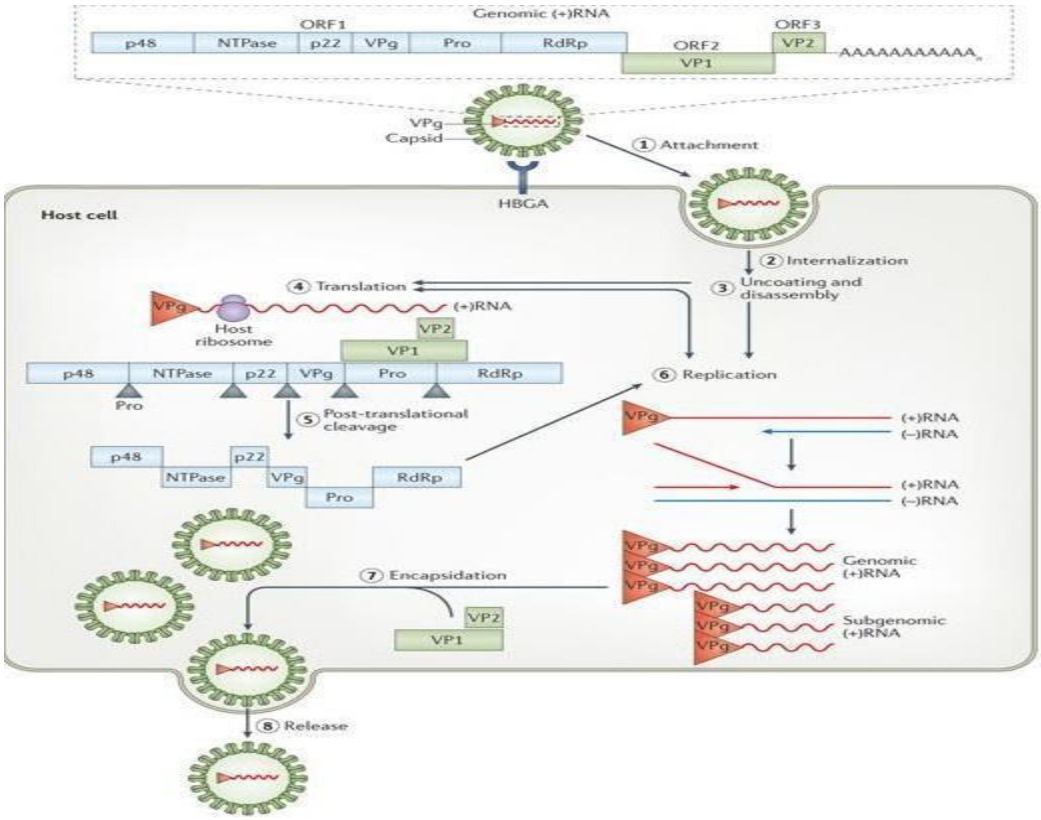


Figure 2.5: Life cycle of human norovirus

2.5 Pathogenesis of Norovirus to cause Diarrhoea

Commonly, viruses use the negatively charged sialic acid and heparan sulfate carbohydrates as cellular receptors, which are expressed on many types of cells and tissues. But norovirus use different types of carbohydrates as their receptor such as, ABO blood group antigens. In small

intestinal gut section, there is villi projecting into the lumen with the crypts beneath the villi and the highest expression of ABH and Lewis carbohydrate antigens on enterocytes at villi tips (Hutson et al., 2004). These carbohydrates play role in cell-cell interactions, self and non-self-identification, and in protection from the environment and pathogens. It was found that, norovirus infection results in broadening and blunting of the intestinal villi, cytoplasmic vacuolization, crypt-cell hyperplasia, infiltration of both polymorphonuclear and mononuclear cells into the lamina propria (Levy et al., 1976), but the mucosa itself remaining intact.

Mild steatorrhea and transient carbohydrates malabsorption have been risen due to decreased enzymatic activity at the brush border of small intestine (Agus et al., 1973). Histological changes occur due to gastric secretion of hydrochloric acid and pepsin. Norovirus infection results in reduced gastric motility which is esponsible for the nausea and vomiting associated with this gastroenteritis (Glass et al., 2009).

2.6 Clinical Manifestations

The Symptoms and the course of infection of this virus can possess variability at the level and severity between different age groups. The incubation period of norovirus is 24-48 hours and symptoms range from vomiting, diarrhoea, low-grade fever and malaise. The gastrointestinal symptoms are normally waning within 12-72 hours of infection. But in cases of infants and children, symptoms may last for up to 6 weeks (Estes et al., 2000, Karst, 2010, Kirkwood and Streitberg, 2008, Murata et al., 2007). In immunocompromised or immunosuppressed patients, such as transplant patients, the symptoms may be prolonged, sometimes to up to 2 years (Gallimore et al., 2004, lass et al., 2009, Kaufman et al., 2003, Kaufman et al., 2005, Nilsson et al., 2003, Westhoff et al., 2009) and in some patients, especially in the elderly, infection by the virus may even cause death (Karst, 2010, Lopman et al., 2004, Lopman et al., 2003). Maybe the failure of host immune system to eradicate the virus efficiently from the body is responsible behind prolonged infections is yet to be completely elucidated. Mother reason may be that antigenic properties of the virus may change, resulting in a cycle of consecutive infection and re-infection with variants, followed by a period of progressive evolution of the virus (Coyne et al., 2007).

2.7 Transmission

Norovirus is the common cause of food-borne disease. Fecal—oral route is the primary mode of transmission for norovirus. In Europe, approximately 10% of all reported outbreaks were associated with contaminated food (Hamano et al., 2005). In Japan, up to 35% of norovirus outbreaks were due to foodborne transmission (Kroneman et al., 2008). In surveillance, it has been found that, 88% norovirus outbreaks are caused by person to person transmission (Doyle et al., 2009) and that is why control measures aimed at reducing the person-to-person spread were most effective against norovirus infection. Noroviruses are highly persistent in the environment. During an outbreak, direct contact with infected individuals and contaminated surfaces, such as fomites, table, door, computer keyboard and by aerosol dispersal following vomiting episodes that subsequently lead to contamination of the surrounding environment. Transmission of norovirus in surrounding environment is associated with viral loads in feces and vomitus during and after the acute phase of infection and also with incubation period. From these surfaces, healthy individuals may get the infection. The chance is higher in hospital settings. Several characteristics of norovirus facilitate their transmission. Such as, first, low infection dose, that means approximately 18 to 1000 viral particles are sufficient to cause infection (Teunis et al., 2008). Second, onset of illness is followed by viral shedding in 30% exposure which increases the chance of secondary spread. Third, the virus can persist on environmental surfaces, in recreational and drinking water, and in a variety of food items as can resist a wide range of temperatures (freezing to 60°C) (Graham et al., 1994). Fourth, because of being RNA virus, Norovirus lack proof reading activity, and that is why prone to undergoes mutation which causes antigenic shift and recombination leading evolution of new strains. Due great diversity of norovirus, they do not provide cross protection and may cause repeated infection throughout life (Glass et al., 2009). Most human NoV infections are found to be caused by Gil strains. GI NoVs are responsible mainly for water-borne outbreaks whereas GII strains are more likely to be involved in food-borne outbreaks or person-to-person transmission (Matthews et al., 2012).

2.8 Prevalence of Disease

NoVs are the most common cause of viral gastroenteritis in developing countries (Brugha et al., 1999). NoV studies include four distinct epidemiological categories such as outbreaks of food-borne and water-borne gastroenteritis, sporadic cases of acute gastroenteritis in children

under 5 years of age, sporadic cases of acute gastroenteritis among adults, infections in the immunocompromised and the elderly (Hoa Tran et al., 2013). Though norovirus infection is self-limiting but in immunocompromised individuals, extended infection and viral shedding has been reported. The number of reported norovirus outbreak has risen sharply and there has also been increased recognition that noroviruses are important causes of childhood hospitalization. It is unclear whether these new observations are due to improved norovirus diagnostics or to the emergence of more virulent norovirus strains. A World Health Organization–commissioned analysis estimated 685 million annual norovirus infections (95% uncertainty interval 491 million–1.1 billion) and 212,000 (95% uncertainty interval 161,00– 278,000) annual norovirus deaths worldwide (Pires et al., 2015). Recent studies testing for norovirus infections in clinical settings, it is estimated that noroviruses cause about 900,000 episodes of gastroenteritis that require a clinic visit and 64,000 hospitalization among children <5 years of age residing in high income countries each year. In developing countries, they cause over one million hospitalizations and 200,000 deaths in young children in developing countries annually (Patel et al., 2008). In India, the percentage of norovirus positive children requiring hospitalization varied between 6.3% and 12.6% hospitalized (Chhabra et al., 2009). A study from Southern India observed the presence of norovirus in 8% of sporadic cases of acute gastroenteritis (Kang et al., 2000). In a report from Vellore and New Delhi, India norovirus was detected in 19.4% and 24% of hospitalized children with acute gastroenteritis (Monica et al., 2007, Rachakonda et al., 2008). In a study conducted in Pakistan, it was found that noroviruses were identified 40.2% cases of acute gastroenteritis in children (Phan et al., 2004). In the United States, about 50% of outbreaks of acute gastroenteritis were caused by norovirus and causes an estimated 800 death and 70,000 hospitalization annually, which could increase by additional 50% during epidemic years (Wikswo and Hall, 2012). NoVs were identified in over 58% of reported food-borne gastroenteritis outbreaks in USA (Scallan et al., 2011). NoVs were also found to be related with nonfood-borne outbreaks in USA, thus accounting for 78% of all illnesses, 46% of hospitalizations and 86% of deaths (Ramani et al., 2014).

In Bangladesh, the first epidemiological study was carried out among hospitalized infant and children with diarrhoea from October 2004 to September 2005 and only 4.5% norovirus positive cases were found (Dey et al., 2007). From January 2004 to December 2005, another study was conducted among hospitalized diarrhoea patients and the estimated overall norovirus detection rate was 8.5% (Rahman et al., 2010). A recent study carried out in Bangladesh, using real time RT-PCR found that the prevalence of norovirus in hospitalized diarrhoeal patients

was 28.4%. In another study, a novel inter-genotype recombinant norovirus strain, Dhalca85/2011/BGD, collected from a stool specimen of a nine-month-old infant who was hospitalized with diarrhoea. Molecular investigation and phylogenetic analysis classified its RNA polymerase gene as GII.4-like, which commonly circulates in humans. The capsid gene was classified as GII.21-like, most likely originated from water. Another novel intergenotype human norovirus recombinant GII.16/GII.3 was identified in the fecal specimens of two male infants with acute diarrhoea in Bangladesh, which was the first report worldwide (Nahar et al., 2013). NoV GII.4 variant Sydney/2012 was identified through hospital surveillance on diarrhoea etiology in Bangladesh in December 2011 and throughout 2012, which was first reported from Australia in March 2012 and, subsequently, in the United States, Belgium, Denmark, Scotland, and Japan (Rahman et al., 2013). In Bangladesh, among GII genogroup, GII.4 was the most prevalent genotype followed by GII.3, GII.6, GII.7, and GII.21. GII.4 and GII.3 strains were frequently identified in children <2 years of age and less commonly in adults more than 18 years of age (Rahman et al., 2016). The findings of a cohort study which conducted in Bangladesh suggested that the variability of norovirus strains is much higher on Bangladeshi children. They found six GI genotypes (GI.3–GI.8) and 18 GII genotypes (GII.1–GII.10, GII.12– GII.14, GII.17, GII.20–GII.22, and GII.25) and among them the globally dominant GII.4 genotype was identified most frequently (~20% of sequences), including the GII.4-New Orleans (13%) and GII.4-Sydney (7%) variants. They also suggest that approximately 80% of viruses belonged to 1 of 23 other genotypes, which span almost the entire known genetic diversity of GI and GII noroviruses in humans (Nelson et al., 2018).

2.9 Genotypic distribution of the global circulating NoV strains

A recent report from the United Sates over 5 years period indicated that most of the norovirus gastroenteritis outbreaks were caused by GII and rarely by GI. In some Asian and African countries, however, GI accounts for almost one third of the total strains. Among GII, most frequently detected strains during the last decade were GII.4 and GII.b, which accounted for 76% of the global strains. In contrast, several Asian countries reported that GII.3 as high as 41-53%. The distribution of norovirus genotypes changes rapidly over time and new variants are introduced at regular intervals. During the last decade, the most important variants circulating worldwide include GII.4/2002 (20022006), GI1.4/2004 (2003 2008), GII.4/ 2006a (2006-2009), GII.4/2006b (2006-2010), GII.4/ New Orleans/2009, and GII.4/Sydney/2012. From

2014, China, Japan, and several other Asian countries observed an unusual emergence of a rare genotype GII.17 (Kabue, Meader et al. 2017).

While noroviruses are divided into seven genogroups and further into more than 30 genotypes based on capsid sequence, the majority of cases and outbreaks are caused by viruses associated with a single capsid genotype, GII.4, which has also caused six pandemics of gastroenteritis since 1995. Each pandemic has been caused by a distinct strain of GII.4. A novel norovirus lineage containing the GII.P16 polymerase and pandemic GII.4 Sydney 2012 capsid was recently detected in Asia and Germany. The emerging GII.P16-GII.4 Sydney 2012 norovirus lineage is circulating worldwide, arose by late-2014 and contains polymerase changes that may increase irus transmission (Ruis, Roy et al. 2017).

2.10 Prevention and Control

The simple effective advice to avoid norovirus infection include, wash hand using soap and hot water for 20 second, drink bottle water when sanitation is in question and eat food that are thoroughly cooked. Control is the mainstay to prevent norovirus transmission. e high prevalence of norovirus infection among person of all age group and limited way to prevent outbreak have led some investigators to consider the potential role of vaccines in controlling the disease. In preclinical trials, VLPs used as vaccine in mice and immune responses as achieved (Estes et al., 2000). The vaccine was well tolerated, showed no severe adverse effects and was immunogenic, showing a dose-dependent increase in serum antibody titers. Healthy, secretor-positive adults receiving two doses of vaccine in 3 weeks apart, were challenged with homotypic live virus and required approximately 10 times the dose required to effect 50% of the participants (Atmar et al., 2011). These studies are important for showing that a NoV VLP vaccine can induce protective immunity. The first studies on administration of bivalent vaccines in adult volunteers were introduced via intramuscular route. No severe adverse events were reported. The vaccine was immunogenic, with IgA and IgG responses to both VLPs seen 7 days after the first dose of vaccine. But the second dose did not further boost the antibody response. However, many challenges to develop norovirus vaccines remain, including an incomplete understanding of the immune correlates of protection, the lack of good long-term immunity and heterotypic protection against antigenically distinct strains, and the existence of multiple genetic and antigenic types of virus. The control of norovirus outbreak has been

extremely challenging because outbreaks that begin with a single common exposure to contaminated food or water can rapidly spread by person-toperson contact.

2.11 Treatment

There is no specific antiviral drug available for norovirus gastroenteritis and vaccines against human norovirus are currently under development (Bartsch et al., 2012). Oral rehydration therapy or intravenous fluid replacement is the choice of treatment if dehydration is severe. Symptoms treatment for headache, myalgias and nausea can be provided using analgesics and antiemetic (Treanor JJ, 2000). Viral polymerase and proteases can be of potential target for the development of drug as these structures are the binding site of histo-blood group antigens. Interferon and ribavirin were found to inhibit replication of Norwalk virus (Chang and George, 2007). But the potential therapeutic value of these drugs, are needed to be further evaluated.

Chapter III: Methodology

3.1 Study Population and Sample Collection

The study population comes from different population as samples were collected from nine different hospitals around Bangladesh. Those hospitals are RMCH, JIMCH, JRRMCH, JGH, SBMCH, BGH, RpMCH, RGH, and CMCH. For this study, fecal samples were collected from both hospitalized patients admitted into these hospitals with acute diarrhea on the month of October and other patients who stayed on the hospital for a prolonged time like surgery patients. Informed consent is obtained from legal guardians of all patients and normal people before filling up the relative questionnaire and taking fecal samples for lab examinations.

Study specimens

This case-control study was conducted by collecting sample from a total of n=140 people. From which n=71 samples were collected from diarrhea patients which further denoted as case and n=69 samples were collected from non- diarrheal people which further denoted as control.

3.2 Experimental Design

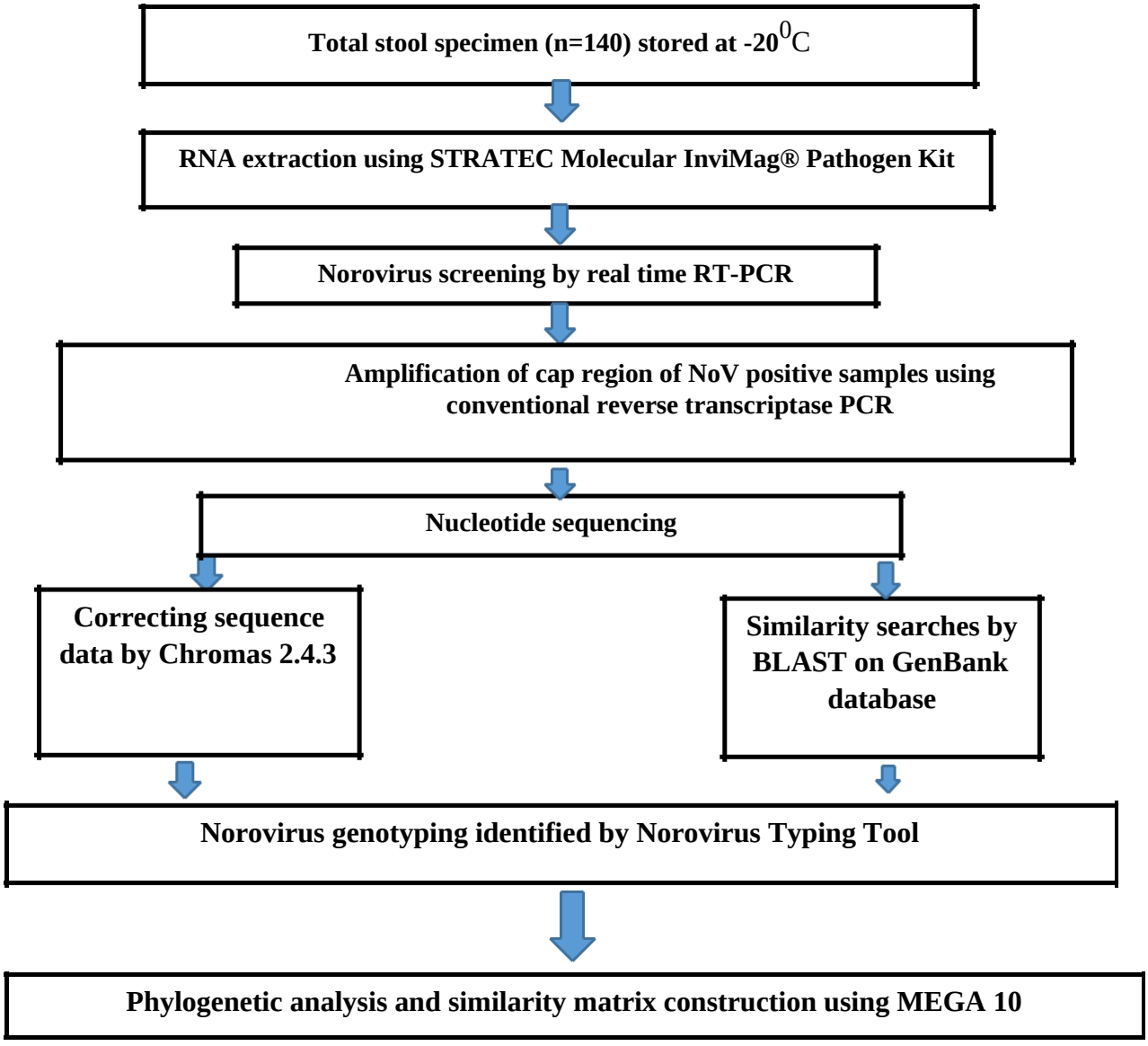


Figure 3.1: Flowchart of laboratory experimental design

3.3 Sample Processing

Stool specimens contain DNase and RNase which quickly cause DNA and RNA digestion and degradation. So before processing for the further testing, the samples were thawed at room temperature. I00 µl of stool sample was transferred into a 2 ml eppendorf tube and the sample volume was adjusted with nuclease free water to 1 ml i.e. 10 times dilution was performed for each raw stool sample. Then the sample was vigorously vortexed for 30 sec followed by 1 min of centrifuge at 12,000 rpm. 500 p.1 supernatant was collected into each sterile eppendorf tube and then extracted.

3.4 Viral RNA Extraction

Viral RNA was extracted from the viral particle containing supernatant by the Thermo Scientific"" KingFishern4 Flex Magnetic Particle Processor using the STRATEC Molecular InviMag® Pathogen Kit/KF96 according to the manufacturer's instructions. Reagents were kept at room temperature (20-25 °C) prior to use. Wash Buffer R1, R2, Elution Buffer were prepared and used according to manufacturer's protocol. Extraction bottle was resuspended with resuspension buffer. The procedure comprises following steps: (i) Preparation of the Lysis Buffer in a prefilled Extraction Bottle (ii) Lysis of the virus particles in the lysis buffer in a 96 well Deep Well plate (samples are usually lysed under denaturing conditions at elevated temperatures). (iii) Binding of the viral nucleic acids (NA) to the magnetic beads (iv) Washing of the bead bound viral NA and evaporation of ethanol (v) Elution of the viral nucleic acids

3.4.1 Preparation of Buffers

- Extraction buffer (Lysis buffer, Lysozyme, Carrier RNA and Proteinase K): 10 mL resuspension buffer-R was added to each extraction bottle 50 and mixed thoroughly
- Wash Buffer R1: 80 mL of 96-100% ethanol was added to each bottle of Wash Buffer R1 and mixed thoroughly
- Wash Butler R2: 160 ml, of 96-100% ethanol was added to each bottle of Wash Buffer R2 and mixed thoroughly

3.4.2 Procedure of Extraction

Steps of automated extraction are given below:

1. Each extraction bottle 50 was thoroughly mixed with 10 mL of resuspension buffer-R.
2. Microtitter Deep Well 96 Plate (2 mL) was used and labeled as "Lysis Plate" and 200 lysis solutions (Extraction buffer) from the Extraction Bottle was transferred into each well of "Lysis Plate".
3. Addition of 200 µl supernatant into each well of the "Lysis Plate:"
4. Another Microtitter Deep Well plate was marked as "Tip Plate"and a tip comb was kept on it.
5. Now the Tip Plate and the Lysis plate were loaded in their corresponding loading positions of the Kingfisher surface. The "NA Lysis" program was chosen on the instrument and the lysis incubation step was started after the "Start" button switched on.
6. Three another Deep Well Plates were labeled as "Washing plate 1", "Washing plate 2", "Washing plate 3". One Kingfisher96 KF plate was labelled as "Elution plate".
7. All plates were prefilled with specific buffers (as labeled) with appropriate volume:

- Washing plate 1: addition of 700 µl Wash Buffer R1
- Washing plate 2: addition of 800 µl Wash Buffer R2
- Washing plate 3: addition of 800 µl Wash Buffer R2
- Elution plate: addition of 100 µl Elution Buffer R

8. Lysis Plate was carefully taken out after lysis and 500 µl Binding solution (isopropanol) and 20 µl SNAP solution were added into each usede cavity of the Lysis plate. Before addition of the SNAP solution (kept at 4°C), it was vigorously vortexed.
9. All the plates were reinserted in their corresponding positions inside the instrument. The "NA Extraction" program was chosen and the instrument continued the purification process after pressing the "Start" button.
10. At the end of automated extraction, the Elution Plate was carefully removed and then covered with a foil.
11. Other plates were collected one after another and discarded after being treated with 0.5% Hypochlorite.
12. The elution plate was centrifuged at 2000 rpm for 2 minute and 100 µl elutes were transferred into sterile eppendorf tubes and these tubes were kept at -20°C until being used.

3.5 Multiplex Real-time Reverse Transcription Polymerase Chain Reaction

The multiplex real-time RT-PCR was carried out using the iTaq™ Universal Probes One-Step Kit (B**io-Rad Laboratories, Inc., USA**). The target is amplified using iTaq™ Universal Probes One-Step Kit on Applied Biosystems® 7500 Real-Time pCR (Applied Biosystem, Foster City, CA). The final reaction mixture (20 ul) contains RNA, iScript advanced reverse transcriptase, iTaq universal probes reaction mix (2x), primer and probe. The composition of master mixture is given in table 2.1.

Table 3.1 Composition of multiplex real-time RT-PCR master mixture

Reagent	**Amount (µl)**
iTaq universal probes reaction mix (2x)	10 µl
iScript advanced reverse transcriptase	0.5 µl
Forward primer- 1 (Cog 1F)	0.5 µl
Reverse primer- 1(Cog 1R)	0.5 µl
Probe- 1(Ring 1E)	0.5 µl
Forward primer- 2(Cog 2F)	0.5 µl
Reverse primer- 2(Cog 2R)	0.5 µl
Probe- 2 (Ring2)	0.5 µl
RNA (add at step 4)	5 µl
Nuclease free Water	1.5 µl
Total reaction setup volume	**20 µl**

In case of NoV GI two probes were added at amount 0.5 µl so nuclease free water is added at an amount of 0.5 pl. For genogrouping, 10 picomol/ µl of corresponding primer and probe were used. For real-time RT-PCR, the primer pair Cog1R/CoglF and the probe RinglE were used for GI. For GII, the primer pair Cog2R/Cog2F and the probe Ring2 were used. Corresponding primer and probes are listed in Table 2.3 (Kageyama et al., 2003; Trujillo et al., 2006). The sample is added at a volume of 5 µl.

Table 3.2: Thermal Cycling Program used in Real-Time RT-PCR

Steps	Temperature(^{0}C)	Time
Reverse Transcription	50^0 C	10 minutes
Denaturation	95^0 C	1-3 minutes
2-step Cycling (40 cycle)		
Denaturation	95^0 C	15 seconds
Annealing-Extension	60^0 C	1 minutes

The reaction was run in Applied Biosystems® 7500 Real-Time PCR (Applied Biosystem, Foster City, CA) and amplification plot was analyzed by Bio-Rad CFX manager software version. The noise and was set to a value above the background fluorescence. A test result was considered as positive if the cycle threshold (ct) value is less than 35 cycles along with expected value of positive and negative control.

Table 3.3: Primers and Probes used in real time RT-PCR

Genogroup	Primer or probe	DNA Sequence (5′-3′)
GI	Cog 1F	CGY TGG ATG CGI TIT CAT GA
	Cog 1R	CTT AGA CGC CAT CAT CAT TYA C
	Ring lE	FAM — TGG ACA GGR GAY CGC — MGB
GII	Cog 2F	CAR GAR BCN ATG TTY AGR TGG ATG AG
	Cog 2R	TCG ACG CCA TCT TCA TTC ACA
	Ring 2	CY5 TGG GAG GGC GAT CGC AAT CT - BHQ2

3.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

In Norovirus positive samples, cap gene was amplified by using Qiagen One-Step RT-PCR kit. MON432/G1SKR and MON431/G2SKR primers pair were used to amplify polymerase and capsid region of GI and Gil norovirus, respectively. The final reaction mixture (25 µl) contains nuclease free water, dNTP, taq polymerase, forward primer, reverse primer and buffer. The

primers are added at an amount of 0.5 µl to each reaction and concentration of the corresponding primers is 40 picomol/pl. 5 µl of samples are added to each reaction.

Table 3.4: Composition of RT-PCR mixture

Components	Volume per reaction (µl)
5x RT-PCR Buffer	5 µl
Forward primer	0.5 µl for both GI and GII
Reverse primer	0.5 µl for both GI and GII
dNTP mix	1 µl
Enzyme mix	1 µl
RNase-free water Total	12 µl
Template RNA	5 µl
Total	25 µl

Table 3.5: primer used in RT-PCR

Conventional RT-PCR Primers for cap	Location and region	DNA sequence (5′-3′)	Amplicon size	References
MON431 (GII)	5093 ORF1/B	TGG ACI AGR GGI CCY AAY CA	213 bp	(Richards et al., 2004)
MON432 (GI)	5093 ORF1/B	TGG ACI CGY GGI CCY AAY CA	213 bp	(Richards et al., 2004)
G1SKR (GI)	5671 ORF2/C	CCA ACC CAR CCA TTR TAC T	329 bp	(Kojima et al., 2002)
G2SKR (GII)	5401 ORF2/C	CCR CCN GCA TRH CCR TTR TAC AT	343bp	(Kojima et al., 2002)
The positions of the primers are relative to the GI Norwalk M87661 and GII Lordsdale X86557 strains. Y=C or T, R=A or G, H=A or C or T, N=Any				

Table 3.6: RT-PCR program

Steps	Temperature(^{0}C)	Time
Reverse Transcription	42^{0}C	30 minutes
Initial PCR activation step	95^{0}C	15 minutes
3-step cycling (40 cycles)		
Denaturation	95^{0}C	30 seconds
Annealing	54^{0}C	30 seconds
Extension	72^{0}C	1 minutes
Final Extension	72^{0}C	10 minutes

3.7 Gel Electrophoresis

A 1.5% agarose gel was prepared and PCR products were run on it. Then the gel had been examined through gel doc to determine the band sizes of obtaining samples.

3.8 Nucleotide sequencing

3.8.1 Wash

The PCR products were purified with ExoSAP-ITO PCR product cleanup kit (Affymetrix, INC, eland, Ohio, USA). 5 µl of PCR product is added with 2 µl of exosap and placed in a thereto cycler at 37°C for 15minutes followed by 80°C for 15 minutes.

3.8.2 Sequencing

BigDye Terminator cycle sequencing reaction kit was used for cycle sequencing and the master mix for cycle sequencing isprepared with following composition.

Table 3.7 Composition of cycle sequencing master mixture

Reagent	Amount (µl)
Buffer	1 µl
Big dye	0.5 µl
Water	6.5 µl
Forward primer	1 (5 picomole/ µl)
Total	9 µl

2 µl of ExoSAP treated PCR product was added and placed in thermocycler at appropriate conditions. The PCR program initiated with Denaturation of template at **96°C for 1 minute**, which is followed by **96°C for 10 seconds**, annealing at **50°C for 5 seconds** and extension at **60°C for 4 minutes.** These three steps were repeated by **24 cycles**.

3.8.3 Final Wash

After cycle sequencing, SAM solution and X-terminator are added for final product purification and then placed in sequencing machine. Sequencing dideoxynucleotide chain termination was done for forward direction using method in an automated genetic analyzer ABI 3500 xL.

3.9 Nucleotide Sequencing Analysis and Genotyping

Chromas 2.6.5 was used for scrutiny of chromatogram sequencing files. Nucleotide sequence similarity was searched by using the national Center for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool) server on GenBank database.

For Norovirus genotyping, partial sequence of cap and pol genes searched for similarities by using the automated norovirus genotype prediction server Norovirus Genotyping Tool version 2.0, (https://www.rivm.nl/mpf/typingtool/norovirus/).

3.10 Phylogenetic Analysis

Phylogenetic analysis and molecular evolutionary analysis were conducted using MEGA X software package. The dendrogram with bootstrap analysis from 500 replicas were constructed using the neighbor-joining method (Tamura et al., 2011).

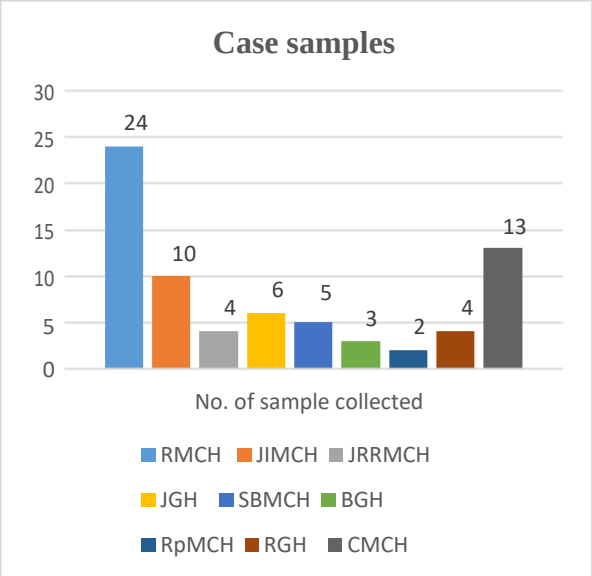
3.11 Construction of similarity matrix

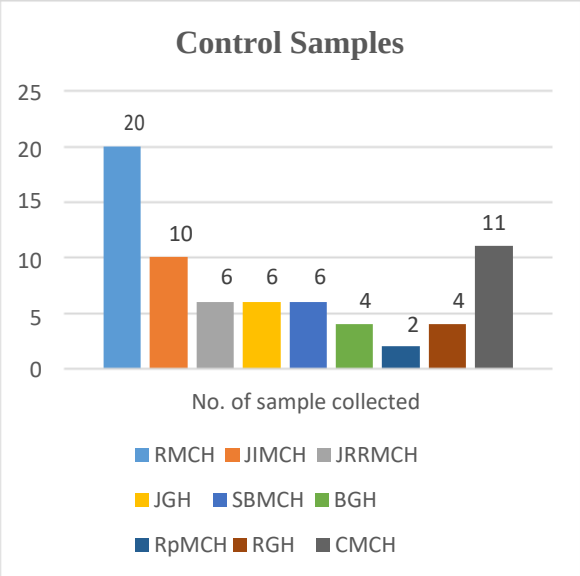
In order to establish the inner relationship among different NoV strains described in this study and their relationship with other published NoV strains, similarity matrix tables were constructed by pairwise comparisons on the nucleotide level. The similarity percentages between nucleotide sequences were calculated by the p-distance model using MEGA X software packages.

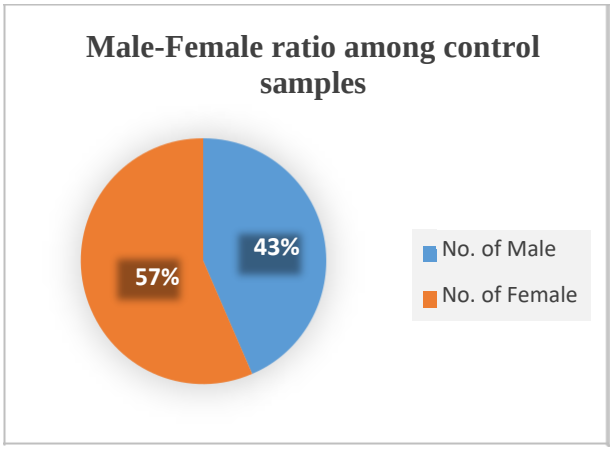
Chapter IV: Results

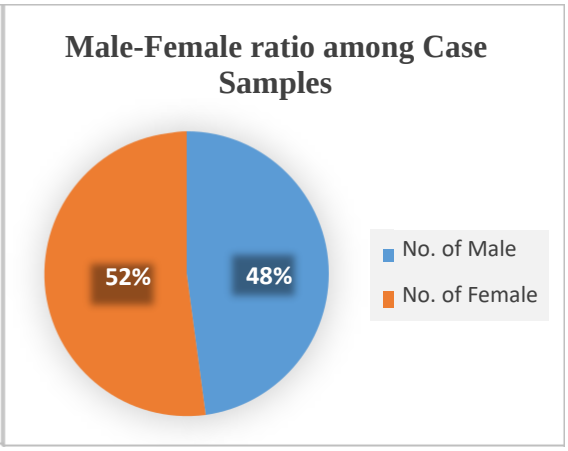
4.1 Sample Demography:

In the month of October, a total of 140 samples were collected from different hospitals around Bangladesh. From those samples, n=71 have diarrhea like symptoms and considered as case sample. On the other hand, other n=69 samples are collected from people who stayed in hospital for a prolonged time. Figure () and () show the number of samples were collected from different hospitals respectively. Among n=71 case samples, 52% are male and 48% are female. And among n=69 control samples, 57% are male and 43% are female. Among all case patients, 41%, 10% and 49% are belongs to 0-5 years, 5-18 years and .18 years respectively, and among control people, 45%, 46% and 9% are belongs to 0-5 years, 5-18 years and .18 years respectively









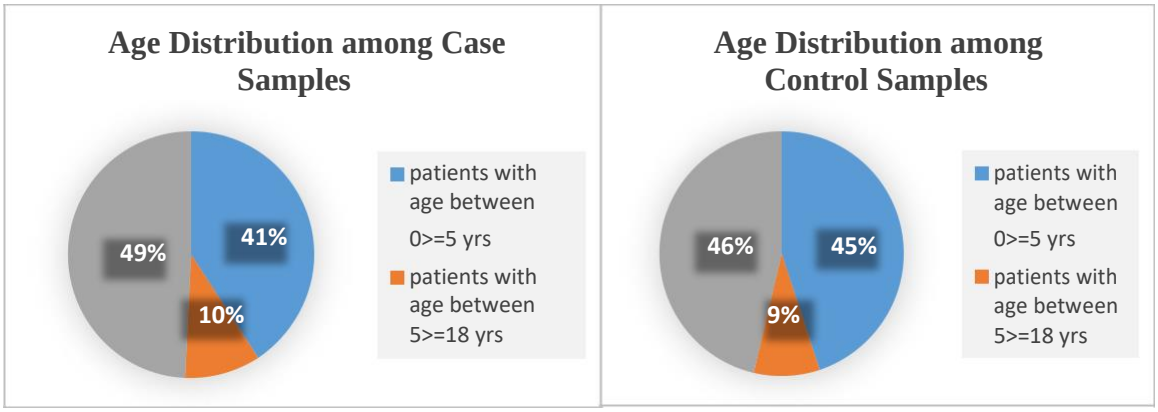


Figure 4.1: Sample Demography of collected samples according to their collected location, percentage of male and female participants and age groups.

4.2 Detection and Final Genogrouping of norovirus by real time RT-PCR:

Table 4.1: RT-PCR results of norovirus with respective Ct value

Specimen ID	Sample Group	NoV_GI_ct	NoV_GII_ct	Final_NoV_genogrp	RT-result
NS001	**Case**		**18.92**	**GII**	**Positive**
NS002	**Case**		**18.92**	**GII**	**Positive**
NS003	Case		33.55	GII	Positive
NS004	Case	34.04		GI	Positive
NS005	Case	32.9		GI	Positive
NS006	Case		32.72	GII	Positive
NS007	Case		34.33	GII	Positive
NS008	Case		33.63	GII	Positive
NS009	Case		32.26	GII	Positive
NC001	**Control**		**16.79**	**GII**	**Positive**
NC002	**Control**		**20.47**	**GII**	**Positive**
NC003	Control		28.84	GII	Positive
NC004	Control	34.43		GI	Positive
NC005	Control		28.87	GII	Positive
NC006	Control		24.35	GII	Positive
NC007	Control		25.44	GII	Positive

According to Real Time RT-PCR, a total of sixteen (12%) fecal samples were confirmed as norovirus positive out of 140 study samples and the distribution of number of positive samples from case and control are 9 and 7 respectively. Among these 16 positives, the ratio of GI and GII genogroup was 3:13.

4.3 Confirmation of Genogroup by RT-PCR:

According to RT-PCR, four samples were confirmed as they are belonging to GII genogroup. Among four positive, two samples were from case sample (NS001 and NS002) and other two from control patients (NC001 and NC002). All positive samples were belonging to genogroup GII. This confirmation was obtained by agarose gel electrophoresis of PCR products.

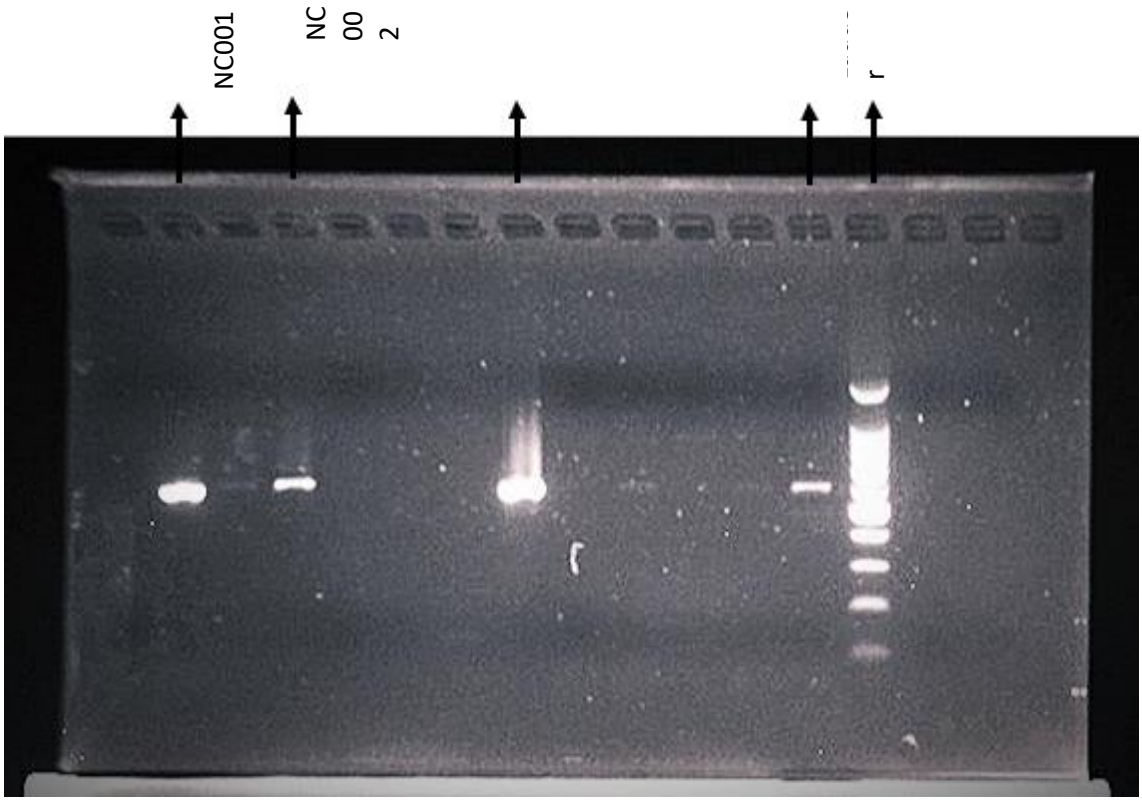


Figure 4.2: Image of RT-PCR products on agarose gel captured from gel documentation

4.4 Genogroups and genotypes:

Among these four samples, genotype GII.4 was more prevalent which found in three samples and another sample was found as belongs to the genotype GII.3. Typing of pol gene revealed that everyone belongs to the polymerase type GII.P16. According to results of cap and pol genotype, the ratio of dual genotype GII.P16_GII.4 and GII.P16_GII.3 were 3:1.

Table 4.2: Genotype of capsid and polymerase of obtained norovirus sequences

Name	Length	Pol_Genotype	Cap_Genotype	Dual_Genotype
NC001	566	GII.P16	GII.4	GII.P16_GII.4
NC002	561	GII.P16	GII.4	GII.P16_GII.4
NS001	533	GII.P16	GII.3	GII.P16_GII.3
NS002	573	GII.P16	GII.4	GII.P16_GII.4

4.5 Phylogenetic Analysis:

Phylogenetic analysis of partial sequence of the genotypes belongs to the genogroup GII were illustrated in Figure 4.3.

The phylogenetic tree represents that Bangladeshi sequences of GII.4 were clustered together and these clustered sequences create another cluster with the sequences of GII.4 variant Sydney 2012 obtaining from GenBank. On the other hand, the sequences belong to the genotype GII.3 clustered with the sequence of strains from Russia (MG892955 Norovirus_RUS/2017) and UK (KY887606 Norovirus_UK/2016).

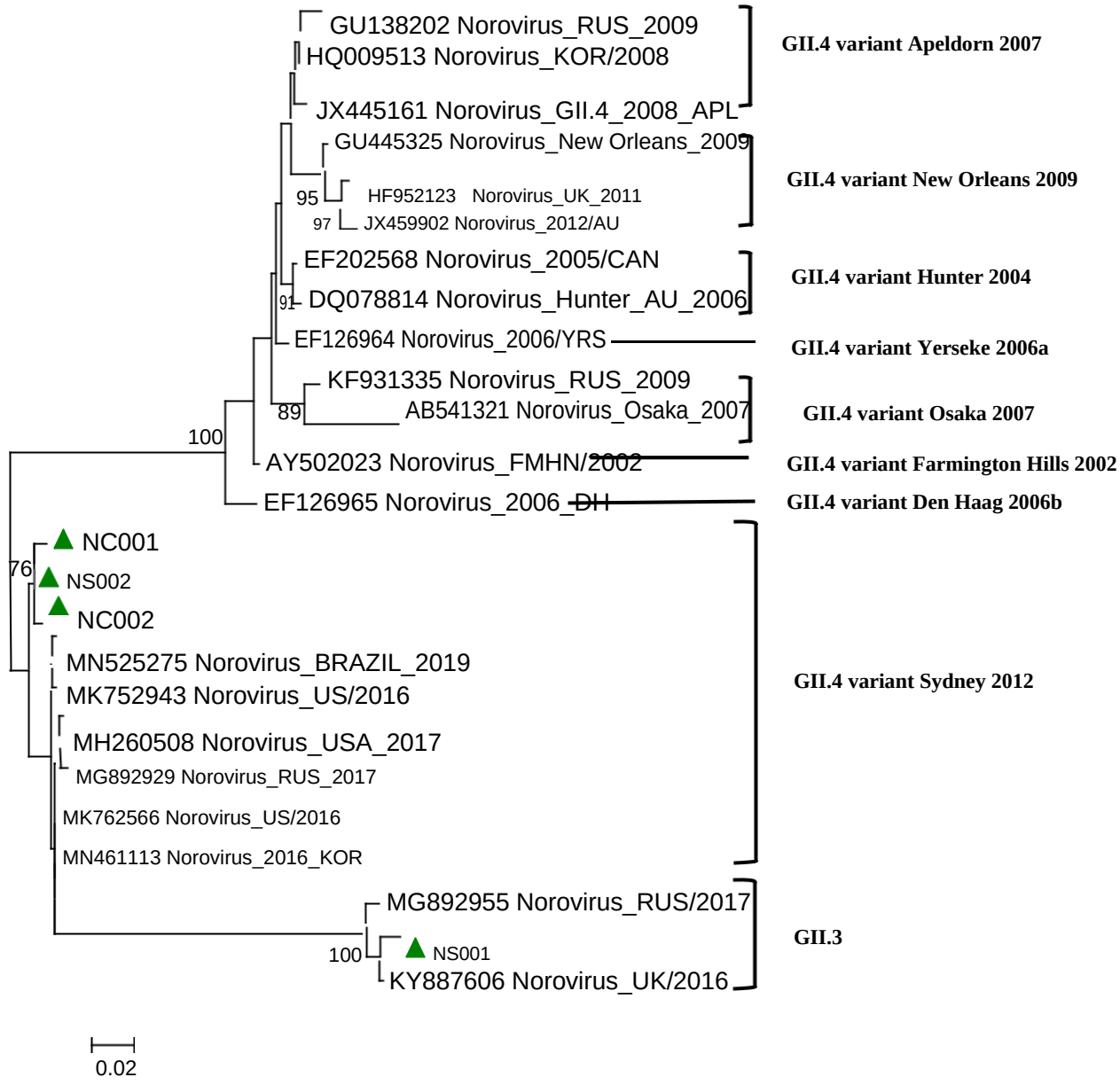


Figure 4.3: Neighbor-joining phylogenetic trees of the partial sequences (550 nt) of NoV GII detected in Bangladesh during October 2019. Bootstrap test (1000 replicates) was used, and bootstrap values 70% are shown at the branch nodes. The evolutionary distances were computed using the Kimura 2-parameter model and scale bar indicates the number of nucleotide substitutions per site. The sequences identified in this study are marked green triangle. The reference strains retrieved from GenBank are shown. Sequences are indicated by GenBank accession number, year, and country of detection. Clusters of capsid genotypes with representatives from this study are shown on the right side of the phylogenetic trees

4.6 Similarity Analysis

To find out the extent of similarity of the NoV genotypes circulating in October, 2019 with other countries, similarity matrix was constructed.

GII.4 strains showed around 97-98% similarity with sequences of GII. Sydney variants and also had more the 84% similarity all other GII.4 variants. The only GII.3 genotypic strain showed 985 and 99% similarity with MG892955_Norovirus_RUS/2017 and KY887606_Norovirus_UK/2016 respectively.

The similarity matrix for NoV genotypes found in this study was shown in Figure 4.4.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	NC001	100																								
2	NS001	84	100																							
3	NC002	99	84	100																						
4	NS002	99	84	100	100																					
5	MN525275_Norovirus_BRAZIL_2019	98	84	98	98	100																				
6	MH260508_Norovirus_USA_2017	98	84	98	98	99	100																			
7	MK762566_Norovirus_US/2016	98	84	98	98	100	100	100																		
8	MG892929_Norovirus_RUS_2017	98	84	98	98	99	99	99	100																	
9	KY887606_Norovirus_UK/2016	84	99	83	84	84	84	85	85	100																
10	MG892955_Norovirus_RUS/2017	84	98	83	84	85	85	85	85	98	100															
11	MN461113_Norovirus_2016_KOR	98	84	98	98	100	100	100	99	85	85	100														
12	MK752943_Norovirus_US/2016	98	84	98	98	100	99	100	99	84	85	100	100													
13	JX445161_Norovirus_GII.4_2008_APL	87	73	87	87	87	87	87	87	73	73	87	87	100												
14	GU138202_Norovirus_RUS_2009	87	71	87	87	87	87	87	87	71	71	87	87	98	100											
15	HQ009513_Norovirus_KOR/2008	87	72	87	87	87	87	87	87	72	72	87	87	99	99	100										
16	EF126965_Norovirus_2006_DH	87	72	87	87	87	87	87	87	72	72	87	87	95	95	96	100									
17	AY502023_Norovirus_FMHN/2002	87	73	87	88	88	88	88	88	72	72	88	88	97	96	97	97	100								
18	EF202568_Norovirus_2005/CAN	87	73	87	87	87	87	87	87	73	73	87	87	98	98	98	96	98	100							
19	DQ078814_Norovirus_Hunter_AU_2006	87	73	87	87	87	87	87	88	73	73	87	87	98	97	98	96	98	99	100						
20	HF952123_Norovirus_UK_2011	86	72	87	87	86	87	87	87	72	72	87	87	97	97	97	94	95	96	96	100					
21	JX459902_Norovirus_2012/AU	87	71	87	87	87	87	87	87	71	71	87	87	96	96	97	93	95	96	96	99	100				
22	GU445325_Norovirus_New_Orleans_2009	87	73	88	88	87	88	88	88	73	73	88	88	98	97	98	95	97	97	97	99	98	100			
23	KF931335_Norovirus_RUS_2009	86	73	86	86	86	86	86	87	73	72	86	86	97	97	97	95	97	97	97	95	94	95	100		
24	AB541321_Norovirus_Osaka_2007	83	71	84	84	83	83	83	83	71	71	83	83	92	92	92	92	93	93	92	91	90	92	95	100	
25	EF126964_Norovirus_2006/YRS	88	73	88	88	88	88	88	88	73	72	88	88	98	98	98	96	98	98	98	97	97	98	97	93	100

Figure 4.4: Nucleotide similarity percentage of capsid region among the obtained strains and strains from GenBank

Chapter V

5.1 Discussion

Diarrheal disease is the fourth most common cause of mortality and second most common cause of morbidity worldwide in children under the age of 5 years. Despite accounting for app. one-fifth of all acute gastroenteritis worldwide across the age range (Bartsch, Loproan et al. 2016), norovirus has received considerably less attention from the press and has fewer program initiatives than other high burden infectious pathogens. Moreover, funding and attention from policy makers for norovirus is not proportional to its disease burden suggested to a systematic review. In context of Bangladesh, the importance of norovirus in daciar7hdoein ag has not been well defined while it is the second most important viral etiological agents of acute gastroenteritis after rotavirus (Rahman et al, 2016, Nahar et al, 2013; Patel et al, 2008). In addition, norovirus is not routinely tested as an etiological agent of gastroenteritis among diarrhoea' patients who are hospitalized (Rahman et al, 2016).

The main objective of this study was to determine the prevalent norovirus strains circulating in Bangladesh in the month of October. In this month, overall, about 12% (16) of all samples tested positive for norovirus in which 13 were positive for GIl and 3 sample were found positive for GI during real time RT-PCR. However, the incidence data reported here falls in the range (8-30%) that has been reported in other low resource countries (Alam et al, 2016; Zeng et al., 2012; Kirby et al., 2011;Yang et al., 2010).

Additionally our result is also in pace with previously reported data that most infections were caused by norovirus GII (Rahman et al., 2016, 2010; Alam et al, 2016, Trang et al, 2014, Nahar et al, 2013; Zeng et al., 2012, Yang et al., 2010). Several studies including the Global Enteric Multicenter Study in Bangladesh site reported that norovirus was frequently identified as co-infections with other diarrhoeal pathogens (Kotloff et al., 2013). It is possible that co-infections of norovirus with rotavirus and bacterial pathogens could be the results from nosocomial transmission due to the fact that norovirus is highly infectious and easily transmissible within closed hospital settings.

Multiple pandemic variants of the GII.4 of NoV have attracted great attention from researchers worldwide. The study findings showed the predominance of the GII.4 Sydney 2012 variant, which has been widely associated with an increased NoV activity and outbreaks across the globe (pan, Xue et al. 2016). The pandemic GII.4 Sydney 2012 variant was first reported in

2012 and soon became the predominant circulating NoV strain globally. In Bangladesh, the NoV 011.4 variant Sydney/2012 was identified through hospital surveillance on diarrhea etiology in December 2011, then throughout 2012 and continued till then. These findings indicate that future emerging strains may circulate at a lower level in sporadic cases, a few years prior to emergence. Therefore, standardized continuous strain surveillance in this part of the world be help to predict which strains may emerge in future years. Despite the broad genetic diversity, norovirus GII.4 has predominated since 1996.

Phylogenetic analysis indicated that the Bangladeshi strains did not cluster with strains from a particular region; instead, strains from different countries clustered in the same branch. This indicates that the global dispersion of the viruses occurred between different geographical locations. Since we analyzed a small locations, portion of the capsid and pol gene, complete genome sequence analysis would be required for detailed characterization of this strain.

According to previous study, C11.4 was most prevalent genotype followed by 611.3 strains in (47%), GILl (11%) and GII.17 (I I %) Bangladesh (Rahman et al. 20 16) and in this study although G11.4 genotype was most prevalent among hospitalized diarrhoeal patients in Bangladesh in October, 2019. Emphasizing on continuous routine surveillance of norovirus is necessary to unravel the genetic diversity of norovirus. This study had some limitations. First, this study was conducted only for one month with very small sample sizes. Longitudinal studies are required to get clear knowledge on issues such as norovirus seasonality and genetic variability and to monitor the spread and persistence of the various genotypes circulating in Bangladesh. Second, only hospitalized patients were included in this study. For these reasons, community-based studies are required to investigate the true burden of the disease caused by the pathogen. Finally, a noteworthy amount (-33%) of norovirus real time positive specimens could not be genotyped as the routine primers failed to amplify norovirus RNA. Therefore, to characterize those untypeable strains, an alternate and updated primer set is required.

5.2. Concluding Remarks

Diarrhoea is an important health concern in Bangladesh like other developing countries and norovirus is one of the notorious enteric viral pathogen responsible for acute gastroenteritis in both children and adults. In 2015, norovirus was responsible for one-fifth of all diarrhoeal cases among hospitalized patients Efforts should be made to introduce the clinical diagnosis of the virus due to its impact on the community as well as health care institutions. Replacement of the previously predominant variants by the newly emergent norovirus genotypes indicates the

evidence of high degree of genetic diversity of noroviruses in Bangladesh like in other countries, thus, posing a major challenge of vaccine development against the circulating strains of Bangladesh. Therefore, continuous routine strain surveillance is necessary in this third world country to identify changes in prevalent strains. In conclusion, this study may not be able to provide such broad-spectrum data but this will help to monitor the prevalence genotypic strains of norovirus circulating in Bangladesh.

Chapter VI: References

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Appendix

Acc No.	Name	Country
MN525275	Norovirus_BRAZIL_2019	Brazil
MH260508	Norovirus_USA_2017	USA
MK762566	Norovirus_US/2016	USA
MG892929	Norovirus_RUS_2017	Russia
KY887606	Norovirus_UK/2016	UK
MG892955	Norovirus_RUS/2017	Russia
MN461113	Norovirus_2016_KOR	Korea
MK752943	Norovirus_US/2016	USA
JX445161	Norovirus_GII.4_2008_APL	Apeldron
GU138202	Norovirus_RUS_2009	Russia
HQ009513	Norovirus_KOR/2008	Korea
EF126965	Norovirus_2006_DH	Dan haag
AY502023	Norovirus_FMHN/2002	Farmington Hills
EF202568	Norovirus_2005/CAN	Canada
DQ078814	Norovirus_Hunter_AU_2006	Australia
HF952123	Norovirus_UK_2011	UK
JX459902	Norovirus_2012/AU	Australia
GU445325	Norovirus_New Orleans_2009	New Orleans
KF931335	Norovirus_RUS_2009	Russia
AB541321	Norovirus_Osaka_2007	Osaka
EF126964	Norovirus_2006/YRS	Yerseke