DETECTION AND GENOTYPING OF ROTAVIRUS A FROM WASTEWATER OF DHAKA AND COX'S BAZAR CITY

By

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A thesis submitted to the Department of Mathematics & Natural Science in partial fulfillment 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/project titled "DETECTION AND GENOTYPING OF ROTAVIRUS A FROM WASTEWATER OF DHAKA AND COX'S BAZAR CITY" submitted by
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of Fall, 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology in Summer 2023.

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Abstract

Around the world, Rotavirus (RV) is a prominent factor in severe diarrhea. This study investigated the diversity of RV genotypes in the wastewater of several defined locations that reflect the holistic comprehension of two concerned cities- Dhaka and Cox's Bazar.

Our procedures comprised of RNA extraction, real-time one-step RT-qPCR, Sanger Sequencing and genomic analysis to define RV strains. Our findings showed the highest frequency of Wa-like Rotavirus strains throughout the research areas. However, a unique sequence, G2P[4] was discovered solely in the Cox's Bazar region. Notably, there were regional differences in genotypic dominance, with G1 (53%; 8/15) prevailing in Dhaka and G3 (72%; 10/14) in Cox's Bazar. While investigating the merged genotypes, G1P[8] (44%; 4/9) had higher incidence in Dhaka whereas G3P[8] (80%; 4/5) was prevalent in Cox's Bazar.

By highlighting the need of wastewater surveillance in combating future outbreaks, this study can advance our understanding of RV in broad geographic contexts.

Chapter-1: Introduction

1.1 Background Information:

Rotavirus is the leading cause of diarrheal disease and child mortality worldwide. According to WHO estimates, 611,000 deaths from severe acute diarrhea occurs to the children who are 0-5 years old where rotavirus is responsible every year, many of them in underdeveloped nations (Parashar et al., 2006). Due to a lack of timely and efficient dehydration treatment, the amount of these fatalities occurrence is 85% in the least developed countries like Africa or Asia, which the World Bank classifies as "low-income" countries (Patel et al., 2009). Studies show that among all diarrheal episodes 8% of it is caused by rotavirus, 28% of clinical appointment for diarrhea, as well as 34% of hospitalizations for younger children with diarrhea in underdeveloped countries (Leung et al., n.d.).

Rotavirus is a member of the Reoviridae family, which is triple layered, non-enveloped virus. It has 11 segments of double-stranded RNA genome which encode for six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-structural viral proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) (Hu et al, 2012). The virus's segmented genome creates the possibility of genetic reassortment, which might lead to a novel gene combination with a variable level of infectivity (Maunula & von Bonsdorff, 2002). According to a commonly used dual classification scheme that defines G types and P types, respectively, the majority of research that is currently ongoing on RVs focusing on the reassortment of two proteins that are situated on the capsid's outer side, they are VP7 and is called glycoprotein, the other one is VP4 or protease protein where 36 types of VP7 and 51 types of VP4 is discovered. (Matthijnssens et al., 2008). Additionally, strains with uncommon combinations of typical G and P types or antigen types have been found, highlighting significant variations in specific geographic regions (Hossain et al., 2020).

Rotavirus is among the major causes of viral acute gastroenteritis in the world. This enteric virus is one of the most prevalent because it can survive in unfavorable settings and during the wastewater treatment process (Nick et al., 2020). As a result, this wastewater surveillance has attracted a lot of interest in order to find several microorganisms that cause gastrointestinal and other deadly diseases. This helps to track wastewater discharged from home septic systems to public drains, surface waters, and sewage treatment facilities, capturing trends in pathogens that are present (Ahmed et al., 2022).

In Bangladesh, there is a significant social and environmental concern related to the ethical disposal of fecal waste produced in urban areas. With 47,400 people per square kilometer, Bangladesh's capital city Dhaka, is known for having it's an overpopulated density more than any other city in the nation.

However, the management of fecal sludge has become quite difficult due to a lack of sewage treatment. Pathogens that are crucial to public health could not be eliminated or neutralized as a result of poor on-site sanitation (OSS) performance, and weak links in the local sludge management (FSM) service chain caused them to contaminate the environment and surface water. 20% of the city's wastewater is treated by a single wastewater treatment facility in Dhaka. (Haque et al., 2022). In locations with inadequate drainage systems, frequent flooding has far-reaching negative effects on human health (Amin et al., 2019). Cox's Bazar is another well-known city with a problem similar to that of Dhaka. The arrival of Rohingya refugees raised the amount of waste and the immediate demand for shelter, food, sanitization, and other medical services. The WASH service's hygienic characteristics weren't sufficient. The prevalence of diarrheal illnesses has increased in Cox's Bazar due to ongoing issues with inadequate water sources. The wastewater may be used to determine the source of these disease-causing microorganisms, and soon after the Rohingya immigrants arrived, their numbers began to rise (Faruque et al., 2022).

1.2 Objective of the study:

1. General objective:

This study's objective was to identify and characterize rotavirus from wastewater samples collected in urban regions of Bangladesh's Dhaka and Cox's Bazar. The following goals must be met in order to achieve this: 2. Specific objectives:

i. To detect rotavirus in wastewater samples using Real-time RT-PCR.
ii. To detection of vaccine strain of rotavirus in wastewater samples using conventional RT-PCR.
iii. To do genetic characterization and phylogenetic relationship of local sequences with globally circulating strains using bioinformatics.

Chapter-2: Literature Review

2.1 Rotavirus

2.1.1 Discovery of Rotavirus

An Australian research team lead by Dr. Ruth Bishop discovered the first rotavirus that was made in the duodenal biopsies of a child who was suffering from acute diarrhea in 1973. Duovirus is the name given to the virus. (Walker-Smith, 1978).This virus was frequently observed under the direct thin layer electron microscope in a large number in feces and significant antibody titre was shown between acute and convalescent sera from the children by immune electron microscopy. (Walker-Smith, 1978).

The virus's unusual electron microscope appearance, which resembled a wheel (Rota is Latin for "wheel"), led to the adoption of the new name "rotavirus" for it. In mice with diarrhea in 1963, viruses were found in similar morphological appearance in the intestinal tissue (Barthold et al., 1985).

2.1.2 Geographical Distribution and Epidemiology of Rotavirus

Everywhere in the world, rotavirus can be found. Even with the practice of hygiene or the availability of pure water, almost all the children on the planet will get infected with rotavirus before they are five. (Parashar et al, 2003 2, n.d.). The largest number of diarrheal deaths caused by rotavirus occur in countries in the Indian subcontinent and Sub-Saharan Africa who are undeveloped and because of that they lack access to medical care. However, depending on the child's residence, the consequences of infection differ greatly. (Gentsch et al., 2005).

All people are capable of contracting the rotavirus, however individuals between the ages of 6 months and 2 years, premature infants, the elderly, and people with weakened immune systems are particularly susceptible to more severe symptoms. Children are especially vulnerable after six months of age, when maternal antibodies offer less protection (Patel et al., 2009). Children hospitalized with rotavirus diarrhea are typically 6 to 9 months old in several African and Asian countries, and up to 80% of them are under a year old (Cunliffe et al., 1998). On the other hand, the typical age in wealthy countries is 13 to 16 months, and most cases begin in when they are 2 years old (Gentsch et al., 2005). By the age of 15 months, most infants have some type of protection after their initial infection (O'Ryan, 2009). However, rotavirus, the primary cause

of severe gastroenteritis in both developed as well as underdeveloped countries and accounts for 40% of hospitalizations globally (CDC, 2008)

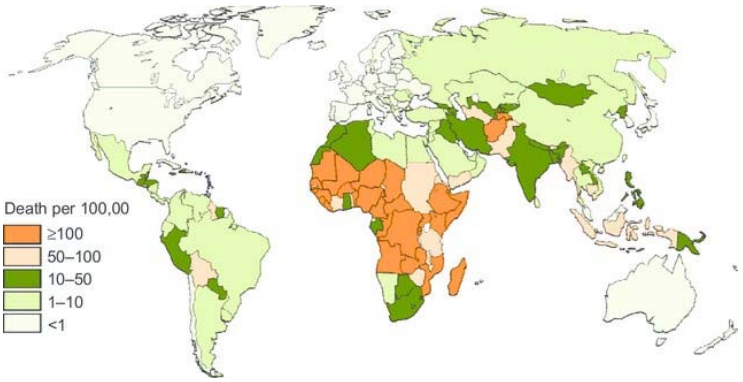


Figure 2.1: Estimated number of rotavirus diarrhea fatalities per 100,000 children under the age of five (Chandran, 2010).

The extremely high viral content in the feces from people with and without symptoms (more than 10^9 virus particles/g) and considering the minimal infection needs for inoculum (10–100 virus particles) have both been connected to high rotavirus transmission rates. With the possible exception of saltwater, extensive viral contamination of numerous water sources and prolonged viral persistence in humans may be to blame for the high prevalence rates of rotavirus infection over the world (Desselberger, 2014; Santos et al., 2005) both surface and ground water (Akhtar, 2012). Winter and early spring are the seasons when rotavirus infections are most common in temperate regions, with other times seeing fewer cases. In tropical areas, rotavirus infections can occur at any time of year, but the number of cases increases during the drier, colder months (Gentsch et al., 2005).

Through molecular epidemiology research, G1, G2, G3, G4, and G9 are the five prevalent rotavirus serotypes that have been identified. Globally, these serotypes are frequently dominant. G1 is the strain that is most prevalent globally, while G9 is the one that is emerging the quickest (Mwenda et al., 2010); (Esona et al., 2010) Additional serotypes like G5, G8, G10, and G12 may also be present and possibly dominant in some environments in underdeveloped countries. The 51 VP4 genotypes have been identified, with the most common genotypes in children being P1A[8], P1B[4], P2A[6], P3[9], P4[10], P5A[3], P8[11], P12[19], P[25], and P[28] (Santos et al., 2005; Volotão et al., 2006) In less developed countries, P[6], P[9], and P[10] genotypes are predominate or circulate at lower levels, similar to VP7 epidemiology. (Santos et al., 2005).

2.1.3 Structure and Biological properties of Rotavirus:

Members of the Reoviridae family, Rotaviruses have an 11-segment double-stranded RNA genome and are non-enveloped viruses (Desselberger, 2014). By using electron microscopy (EM), it is possible to see three different types of rotavirus particles, each of which has a unique morphologic appearance. The overall particles seem like a wheel which has little spokes and a smooth, well-defined rim attached on the outside. The term "rotavirus" (derived from the Latin rota, meaning "wheel") was coined in response to this morphology. (Mcnulty, n.d.). The icosahedral capsid of the virus is composed of three layers, each measuring about 80 nm in diameter: the inner capsid, which houses the virus's RNA segments, and the outer capsid. (Akhtar, 2012a);Van Der Heide et al., 2005).

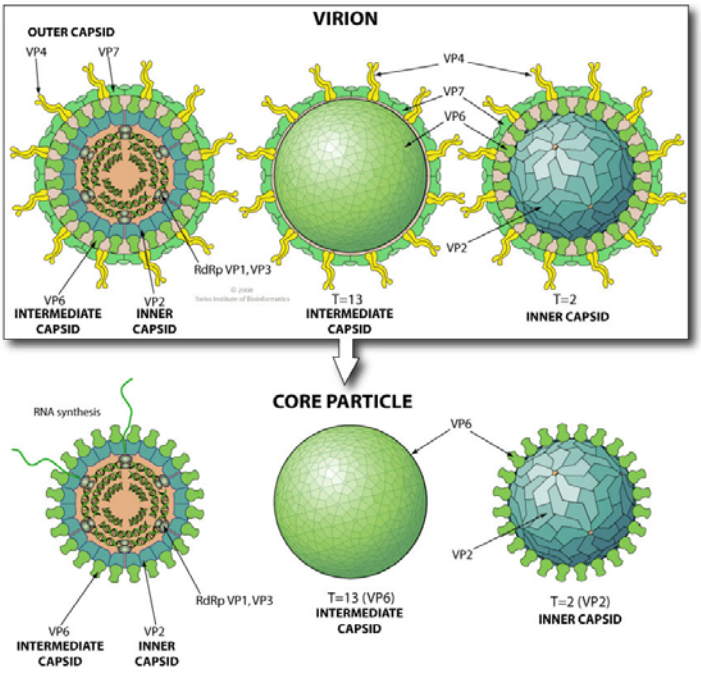


Figure 2.2: Virion Maturation Schemes. Virion has a triple capsid structure, not enclosed, icosahedral, and measuring around 80 nm in diameter. The inner capsid has a T=2 icosahedral symmetry, while the intermediate capsid has a T=13 m one (ViralZone, Swiss Institute of Bioinformatics, 2011).

The whole infectious particles are also known as virions, or triple-layered particles. Because their periphery displays protruding trimeric subunits of the inner capsid, double-layered

particles (DLPs) without the outer shell are referred to as rough particles. Rarely seen single-layered particles (SLPs or cores) are frequently aggregated and devoid of genomic RNA.

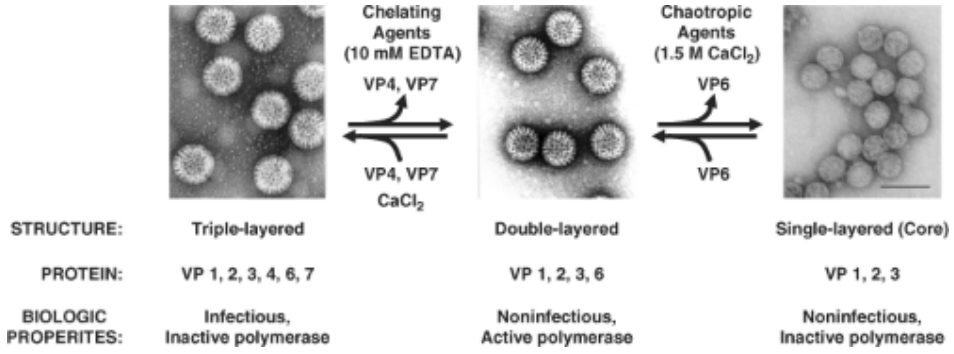


Figure 2.3: Rotavirus particle structural and biological characteristics. After staining with 1% ammonium molybdate, electron micrographs of typical triple-layered particles (TLPs), double-layered particles (DLPs), and single-layered particles (SLPs) (core) are visible. Sequential capsid protein removal (top arrows) or addition (bottom arrows) can be used to create TLPs, DLPs, and core particles, as indicated. The material goes into detail on the particles' proteins and biological characteristics. Bar, 100 nm (Mary K. Estes, Harry B. Greenberg).

2.1.4 Classification

The Rotavirus genus is divided into seven groups, corresponding to distinct Rotavirus species, from A to G based on the VP6 protein. Only the rotaviruses in groups A, B, and C are recognized as being contagious to people. Group A rotavirus is typically referred to when the term "rotavirus" is used due to its greater medical relevance. (*Anderson and Webe, 2004,* n.d.). China, India, and Bangladesh account for the majority of group B rotavirus outbreaks and sporadic infections. (District et al., 2005). Pigs, cows, lambs, and rodents are frequently infected by these viruses, which are common causes of animal illnesses. Group C rotaviruses, which are widely detected in pigs and dogs and other animals and can be the reason for other outbreaks in the human population where the target will probably be the older children, can be detected in up to one-third of adult humans. (Hoshino & Kapikian, 2000).

Based on the subgroup antigens found on the VP6 protein, rotaviruses in group A are divided into subgroups I, II, I+II, nonI, and noni. (Greenberg et al., 1983) Group A rotaviruses are further divided into several serotypes using the two outer capsid proteins VP7 and VP4.

(Martínez-Laso et al., 2009). G serotypes are distinguished using VP7, a glycoprotein that makes up the matrix of the capsid. The P serotypes are determined by VP4 (Because trypsin-mediated cleavage of the P protein is necessary for viral absorption into cells, P protein is protease-sensitive.) (Gentsch et al., 2005).

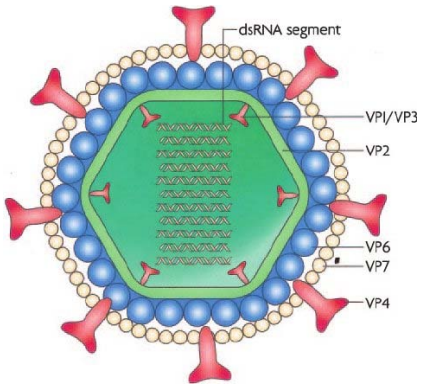


Figure 2.4: Rotavirus virion schematic illustration. The virus consists of three protein shells, an outer capsid, an inner capsid, and an internal core, which cover 11 double-stranded RNA segments. The exterior capsid proteins VP4 and VP7, which act as neutralization antigens, distinguish between the P and G serotypes. The inner capsid's structural protein VP6 serves as the subgroup antigen. (Angel et al., 2007)

Due to a lack of monospecific P antisera, more genotypes than serotypes have been discovered for P types. For G types, the same numbering approach can be used because serotypes (decided by a neutralization test) and genotypes (found by RT-PCR) are essentially identical. As a result, P types are identified as serotypes by Arabic numerals and genotypes by Arabic numbers in square brackets. As a result, the prototype human rotavirus strain Wa has been given the serotype G1P1A[8]. At least 37 P types (P[1]-P[26]) and 27 distinctive G types (G1-G15) have currently been discovered in both humans and animals. (Trojnar et al., 2013). According to an epidemiological research, the five G (G1-G4 and G9) and three P (P[4], P[6], and P[8]) genotypes are the most common ones linked to rotavirus infections in humans worldwide (Santos et al, 2005). The most prevalent G/P genotypes, however, are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]. However, recent data suggest that the P[8] or P[6] genotype may co-develop with the G12 genotype. Matthijnssens et al., 2010).

Table 2.1: Classification strategies for rotavirus ((Maes et al., 2009))

Classification	Basis of classification	Recognized types
Serogrouping	Inner capsid protein VP6	Group A-G
Subgrouping	Inner capsid protein VP6	Subgroup I, II, I and II, non-I and -II
Electropherotyping	Migration pattern of 11 gene segments in gel electrophoresis	Short, Long, Super-short
G serotyping	VP7 (Glycoprotein)	G1-G15
P serotyping	VP4 (Protease sensitive protein)	P1-P14
G genotyping	Sequence of VP7 gene	G1-G27
P genotyping	Sequence of VP4 gene	P[1]-P[35]
Genogrouping	RNA-RNA hybridization of 11 gene segments	Wa-like. DS-1-like, AU-1-like
Full genome-based classification	Nucleotide sequence of 11 gene segments	G1-G19, P1-P27, II-I11, R1-R4, C1-C5, M1-M6, A1-A14, N1-N5, T1-T7, E1-E11, H1-H6

2.1.5 Rotavirus genome:

Eleven double-stranded RNA (dsRNA) segments, which together make up the rotavirus genome, are located in the inner core of the three-layered rotavirus. (Meador et al., 2001). The entire genome contains 18,555 nucleotides. Each section, numbered 1 through 11, stands for a gene. A pattern of RNA migration known as an electropherotype can be seen by employing polyacrylamide gel electrophoresis (PAGE) to separate the segmented genome. Due to the RNA pattern's consistency and uniqueness for a single strain, epidemiological research has used it extensively to trace the channeling and spread of rotavirus. (Steele et al., 2016). The remaining dsRNA segments each code for atleast one of each six structural and nonstructural proteins, where the only segment 11 is the only one that is bicistronic (encodes two proteins) (Anderson and Webe, 2004, n.d.).

2.1.6 Coding assignment of the genes of rotavirus:

Through in vitro translation using denatured dsRNA and mRNA, the protein assignments of the rotavirus genes and the properties of the encoded proteins have been determined. (Table 2.2)

The virus particle (virion) is made up of the six proteins that are viral and they are VP1, 2, 3, 4, 6, and 7. The rotavirus RNA-Dependent RNA Polymerase is known as VP. (Meador et al., 2001)VP3 performs the function of guanylyl transferase, an enzyme that caps mRNA, whereas VP2 serves as an intermediary in replication and binds the RNA genome. (Fresco & Buratowski, 1994). The rotavirus P serotype, host specificity, pathogenicity, and protective immunity are all under the control of VP4 (Maunula & von Bonsdorff, 2002). The G serotype is determined by VP7, a glycoprotein. The rotavirus's A-G groupings and I and II subgroups are determined by VP6 (Gentsch et al., 2005).

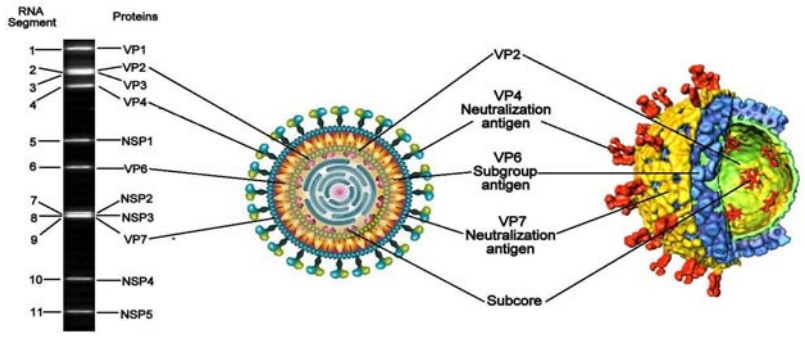


Figure 2.5: Rotavirus Structure of 11 genome RNA segments isolated on polyacrylamide gel exhibiting protein coding assignments (left). The locations of the main structural proteins (VP) are depicted in the schematic design (middle) and the cryoelectron microscopic reproduction of a virion (right). The intermediate protein shell protein, VP6, is the subgroup antigen, while the outer capsid proteins VP4 and VP7 are neutralization antigens that cause neutralizing antibodies. Nonstructural protein, NSP (Reproduced from Fields Virology, 3rd ed.).

The six non-structural proteins (NSP1, 2, 3, 4, 5, and 6) are only produced by rotavirus-infected cells (*Anderson and Webe, 2004*, n.d.; Graff et al., 2002). NSP1 binds to Interferon Regulatory Factor 3, which may decrease the interferon response during rotavirus infection. (Graff et al., 2002). NSP2 collaborates with NSP5 to produce viroplasms, produce and package viral RNA, and replicate viral genomes. NSP3 binds to the 3' end of viral mRNA, enhances viral protein synthesis, and inhibits host cell protein production. When present, the viral enterotoxin NSP4 causes diarrhea. An out of phase open reading frame in gene 11 encodes the RNA-binding protein NSP6. (Rainsford & McCrae, 2007).

Table 2.2: Properties of rotavirus structural and nonstructural proteins ((Chadwick & Goode,2001))

RNA Segment (Gene)	Size (base pairs)	Protein	Molecular weight (kDa)	Location	Function
1	3302	VP1	125	All the vertices of the core	RNA-dependent RNA polymerase
2	2690	VP2	102	Forms inner shell of the core	Stimulates viral RNA replicase
3	2591	VP3	88	At the vertices of the core	Guanylyl transferase mRNA capping enzyme
4	2362	VP4	87	Surface spike	Cell attachment, virulence
5	1611	NSP1	59	Nonstructural	5'RNA binding
6	1356	VP6	45	Inner Capsid	Structural and species-specific antigen
7	1104	NSP3	37	Nonstructural	Enhances viral mRNA activity and shut-offs cellular protein synthesis
8	1059	NSP2	35	Nonstructural	NTPase involved in RNA packaging
9	1062	VP7	38 and 34	Surface	Structural and neutralization antigen
10	751	VP7[1] VP7[2]	20	Nonstructural	Enterotoxin
11	667	NSP5 NSP6	22	Nonstructural	ssRNA and dsRNA binding modulator of NSP2

2.1.7 Replication cycle:

The outer VP4 and VP7 layers are stripped away when rotavirus enters enterocytes, changing triple-layered particles (TLPs) into double-layered particles (DLPsThe 11 viral plus-strand RNAs are created by the RNA-dependent RNA polymerase (RdRp) VP1 of the DLP, which also acts as a transcriptase (Lawton et al., 1997). Channels at the vertices of DLPs are used to extrude plus-strand RNAs, and these channels penetrate both the VP2 and VP6 protein layers. Six structural proteins and six nonstructural proteins are produced during translation of plus-strand RNAs, which lack 3' poly (A) tails and only contain 5' caps. Additionally serving as

templates for the assembly of the dsRNA genome segments are the plus-strand RNAs. The packaging of the 11 genome segments into freshly created cores takes place concurrently with RNA replication, and the two processes are coordinated such that the 11 segments are generated in equimolar proportions. (Patton & Gallegos, 1990). As a result of rotavirus infection, perinuclear, nonmembrane-bound cytoplasmic inclusions (viroplasms) form. Important roles for NSP2 and NSP5 in formation of viroplasm (Lawton et al., 1997). Viroplasms are the suggested sites for DLP assembly, core assembly, and minus-strand synthesis of RNA. The DLPs acquire the third, outer layer as they go to the endoplasmic reticulum (VP7 and VP4 are responsible for producing this layer). The host cell removes the offspring of viruses via lysis (Jayaram et al., 2004).

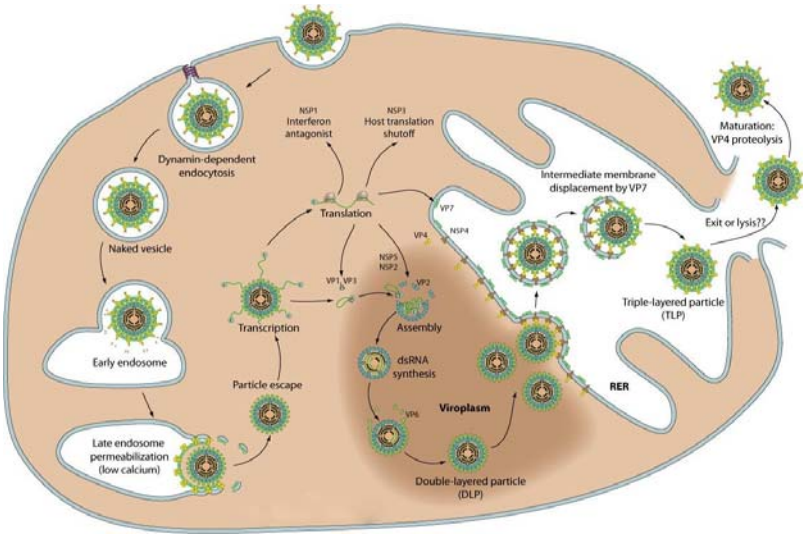


Figure 2.6: Overview of the rotavirus replication cycle ((Papa et al., 2021))

2.1.8 Mechanism of Pathogenesis:

Although some investigations found low titers of virus in respiratory tract secretions and other bodily fluids, suggesting the possibility for airborne and waterborne transmissions, the main method of rotavirus transmission from person to person is fecal-oral (Dennehy, 2000).

The intestinal epithelium undergoes structural alterations as a result of the rotavirus particles' exclusive consumption of adult differentiated enterocytes in the middle and upper parts of the small intestine's villi (Lundgren & Svensson, 2001). In contrast to parvovirus, rotavirus cannot infect either the intestinal enterocytes or the growing villous crypt cells. The VP4 protein is used by rotavirus to attach to its cellular receptors. The virus may enter target cells directly, fusing with enterocytes, or by Ca2+-dependent endocytosis. (Â Francisco Pe Â rez et al., 1998; Jayaram et al., 2004).

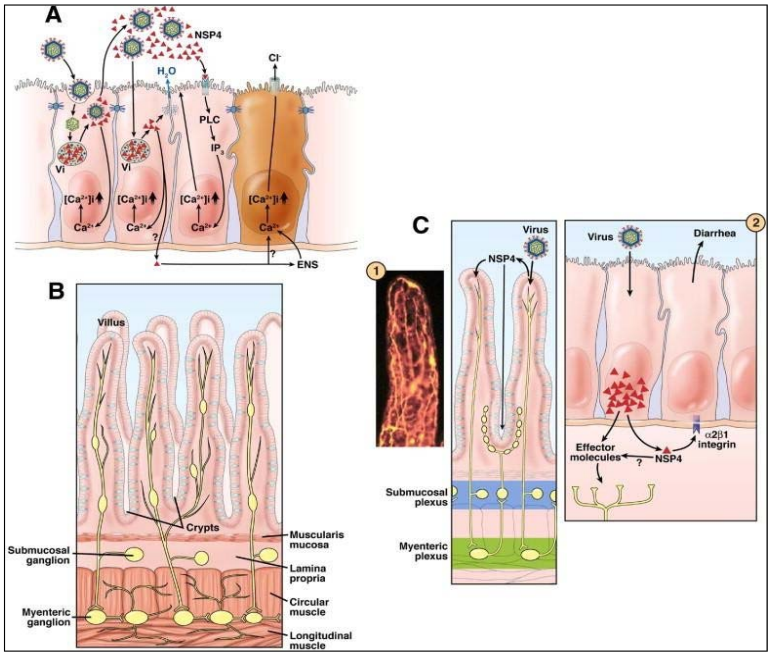


Figure 2.7: Rotavirus-induced diarrhea ((*Ciarlet et al, 2001*, n.d.))

According to research, rotavirus can cause diarrhea in three different ways. First, 12 to 24 hours after infection, enterocytes are still present, but the amounts of brush-border disaccharidases (lactase, maltase, and sucrose) have dramatically decreased. Disaccharides in the diet cannot be digested into monosaccharides and then absorbed, which leads to the development of osmotic diarrhea. (Anderson and Webe, 2004, n.d.). Second, NSP4 influences the capacity of enterocytes to open calcium channels. As a result, there is an outflow of salt and water, which causes secretory diarrhea. (Ciarlet et al, 2001, n.d.) (Figure 2.7). Not to mention, the increasing intra-enterocyte calcium concentration causes enterocytes to die oncotically. Mature enterocytes at the tips of the villi die more quickly than they can develop into immature enterocytes derived from crypt stem cells, which leads to villous blunting and subsequent

malabsorption. (Walker-Smith, 1978). When the virus no longer has access to mature enterocytes that are susceptible and an immune response is established, the infection ends (Lundgren & Svensson, 2001).

2.1.9 Immune Response to Rotavirus:

Production of memory B and T lymphocytes with rotavirus specificity is the result of initial rotavirus infections. (Raú L Velázquez et al., n.d.). Serum IgA is believed to be the most important indicator of rotavirus immunity since IgG has high titers which do not appear to be much protective but IgA has that much titers which is needed against moderate to severe disease in people. These antibody responses are serotype specific, which is one reason why they do not totally prevent. These antibodies are unable to provide complete protection against infection by a different serotype due to the variety of the several rotavirus serotypes. Repeated infections are less hazardous because they increase the number of B cells that may produce cross-reactive antibodies that can recognize a variety of serotypes. To provide a trustworthy defense, any vaccination attempt would need to produce these cross-reactive antibodies. (Rodrigo et al., 2010).

Additionally required for the elimination of rotaviral infection are CD4+ helper T (TH) cells. (VanCott et al., 2001). Because of this, rotavirus immunity is linked to large amounts of cross-reactive secretory IgA as well as serotype-specific serum IgA and IgG, which need a TH cell response particular to rotavirus and a CTL response particular to rotavirus, respectively. (Anderson and Webe, 2004, n.d.). Both maternal antibodies acquired transplacentally and maternal antibodies as well as other components in breast milk appear to protect infants from rotavirus infection. It is interesting that, barring the development of novel serotypes, rotavirus infections in infants often cause quiet diseases. Rotavirus may also covertly spread in newborn words. (Patel et al., 2009).

2.1.10 Clinical Features of Rotavirus Infections:

Rotavirus infections can cause anything from mild, temporary watery diarrhea to severe gastroenteritis with potentially lethal dehydration. A brief one to three-day incubation phase is followed by an abrupt onset of symptoms. The first two to three days of the sickness are characterized by fever, frequent stomachaches, and vomiting. The next three to eight days are then marked by pale, watery, or loose, non-bloody diarrhea. Patients who regularly have diarrhea have 10 to 20 bowel movements per day, which might be a lot. Such severe diarrhea

may be fatal if fluids and electrolytes are not replaced. Another possibility is a brief intolerance to lactose. Although rotavirus gastroenteritis usually includes respiratory symptoms, its cause is unknown. Recent studies have shown that rotavirus gastroenteritis can cause symptoms outside of the gastrointestinal tract, such as viremia. Due to their protracted excretion of the virus, patients may serve as reservoirs for its propagation to other people. (Akhtar, 2012a).
Clinical signs alone cannot distinguish rotavirus gastroenteritis from other viral causes of non-inflammatory diarrhea (Lundgren & Svensson, 2001). Contrarily, diarrhea caused by rotavirus is typically more severe than diarrhea caused by other enteropathogens. The presence of another pathogen does not affect the severity of rotavirus-related illness. (Walker-Smith, 1978).

2.1.11 Diagnosis of Rotavirus:

Using methods including antigen detection tests, electron microscopy (EM), polyacrylamide gel electrophoresis (PAGE), reverse transcription-polymerase chain reaction (RT-PCR), and viral isolation, rotavirus can be detected in a patient's feces. (Uchida et al., 2006). The antigen detection techniques used most frequently in diagnostic laboratories are immunochromatography, latex particle agglutination assays, and enzyme-linked immunosorbent assays (ELISA) (Akhtar, 2012b). These tests are exclusively meant to identify group A rotavirus, despite the fact that they frequently have good sensitivity and specificity. Additionally, beyond day 10 post-infection, when antibody levels in the feces begin to decline, ELISA is not a practical strategy (Greenberg et al., 1983). Additional rotavirus subgroups can be distinguished in cell cultures, however viral culture is only relevant for research. Rotavirus can also be identified by antibody detection; however, this is less common. EM is a quicker technology that is used to identify non-group A rotaviruses. (Mcnulty, n.d.). However, electron microscopes are rarely available in developing nations. When rotavirus RNA has been isolated straight from feces samples, it is simple to find using PAGE. Other rotaviruses than group A can be found using the assay. The technique is simple, straightforward to use, and has good specificity as well as sensitivity. Additionally, depending on the electrophoretic migration pattern of the 11 dsRNA segments, this test gives epidemiological data (Jiang et al., 1995). In research settings, RT-PCR is frequently recognized as the gold standard for virus detection (Gouvea et al., 1990). The method offers details on the G and P genotypes of the rotavirus strains that are in circulation as well as the length of viral shedding in the stool ((Fischer & Gentsch, 2004)).

2.1.12 Management, Prevention and Control of Rotavirus Infection:

There is no known cure for the rotavirus. As a result, the cornerstone of therapy is oral rehydration, which replaces lost fluid with fluids that have a precise composition of electrolytes and glucose (Anderson and Webe, 2004, n.d.). Patients with severe dehydration, shock, or altered degrees of consciousness require intravenous rehydration treatment. Hyperimmune human serum immunoglobulin and human or bovine colostrum may be used to treat persistent rotavirus infection in children with compromised immune systems. Probiotics like Lactobacillus casei GG may be administered for extra benefits. Anti-diarrheal medications shouldn't be taken as they can make the infection last longer. (Leung et al., n.d.).

Vaccines are being developed as the first line of defense against rotavirus infections due to the severity of diseases caused by rotavirus infections and the likelihood that public health attempts to improve sanitation would not reduce the incidence and burden of this disease. (O'Ryan, 2009; Rodrigo et al., 2010). Numerous pediatric clinical trials, including well designed cohort studies, have decisively shown that while rotavirus infection naturally provides some protection against reinfection, this protection is inadequate. (Aúl et al., 1022; Fischer et al., n.d.). The majority of children (and possibly adults) only have one moderate to severe clinical episode after their initial exposure to the virus, despite the possibility that they could become infected again in the future. This clinical result laid the groundwork for the hypothesis that a disease may induce protective immunity, which in turn gave rise to the hypothesis that protective immunity might be acquired through vaccination. (Rodrigo et al., 2010).

2.1.13 Rotavirus Vaccines:

The rhesus-human reassortant vaccine RotaShield^{TM}, which contains three RRV-based reassortant (G1, G3, and G4) strains, was the first multivalent live-attenuated oral rotavirus immunization. The vaccine's efficacy against serious sickness ranged from 70 to 95 percent, while its efficacy against all rotavirus gastroenteritis was 49 to 83 percent (Heaton & Ciarlet, 2007). RotaShield^{TM} was approved by the US Food and Drug Administration in 1998 and included in the US baby immunization program after more than 15 years of development and clinical testing. The Centers for Disease Control advised deferring future RotaShield^{TM} delivery in July 1999 due to the potential for intussusceptions—a bowel obstruction in which one segment of the intestine becomes enfolded within another segment. In October 1999, RotaShield^{TM} was subsequently removed from sale. (Heaton & Ciarlet, 2007; The

Immunological Basis for Immunization Series, 2011).

RotaTeq™ (Merck & Co., Inc. Whitehouse Station, New Jersey) and Rotarix™ (GlaxoSmith Kline [GSK], Rixensart, Belgium) were created and are currently licenced in a number of nations throughout the world despite the observation of intussusceptions with RotaShield™. Very extensive Phase III clinical trials were required to show that these vaccines were safe, well tolerated, and that there was no link to intussusceptions (Ruiz-Palacios et al., 2006). Five live-attenuated rotavirus strains with serotypes G1, G2, G3, G4, and P1A [8] and G6 and P7 in cattle make up the pentavalent combination vaccination RotaTeq™. (Heaton & Ciarlet, 2007). The attenuated human G1P1A [8] rotavirus strain is the basis for Rotarix™ ((Ward, 2008)). In affluent nations and Latin America, studies have demonstrated the excellent efficacy of both RotaTeq™ and Rotarix™ (Heaton & Ciarlet, 2007; Ruiz-Palacios et al., 2006; Ward, 2008)). Through the first season following vaccination, RitaTeq has shown to be effective and has provided 98% protection against severe rotavirus gastroenteritis, such as fever, vomiting, and diarrhea, and 74% protection against gastroenteritis of any severity, such as inflammation of the stomach and intestines. However, within the first two years following immunization, babies who received the RotaTeq vaccine had a 94% lower likelihood of visiting an ER and a 96% lower likelihood of being hospitalized. Through two seasons (December through June), two clinical studies showed Rotarix to offer 85% to 96% protection against severe rotavirus. (CDC)

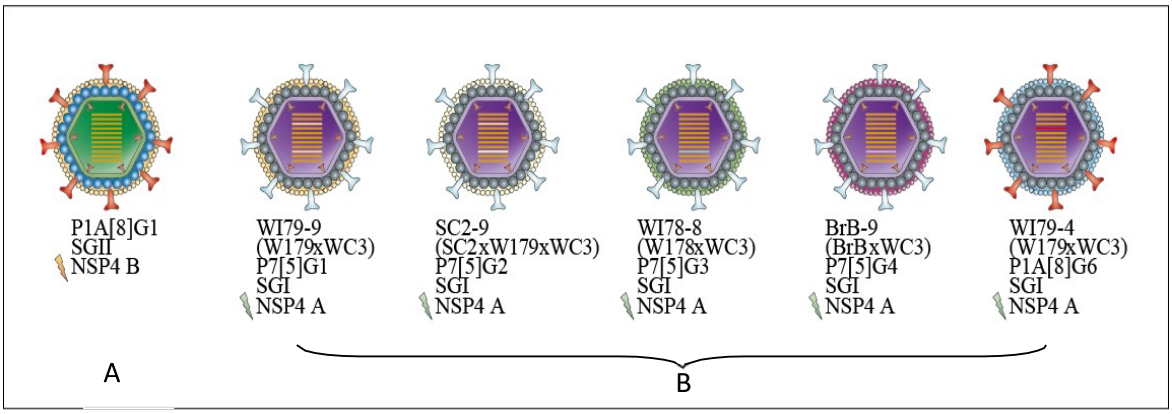


Figure 2.8: **Rotavirus vaccine**. A) Rotarix is an attenuated human rotavirus vaccine made of a tissue-culture-adapted human P1A[8]G1, VP6 subgroup II and NSP4 geno-group B strain. B) RotaTeq is a bovine (WC3)- human reassortant vaccine composed of the five strains shown, each containing a human rotavirus gene encoding the VP7 neutralizing protein from different serotypes ((Angel et al., 2007)).

2.2 Wastewater:

Waste disposal uses water. Chemicals, food scraps, oils, soaps, and human waste are among its components. It includes the water used in the home's sinks, showers, washing machines, dishwashers, and toilets. Another way to describe water is water that cannot be used for industrial or home purposes. This water can be found in lateral sewers, which connect the drains from homes, offices, and companies to branch sewers, which then transport the water to main sewers, where it is treated, before being appropriately disposed of in septic treatment facilities. Domestic sewage contains undigested food that is nevertheless rich in a variety of organic components, including a wide range of fat, protein, and carbs, as well as bacterial cells. These offer the bacteria a fantastic nutritional environment for growth. Sewage's microbiological makeup and feces concentration are not constant; they fluctuate daily and over time, depending on the climate and human activity patterns.

Wastewater contains both harmful and beneficial microbes. Despite the fact that there are far more harmless microbes than hazardous microbes, people who come into contact with wastewater may get gastrointestinal issues or other disorders as a result of the disease-causing bacteria present in the wastewater. Rotavirus, enteric adenoviruses, and the hepatitis virus are also included in this. (De Serres et al., n.d.). Especially enterohaemorrhagic and enteropathogenic *Escherichia coli* strains, Salmonella, and Shigella spp. are pathogenic bacteria that are present in feces (Percival et al., n.d.), *Campylobacter jejune* and *Vibrio cholera*, represent potentially fatal threats to our health (Seto et al., 2016). Cyst producing protozoa like *Entamoeba histolytica*, Cryptosporidium spp. and Giardia lamblia (TeAM YYeP G, n.d.) have become increasingly of concern, while intestinal helminth parasites including tapeworms, flatworms and roundworms may also be present. Pathogenic fungi including Aspergillus and Candida spp. Also have been detected.

Because the health problems brought on by a single incident involving any water borne pathogen will unavoidably have catastrophic results, simultaneously affecting some viruses, we now have quick, sensitive, and targeted molecular based methods for their detection and in some cases, quantification from wastewater (Carducci et al., 2009; Lemarchand et al., 2004).

Bangladesh is one of many low- and middle-income countries with a mixed sanitation system, which produces sludge with a range of unique qualities. As a result, the techniques used in high-income countries for on-site sanitation, direct feces disposal into the environment, and shared and private latrines will need to be adjusted. Wastewater samples collected from drains, surface water, and septic tank effluent allowed for the successful identification and quantification of viral RNA. Results from wastewater monitoring ought to reflect combined rather than independent research findings. The survival, degradation, and detection of viruses in the environment can all be impacted by temperature, humidity, and rainfall, making it crucial to look at how climatic variability affects wastewater-based surveillance in subtropical regions. Depending on the public health goals, for the observational program of a disease in a particular community, the number of samples needed to cover that community can represent the overall health of that community. Viral nucleic acid collected from those aggregated wastewater samples can indicate the presence of the variants of concern through this. (Rouchka et al., 2021)

Chapter-3: Methodology

3.1 Study Population

In this study, the wastewater samples were collected from two different urban areas of Bangladesh. A total of 150 wastewater samples were collected from effluent of these two districts. The samples are from urban areas of Dhaka and Cox's Bazar.

3.2 Sample collection:

Around 300ml of environmental sample was collected in sterile double zipper bag and was sealed inside another plastic zipper bag. The field sample collectors of One Health Laboratory were appointed to collect them and deliver them. Sample was collected from 16th October 2023 to 5th December, 2023. During sample collection sample ID, date of collection and time, delivery time and date and site of collection were recorded properly. The samples were transported to the one health laboratory in cooler box and all the samples had been preserved in 4^{0}C until they are aliquoted.

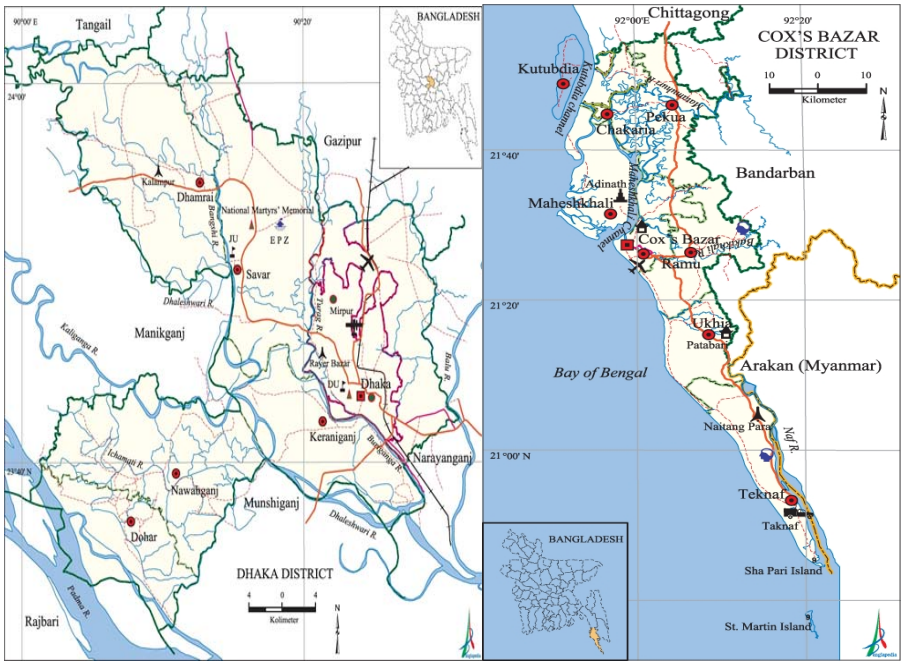
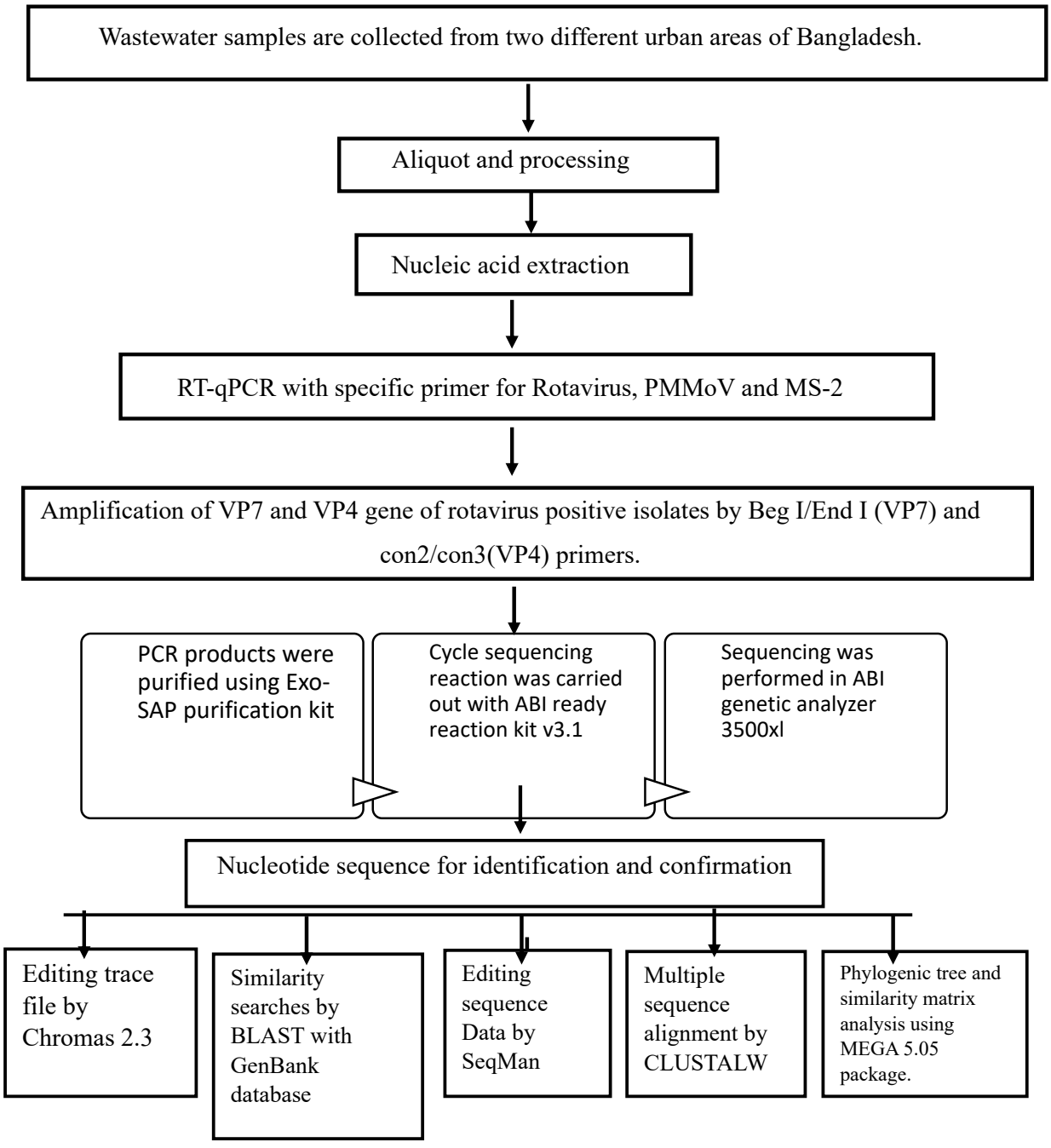


Figure3.1: Areas of sample collection (Dhaka & Cox's Bazar)

Table 3.1: Experiment design



3.4 Processing:

Taking 10ml of sample from the container, all the cell particles present inside this 10ml is taken inside into 1ml by concentrating through processing.

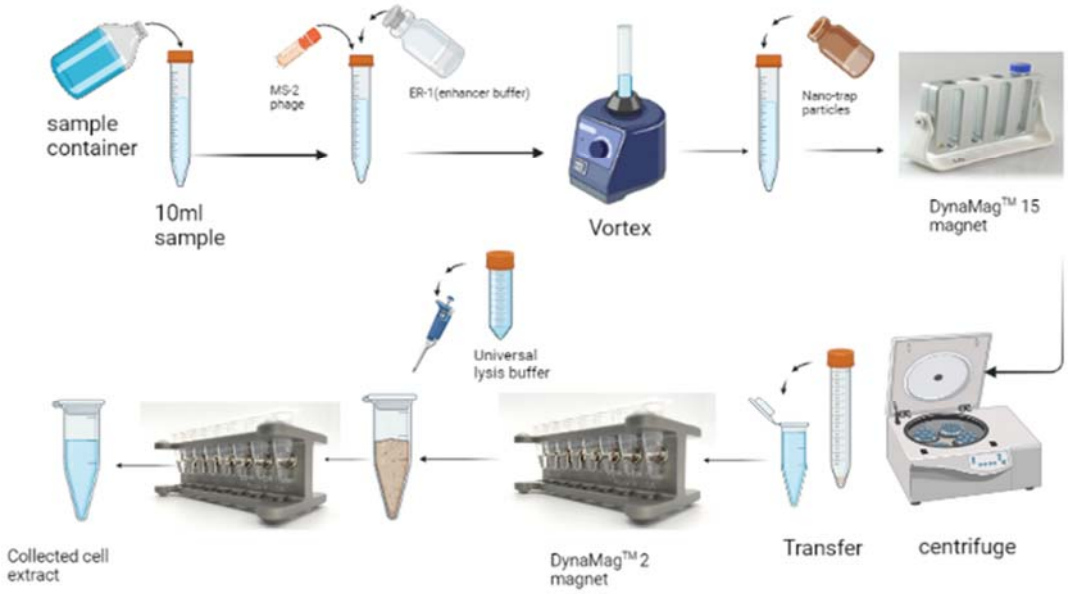


Figure 3.2: Processing to concentrate the sample.

3.4.1 Equipment:

- 15ml conical centrifuge tubes
- Vortex machine
- Pipette
- DynaMag™ 15 magnet
- DynaMag™ 2 magnet
- Centrifuge machine
- Autoclaved Eppendorf set

3.4.2 Reagents:

- MS-2 phage
- ER-1 (enhancer buffer)
- Nano-trap particles

- Nuclease free water
- Universal lysis buffer

1. For the aliquot, 15ml conical centrifuge tubes are taken and each of them are marked with ENV and ID number. Matching the number of conical centrifuge tubes with the sample container, the zipper bags are taken out and cut at the edge of the zip to make sure the sample doesn't spill off and they are poured in the 15ml conical centrifuge tube. 10ml of the sample was taken and the remaining sample was discarded.
2. 10µL MS-2 as internal control and 100µL ER-1 which is enhancer buffer for enhancing the activity of Nano-trap particles are added in each tube. All the tubes are vortexed and kept for 10 minutes at room temperature.
3. When the 10 minutes is up, 150µL of nano-trap particle is added to each tube and inverted. Again, they are kept for 10 minutes but in between these 10 minutes, after every 5 minutes they are inverted. After these 10 minutes, they are prepared to be kept in the DynaMag^{TM} 15 magnetic rack. Before keeping them, they are again inverted. The nano-trap particles trap all the cells present in the sample. The nano-trap particles have magnetic force of their own, which makes them attract toward any magnet. So, the magnet will attract these particles and make them attach with the surface facing magnet. After a certain period of time, when all the particles have gathered at the edge of the wall facing magnet and the water is almost transparent, the water is discarded.
4. 1ml of distilled or nuclease free water is added in the tube by using pipette and shaken vigorously to get all the particles mixed with the water. The tubes are centrifuged the get all the nanoparticles and water at the bottom of the tube. Using a pipette, the water is transferred in the 1.5ml low bind conical centrifuge tubes.
5. All the particles that were present in the 10 ml water are now in this 1ml of water. The tubes are kept in a magnetic rack. After a certain time, all the particles will attach with the wall facing the magnet and the water that is left is discarded.
6. The tubes are taken out and kept in the rack and they are mixed with 500µL universal lysis buffer with the help of pipette. After 10 minutes they are placed in the magnetic rack. After a certain period of time, all the nano-trap beads are gathered at the wall facing the magnet by separating from the supernatant. Then with a pipette, 400µL supernatant is taken out and kept in autoclaved 1.5ml centrifuge tubes.
7. Until further process, the supernatant is kept in 4^0C.

3.5 Extraction:

The extracted supernatant goes through nucleic acid extraction and wash. This extraction was done by the magnetic bead extraction technique.

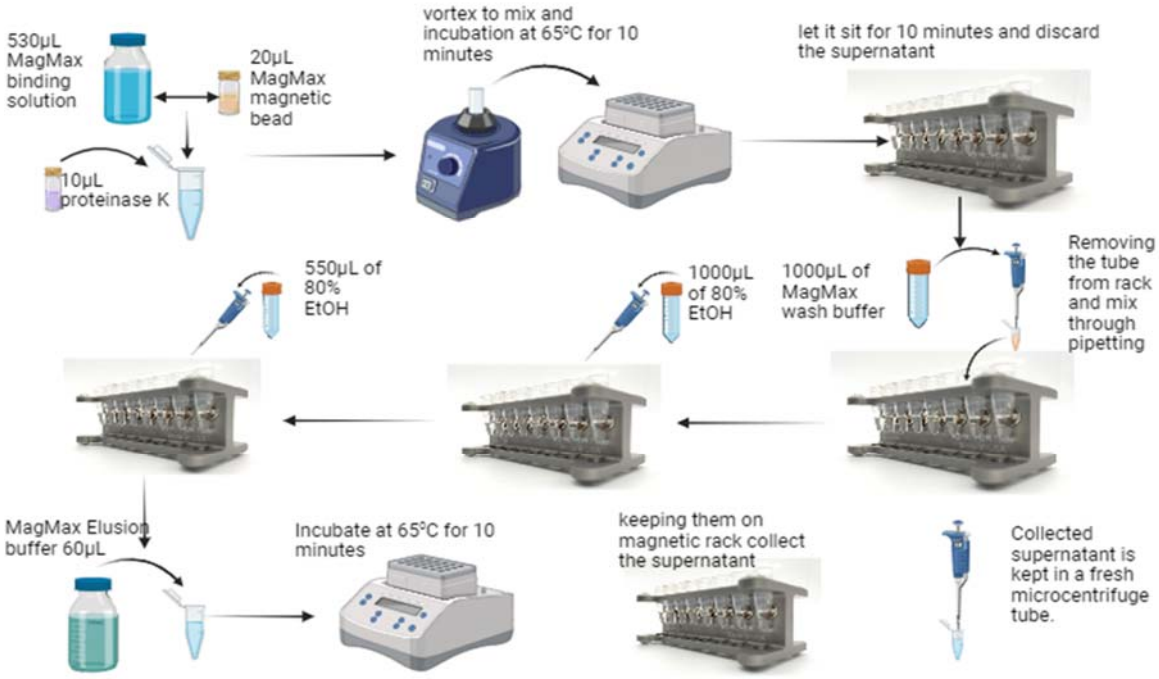


Figure 3.3: Magnetic bead nucleic acid extraction

3.5.1 Equipment:

- Centrifuge machine
- Vortex machine
- DymaMegTH 2 magnet
- Pipette
- Incubator
- Autoclaved Eppendorf set

3.5.2 Reagents:

- Proteinase K
- Binding solution
- Magnetic beads
- 80% EtOH

- Elusion buffer
- Wash buffer

1. After taking out the supernatant containing tubes 10µL of proteinase K to them.
2. 550µL of binding bead along with magnetic beads were added to them and vortexed until they mixed properly. Then they were incubated at 65^0C for 10 minutes.
3. After that they were placed on the DynaMag^{TM} 2 magnetic rack for 10 minutes. The magnetic beads would gather at the wall of Eppendorf facing the magnet and separate themselves from the supernatant. Then the supernatants were discarded.
4. Removing the tubes from the magnetic rack, 1ml of wash buffer was added and mixed properly with the help of pipette.
5. Again, the tubes were placed on the magnetic stand for 2 minutes. After that 2 minute, the supernatants were discarded.
6. Without removing the tubes, 1ml 80% ethanol was added in them and without keeping them for long it was again discarded. Then again 550µL of ethanol was added in the tubes and discarded. During this time of removing the wash buffer supernatant and ethanol, the bead was still attached with the wall facing magnet. The nucleic acids were still attached to the beads. These wash buffer and ethanol only washed them but didn't wash them off because of the salt concentration and pH of these reagents the nucleic acids binding affinity was very less with them.
7. Removing the excess ethanol from the tubes, the tubes were removed from the magnetic rack and 65µL of elusion buffer was added to each of them and were mixed by using pipette.
8. They were again placed in the incubator at 65^0C for 10 minutes and at 1000rpm.
9. After taking them out they were pulse centrifuged and were again placed in the magnetic rack.
10. After 15 minutes, all the magnetic beads separate themselves from the nucleic acid containing supernatant and gather themselves at the side of the wall facing the magnet. The nucleic acid containing supernatant is taken with the help pf pipette carefully by making sure that the tip of the pipette has not touched the beads and the supernatant is kept in another set of autoclaved Eppendorf and preserved in -20^0C.

3.6 Purification:

To remove the PCR inhibitor like humic acid the PCR inhibitor removal kit was used, and the extracted nucleic acid was purified. As this nucleic acid was extracted from environmental effluent samples, they may contain soil particles that may contain humic acid which is a PCR inhibitor.

1. The PCR inhibitor powder containing columns are positioned inside the collection tubes and 600µL of prep solution is added inside each column and they are centrifuged at 8000x g for 3 minutes.
2. Then they are taken out and the collection tubes are discarded. The column filters are positioned inside a set of autoclaved Eppendorf.
3. Then the extracted nucleic are added in each column and centrifuged at 18000x g for 3 minutes and the supernatant inside the Eppendorf is PCR inhibitor free.

3.7 Rotavirus, PMMoV and MS-2 phage detected by real time RT-qPCR:

Quantitative PCR was carried out using the Taq-path RT-qPCR system according to the manufacturer's instructions.

3.7.1Preparation of positive control

Table 3.2: RVA/PMMoV/MS2 standard preparation

Plasmid controls were prepared according to the following.

Dilution	Name	Plasmid (µL)	Molecular Water (µL)	Final Volume(µL)	gc/µL*	gc/5µL
1	Dilution D4	100	-	100	180000	900000
2	Dilution D5	10	90	100	18000	90000
3	Dilution D6	10	90	100	1800	9000
4	Dilution D7	10	90	100	180	900

3.7.2 Primer and Probe Sequences

A specific oligonucleotide primer was used for the amplification of the VP6 coding gene for the detection of Rotavirus A in the samples. The primers and probes used in this study are given in the following table. Primers and probes for real-time PCR were purchased from CDC (Centers for Disease Control and Prevention); at the 5` ends, they were conjugated to the 6-carboxy fluorescein (6-FAM) and the 3` end to the Quincher-1:

Table 3.3: Primer and Probe Sequences

Reaction (Target gene)	**Primer and probe**	**Sequence (5`-3`)**
Rotavirus A (Jothikumar et al., 2009)	JVKF	CAGTGGTTGATGCTCAAGATGGA
	JVKR	TCATTGTAATCATATTGAATACCCA
	JVKP	FAM-ACAACTGCAGCTTCAAAAGAA-BHQ
PMMoV (Haramoto et al., 2013; Zhang et al., 2006)	Forward	GAGTGGTTTGACCTTAACGTTTGA
	Reverse	TTGTCGGTTGCAATGCAAGT
	Probe	Cy5-CCTACCGAAGCAAATG-BHQ
MS2 Phage (Gautam et al., 2016)	MSF.F	TGGCACTACCCCTCTCCGTATTCACG
	MSF.R	GTACGGGCGACCCCACGATGAC
	MSF.P	HEX-CACATCGATAGATCAAGGTGCCTACAAGC-BHQ

3.7.3 Preparation of PCR master-mix:

Components of the one step Real Time RT-PCR kit (Master Mix, Probe, Reverse Transcriptase enzyme mix, Nuclease free water) were mixed with the selected primers to prepare a volume of 15µL for each reaction tube is given below:

Table 3.4: Master-mix preparation

Composition	***Volume (µL/well)***	***Final concentration/well***
4X TaqPath Master Mix	5.0	1×
JVK-Forward (10µM)	0.4	200nM
JVK-Reverse (10µM)	0.4	200nM
JVK-Probe (10µM)	0.2	100nM
PMMoV-Forward (10µM)	0.8	400nM
PMMoV -Reverse (10µM)	0.8	400nM
PMMoV -Probe (10µM)	0.4	200nM
MS2-Forward (10µM)	0.8	400nM
MS2-Reverse (10µM)	0.8	400nM

MS2-Probe (10µM)	0.4	200nM
Molecular grade water	5.0	-
Total	**15**	
Template	5	

The mixture was gently mixed and centrifuged before the addition of 5µM extracted template RNA.

3.8 Thermal Cycling Protocol of Real Time PCR:

The Rotavirus genome is double stranded, so denaturation is carried out for these viral dsRNA at 95^0C for 5 minutes. After denaturation, RNA samples were added to the 15µM of master-mix taken in the reaction tube. The tubes were then centrifuged and transferred to the CFX Opus 96.

Real time PCR Assay:

Real-time quantitative polymerase chain reaction was performed in CFX Opus96 according to PCR condition detailed in following table. A reagent bank, which contained all components of reaction mixture and sterile water instead of the template RNA was included in every PCR procedure. A positive and negative control was also used in every PCR.

Table 3.5: Thermal cycling program

Step	Time	Temperature	Cycles
Polymerase Activation	2 min	95°C	1
Denaturation	15 sec	95°C	**45**
Image Capture	1 min	56.5°C	

3.9 Quantification by standard curve

From each RT-qPCR run with serially diluted standards, we constructed a standard curve based on the log copy number of each standard dilution and obtained the average Ct value of the particular standard dilution. From the standard curve, we obtained the linear regression value (R2) and the slope-intercept form of the equation of the straight line (y = mx + c), where m is the gradient of the line (how steep the line is), and c is the **y-intercept**. Using the intercept and slope value, we calculated the input RNA copy number per microliter template from the equation, $Nn = 10^{((Ct-C)/m)}$, where Nn is the input template copy number. After obtaining the RNA copy number in per µL of reaction volume and input template, we back calculated the viral RNA copy numbers per ml of a sample using the formula copy/mL = (copies/µL × total elute)/sample (mL). Initially, the gene copy number was quantified per µL of sample input RNA as described above, and the back-calculation was performed based on the initial gene copy number, total eluted RNA, and total concentrate, extracted concentrate of wastewater.

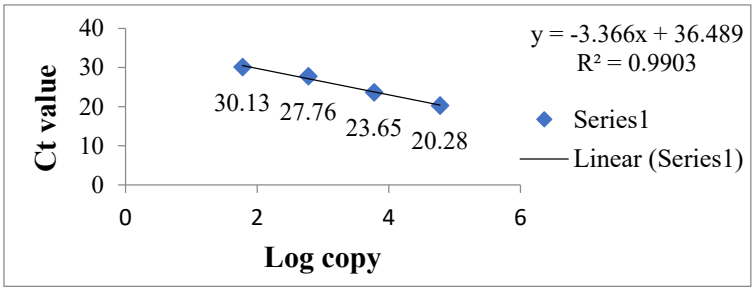


Figure 3.4: Standard curve calculation

3.10 RT-PCR:

Table 3.6: Genotype determined by Reverse transcriptase Polymerase Chain Reaction (RT-PCR):

Primer	Position	Strand	Sequence (5'→3')	Amplicon Size (bp)	References
VP7					
BegI	51-71	Plus	ATGTAGGTATTGAATATACCAC	882 bp	Gouvea *et al.*, 1990
EndI	914-932	Minus	AACTTGCCACCATTTTTTCC		
			VP4		
Con2	868-887	Minus	ATTTCGGACCATTTATAACC	887 bp	Gentsch *et al.*, 1992
Con3	11-32	Plus	TGGCTTCGCCATTTTATAGACA		

1-17F	1-17	Plus			

3.10.1 Preparation of PCR master mixture:

Reverse transcription polymerase chain reaction was carried out using the QIAGEN One-step RT-PCR kit. Components of the One-step RT-PCR kit: 5x buffer, dH_2O, dNTPs and enzyme were mixed with the restrictive primers to make up the volume of 20µL. For each reagent the amounts are given below:

Table 3.7: Master mixture for VP7 gene amplification

Composition	***Volume (µL/well)***	***Final concentration/well***
$MgCl_2$ (25 mM)	2	40
Buffer (5X)	4	80
dNTP (10mM)	0.4	8
*Beg*I (20 µM)	0.5	10
*End*I (20 µM)	0.5	10
Polymerase	0.1	2
H_2O	10.5	210
	18	360

The mixture was gently mixed and centrifuged before addition of 5µL extracted RNA.

Table 3.8: Master mixture for VP4 gene amplification

Composition	***Volume (µL/well)***	***Final concentration/well***
$MgCl_2$ (25 mM)	2	40
Buffer (5X)	4	80
dNTP (10mM)	0.4	8
Con3 (20 µM)	0.5	10
Con2 (20 µM)	0.5	10
1-17F (20 µM)	0.5	10
Polymerase	0.1	2
H_2O	10	200
Total	18	360

3.10.2 Procedure for RT-PCR:

As this virus has dsRNA, they were first denatured by heating at 95^0C for 5 minutes and subsequent rapid cooling on ice. After denaturation RNA sample was added to 20µL of master-mix taken in the PCR plate. This plate was then centrifuged and transferred into the PCR.

3.10.3 RT-PCR Assay:

RT-PCR assay was carried out in T100 Thermal Cycler according to the PCR condition detailed in following table. A reagent blank, which contained all components of the reaction mixture with the negative control were also used in every PCR. PCR condition used for the amplification of VP7 and VP4 encoding gene is given below:

Table 3.9: RT-PCR program

Step	**Time**	**Temperature**	**Cycles**
Reverse Transcription	30 min	50°C	1
Polymerase Activation	15 min	95°C	1
Denaturation	30 sec	95°C	40
Annealing	30 sec	48°C	
Extension	1 min	72°C	
Final extension	7 min	72°C	1
Hold	∞	4°C	1

3.11 Analysis of amplified PCR product:

The amplified product was visualized by 1.5% agarose gel electrophoresis. 6µL sample was mixed with 1µL of 6x GelPilot Loading Dye and loaded in agarose gel with a 100bp plus DNA ladder as marker. Electrophoresis was carried out on a Biometra Horizontal electrophoresis apparatus at 180V for 30 minutes. The DNA bands were then visualized and documented using UV transilluminator.

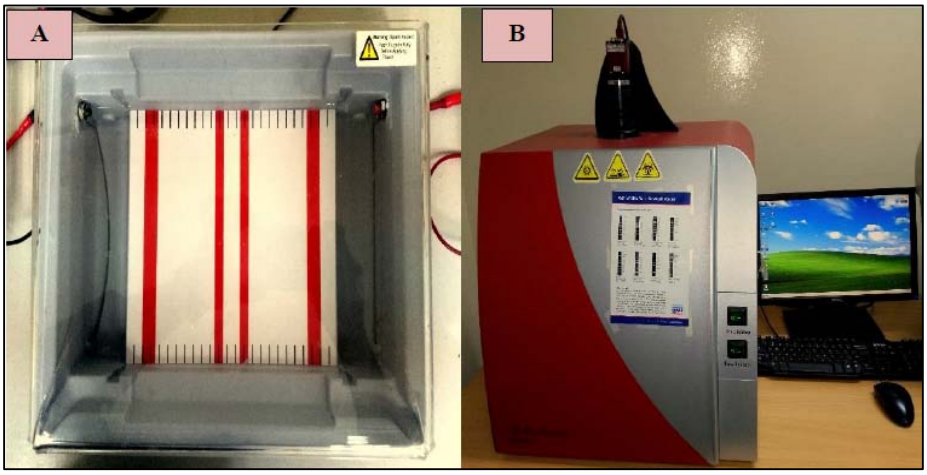


Figure 3.5: Gel apparatus. (A) Apogee gel apparatus. (B) BioDoc gel documentation

3.12 Sequencing of the amplified PCR amplicons:

The ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle sequencing kit was used to sequence this PCR product. The VP7-specific gene was sequenced using the BigI forward and EndI reverse primers, and the VP4-specific gene was sequenced using the Con2 forward and Con3 reverse primers. Sequencing of amplified PCR product includes the following steps:

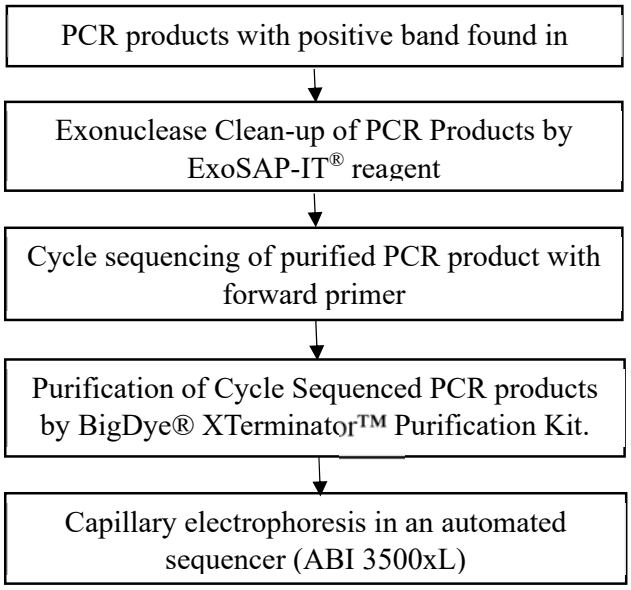


Figure 3.6: the schematic representation of the Sequencing of amplified PCR product

Figure 3.4: The schematic representation of the sequencing process.

3.12.1 Exonuclease Cleanup of PCR products:

ExoSAP-IT® is made to purify PCR products quickly and effectively for use in subsequent processes like sequencing, genotyping, cloning, etc. Comparing ExoSAP-IT® to other clean-up techniques like gel electrophoresis, ethanol precipitation, or column chromatography, neither sample loss nor are time-consuming processes required. In this work, PCR products were cleaned using ExoSAP-IT® procedure before being sequenced. It uses Exonuclease I and Shrimp Alkaline Phosphatase, two hydrolytic enzymes. The Shrimp Alkaline Phosphatase hydrolyzes the unbound dNTPs from the PCR mixture, which would otherwise interfere with the sequencing reaction and result in an unreadable sequencing product. Exonuclease I eliminate any remaining primers and any extraneous ssDNA created by the PCR.

3.12.2 ExoSAP-IT® PCR Clean-up Protocol:

ExoSAP-IT® reagent treats PCR products ranging in size from less than 100bp to over 10kb with absolutely no sample loss by removing unused primers and nucleotides. ExoSAP-IT® PCR product Cleanup is active in commonly used PCR buffers, so no buffer exchange is required. A total of 2µl of ExoSAP-IT® was directly added with 5µl of PCR product. This mixture was further incubated at 37ºC for 15 minutes (Activation of ExoSAP) followed by at 80ºC for 15 minutes (Inactivation of ExoSAP). The treated PCR products are now ready for subsequent analysis in applications that require DNA to be free of excess primers and nucleotides.

3.12.3 Cycle Sequencing:

The most repeatable results for sequencing PCR templates come via cycle sequencing. Cycle sequencing offers multiple opportunities to denature and lengthen the template, ensuring sufficient signal in the sequencing reaction even though PCR fragments can be challenging to denature with conventional sequencing methods. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used to cycle sequence the combination after the PCR product had been purified using ExoSAP-IT®. The master-mix preparation for cycle sequencing is given below:

Table 3.10: Composition of cycle sequencing reaction mixture.

Reagent	Amount (µL)
Buffer (5x)	1.0
Terminator ready reaction mixes (Big dye)	0.5
Primer (Forward/Reverse) (5µM)	1.0
Nuclease free water	6.5
Template cDNA	1.0
Total	**10 µL**

Table 3.11: **Thermal cycling program used for cycle sequence reaction.**

Stage	Temperature	Duration
Initial denaturation	96˚C	1min
3 steps cycling (25 cycle)		
Denaturation	96˚C	10 sec
Annealing	50˚C	5 sec
Extension	60˚C	4 min
Holding Stage	4˚C	∞

3.12.4 Purification of Cycle Sequencing Reaction Products:

The cycle sequencing product was purified by BigDye Xterminator™ purification kit. This kit requires two reagents, which are added sequencially or premixed:

- XTerminator™ solution
- SAM™ solution

The purification workflow is given below:

1. Cycle sequenced reaction products were centrifuged briefly, and then 45µl SAM™ solution was added into each well.
2. After addition of SAM™ solution 10µl XTerminator™ solution was added by using wide bore pipette tip.
3. The plate was then sealed using clear adhesive films.
4. The sealed plate was then shacked in a vortex at 21000 rpm for 30 minutes.
5. Centrifuged at 200x g for a minute.
6. Finally, the plate was transferred to ABI PRISM® 3500xl Genetic Analyzer.
7. In the Data Collection Software Plate Editor, BigDye XTerminator run module was selected and the plate was run for sequencing the product.

3.13 Nucleotide sequence analysis

Chromas 2.3 (Technelysium, Australia) was used to review and alter the chromatogram sequences. ClustalW Multiple Alignment in Bioedit, version 7.1.3, was used to align multiple sequences. Using the MEGA 7 program, phylogenetic and molecular evolutionary studies were performed. The Kimura 2-Parameter technique was used to determine genetic distances. The neighbor-joining approach was used to build the dendrogram.

3.13.1 Sequence similarity analysis

All sequences were submitted to the online BLAST (basic local alignment search tool) program at the National Centre for Biotechnology Information website (found at: https://blast.ncbi.nlm.nih.gov/Blast/) in order to determine how similar the sequences were. The Technelysium DNA sequencing program Chromas version 2.4 (Technelysium, Australia) was used to construct and modify sequences.

3.13.2 Multiple Sequence Alignment

Using ClustalW Multiple Sequence Alignment in the BioEdit program, version 7.1.3, sequences were aligned with the corresponding reference sequences and the sequences of the globally circulating strain.

3.14 Phylogenetic analysis

MEGA 7 (Molecular Evolutionary Genetics study) was used to do a phylogenetic study of the VP7 and VP4 genes of the Rota virus A utilizing avian influenza A virus strains that are currently circulating worldwide. Using Kimura 2-Parameter and the Neighbor-Joining approach, evolutionary history was deduced. Only in pair-wise sequence comparisons were all places with alignment gaps and missing data removed. Bootstrap analysis with 500 replications was used to statistically evaluate the reliability of the neighbor-joining (NJ) trees.

Chapter 4: Result

A total of 150 effluent environmental samples from urban Dhaka and Cox's Bazar were collected between October 2022 to December 2022. To investigate the presence of Rotavirus in these samples were processed using several molecular techniques.

- All the samples were screened out by Real-Time RT-qPCR technique to detect the presence and amount of Rotavirus A.
- The samples were screened out by Real-Time RT-PCR to detect the presence of Rotavirus A positive isolates.
- Samples with CT values <30 (29 samples) was selected for the Rotavirus A genotyping.
- The genotyping of Rotavirus was performed by nucleotide sequencing of both VP7 and VP4 segments.
- In addition, phylogenic analysis was performed to identify the origin of Rotavirus.

Table 4.1: Number of samples collected from different sites.

Sample collection sites	**No. of samples**
Dhaka	75
Cox's Bazar	75
Total	**150**

4.1 Molecular Detection of Rotavirus by Real-time RT-qPCR:

A total of 145 samples were found positive for group A Rotavirus in one-step real-time RT-qPCR assay out of total 150 samples. So, we found 96.67% samples positive for group A rotavirus in the screening test. Among these positive samples, 71 were from Dhaka and 74 were from Cox's Bazar. MS-2 Phage was used as an internal control. PMMoV was also the target, and its presence ensures that the sample was environmental as its presence indicates fecal contamination and which can only be present in environmental samples.

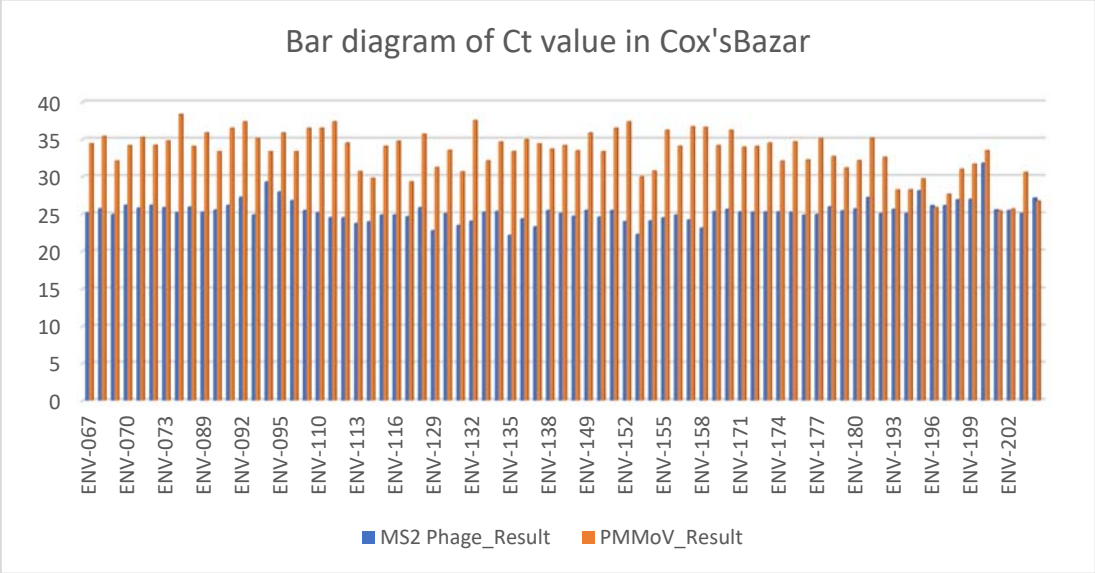


Figure 4.1: Ber diagram of Ct-value of MS-2 & PMMoV per sample in Cox'sBazar

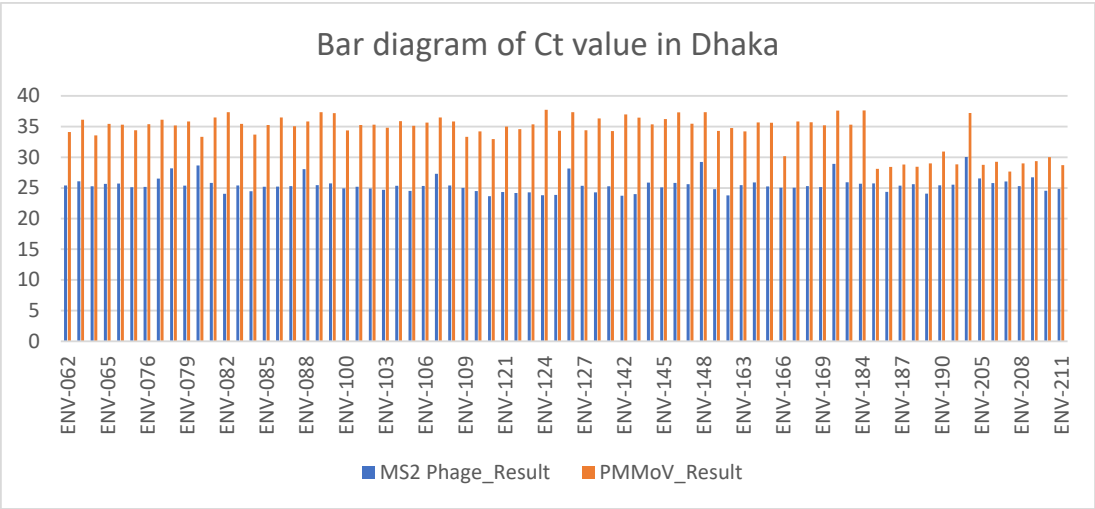


Figure 4.2: Bar diagram of Ct-value of MS-2 & PMMoV per sample in Dhaka

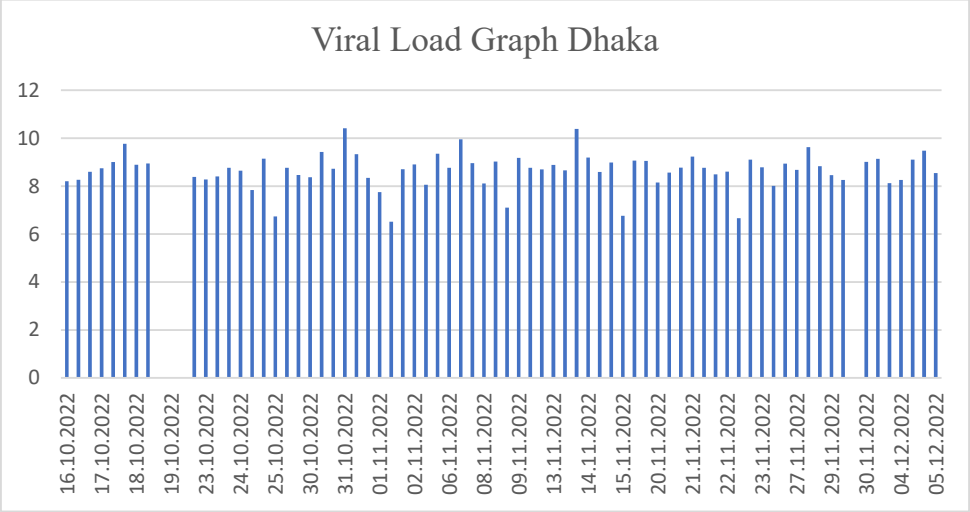


Figure 4.3: Bar diagram showing viral load in Dhaka

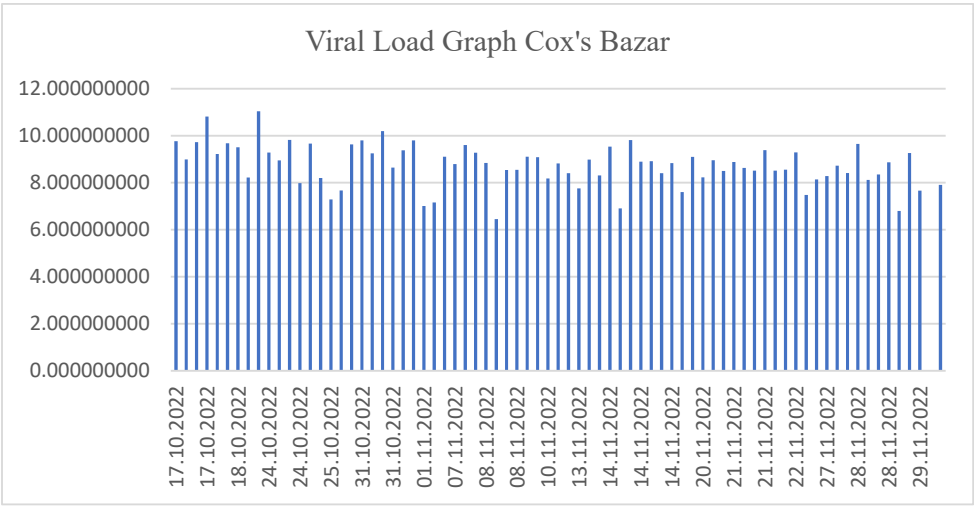


Figure 4.4: Bar diagram showing viral load in Cox's Bazar

4.2 Amplification of VP7 and VP4 genes:

For the amplification of VP7 and VP4 coding gene, RT-PCR was performed of 30 samples that found positive in real time RT-qPCR. RT-PCR was performed by specified primers for the VP7 and VP4 genes. We successfully amplified 29 VP7 and 24 VP4 genes in RT-PCR. The specific amplicon size of VP7 and VP4 outer capsid genes are 882 and 887 bp respectively.

Figure 4.5: Agarose gel showing specific bands for VP7 gene visualized in 1.5% agarose gel with gel red staining. 18-27 samples were all positive and it was detected by 1kb DNA marker.

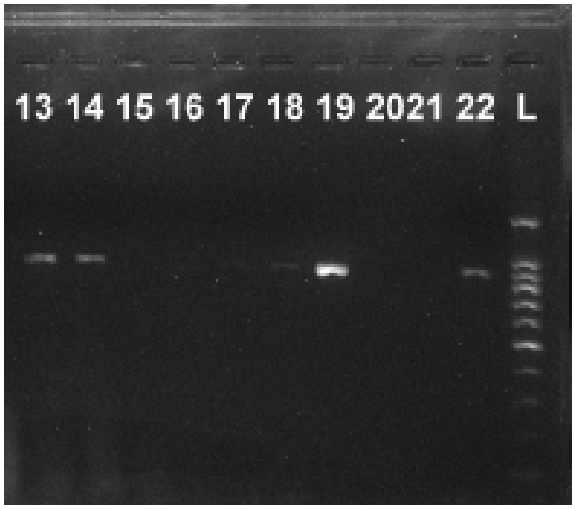


Figure 4.6: Agarose gel showing specific bands for VP4 gene visualized in 1.5 % agarose gel with gel red staining. Sample 13, 14, 18, 19 and 22 are positive samples of amplified VP4 gene.

4.3 Nucleotide Sequencing:

1. Sequencing analysis:

   The ABI PRISM 3500xl automated sequencer's standard methods were used to sequence the PCR products. For this, the complete lengths of the VP7 and VP4 gene segments were amplified using primers that were specific to those genes. Chromas 2.3

software was used to analyze the rotavirus sequences. The four nucleotides were identified by the peaks of their various colors. Chromas were used to fill in the gaps and insertions between nucleotides as well as the unreadable portions of the sequence at the beginning and conclusion.

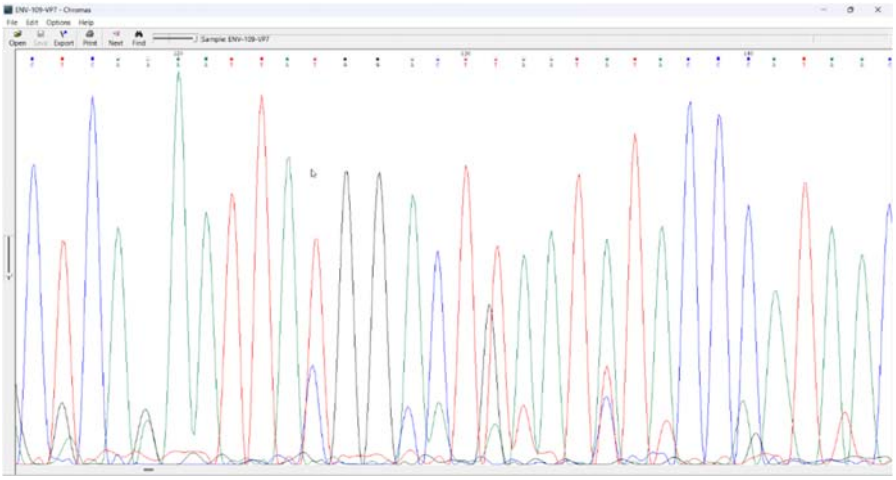


Figure 4.7: Nucleotide sequence analyzed by Chromas.

4.3.1 Blast Search Analysis of the Sequences:

Chromas-edited forward and reverse sequences were concatenated to create full-length sequences (800 bp to 850 bp), which were then compared to the GenBank database of the National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST). Similarity searches were performed separately for all G and P genotypes to find out the most similar rotavirus strains available in GenBank.

Table 4.2: Sequence homology (%) of locally isolated strains of RVA VP7 genes with global strains

Isolate no.	Close similarity to	Accession no.	E Value	%Max identity
ENV-066	RVA/Human-wt/BFA/33/2020/G1P[8] VP7 gene	ON622870.1	0.0	91.31%
ENV-090	RVA/Human-wt/BFA/RV-MMC2019-9/2019/G3P[8] VP7 gene	MN414258.1	0.0	96.19%
ENV-109	RVA/Human-wt/BFA/1401850/2018.G1P[8] VP7 gene	ON622880.1	0.0	98.92%
ENV-139	RVA/Human-wt/IND/IDH-8860/2016/G3P[8] VP7 gene	MF563919.1	0.0	99.17%
ENV-182	RVA/Human-wt/IND/BCH589/G3P[8] VP7 gene	MF563883.1	0.0	90.74%

The top-performing strains of our isolates for VP7 genotyping, together with their corresponding accession numbers and maximal identity percentages, are shown in the table below. Five isolates exhibited similarities to Rotavirus A strains of the G1 and G3 types. The three ENV strains that were most similar to the Bangladeshi Human Rotavirus strain were ENV-066; ENV-090; and ENV-109. The Indian Human Rotavirus strain was most closely related to ENV-139 and ENV-182, respectively. Using Bio-edite's ClustalW sequence alignment software, all sequences were aligned with all other sequences, and Mega version 5.05 was used to build each sequence's corresponding phylogenic tree.

4.4 Distribution of Genotypes:

A total of 29 rotavirus strains were genotyped on the basis of VP7 gene sequences. G3 was the most prominent type among these rotavirus strains of wastewater. G1 and G2 were also found in this study.

Table 4.3: G-types of rotavirus strains found in the study.

#SL	Isolate ID	G type	Area
1	ENV-066	G1	Dhaka
2	ENV-071	G2	Cox's Bazar
3	ENV-081	G1	Dhaka
4	ENV-089	G3	Cox's Bazar
5	ENV-090	G3	Cox's Bazar
6	ENV-097	G2	Cox's Bazar
7	ENV-098	G3	Dhaka
8	ENV-099	G1	Dhaka
9	ENV-104	G3	Dhaka
10	ENV-109	G1	Dhaka
11	ENV-123	G1	Dhaka
12	ENV-128	G2	Dhaka
13	ENV-129	G3	Cox's Bazar
14	ENV-134	G3	Cox's Bazar
15	ENV-139	G3	Cox's Bazar
16	ENV-145	G2	Dhaka
17	ENV-152	G3	Cox's Bazar
18	ENV-157	G2	Cox's Bazar
19	ENV-162	G1	Dhaka
20	ENV-167	G1	Dhaka
21	ENV-172	G3	Cox's Bazar
22	ENV-177	G1	Cox's Bazar
23	ENV-182	G3	Cox's Bazar
24	ENV-184	G2	Dhaka
25	ENV-189	G1	Dhaka
26	ENV-197	G3	Cox's Bazar
27	ENV-201	G3	Cox's Bazar
28	ENV-206	G2	Dhaka
29	ENV-211	G3	Dhaka

A total of 29 rotavirus strains were genotyped on the basis of VP4 gene sequences. P8 was the most prominent type among these rotavirus strains of wastewater. Only one P4 was found. No other types were found, and others were bad sequences.

Table 4.4: P-types of rotaviruses strains found in the study.

Serial No.	Isolate ID	P type	Area
1	ENV-081	P8	Dhaka
2	ENV-089	P8	Cox's Bazar
3	ENV-090	P8	Cox's Bazar
4	ENV-097	P4	Cox's Bazar
5	ENV-098	P8	Dhaka
6	ENV-099	P8	Dhaka
7	ENV-104	P8	Dhaka
8	ENV-109	P8	Dhaka
9	ENV-139	P8	Cox's Bazar
10	ENV-145	P8	Dhaka
11	ENV-167	P8	Dhaka
12	ENV-172	P8	Cox's Bazar
13	ENV-184	P8	Dhaka
14	ENV-211	P8	Dhaka

Single genotypic pie-chart based on location:

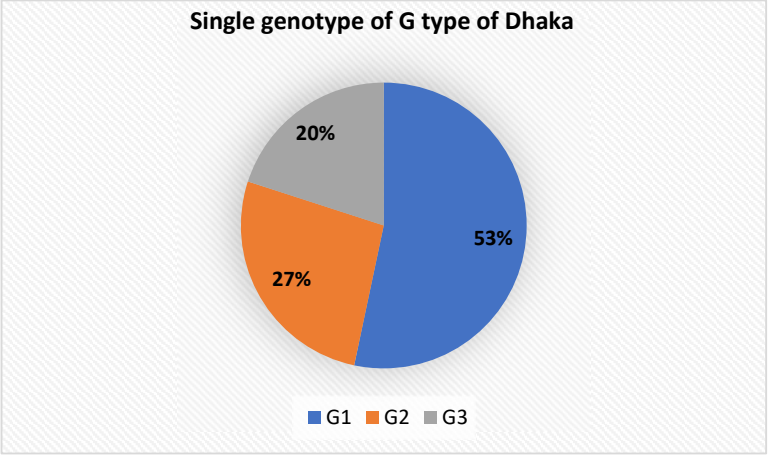


Figure 4.8: The persentage of single genotype from samples collected of Dhaka

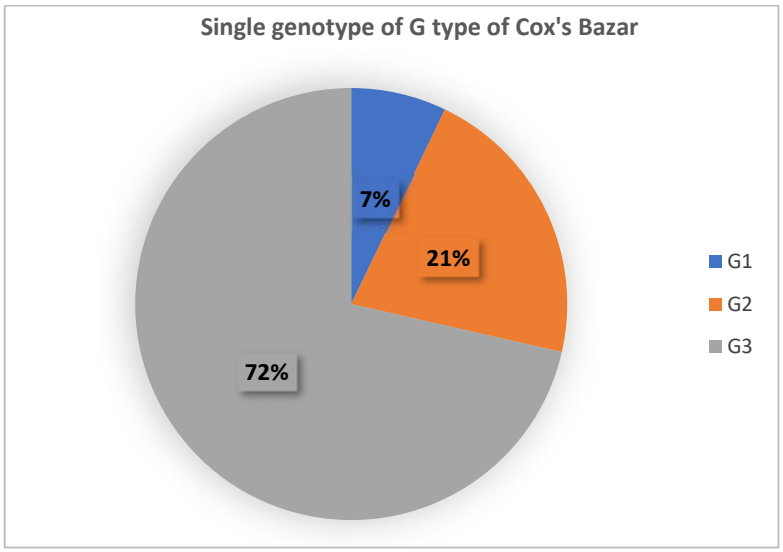


Figure 4.9: The persentage of single genotype from samples of Cox's Bazar

Comparison among different merged genotypes based on location:

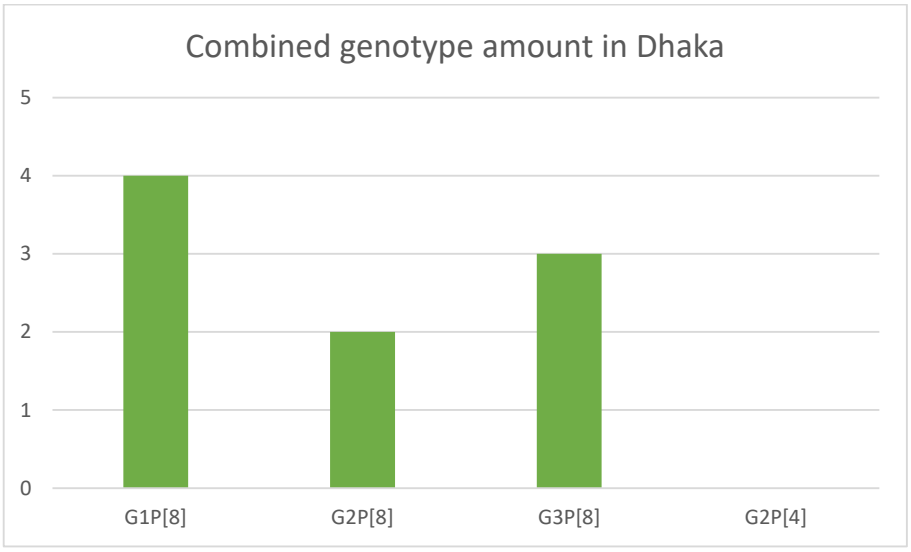


Figure 4.10: The bar chart of combined genotypes found from the samples of Dhaka

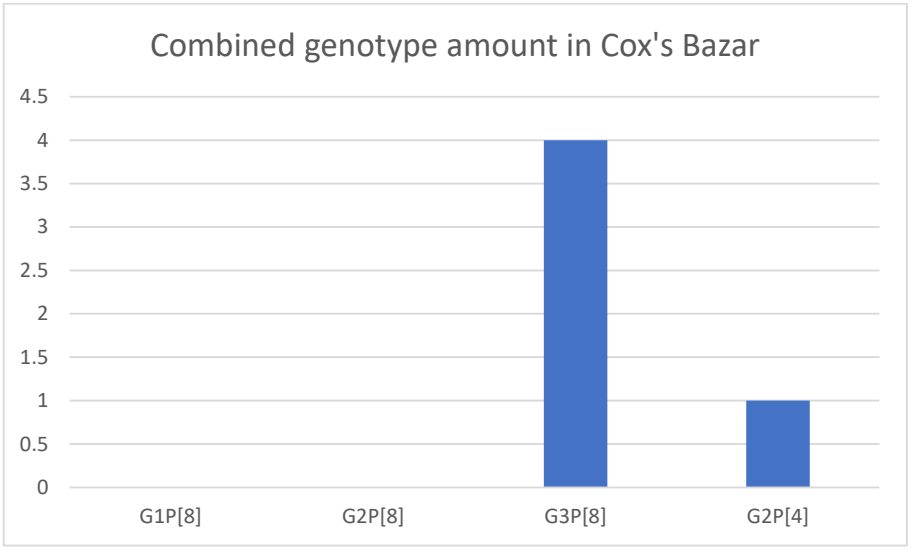


Figure 4.11: The bar chart of combined genotypes found from the samples of Cox's Bazar

4.5 Phylogenetic lineage analysis:

Phylogenetic lineage analysis is an important tool to find out the origin of local strains by clustering them in an array of global sequences. In this study to find out the origin of each rotavirus strain, more detailed phylogenetic analysis of VP7 and VP4 are performed, and two separate phylogenetic trees are constructed. These trees indicate the evolutionary relatedness of our experimental strains within themselves and also with the globally circulating rotavirus strains. The detailed phylogenetic lineage analysis is given below:

4.5.1 Phylogenic tree for VP7 genome segments:

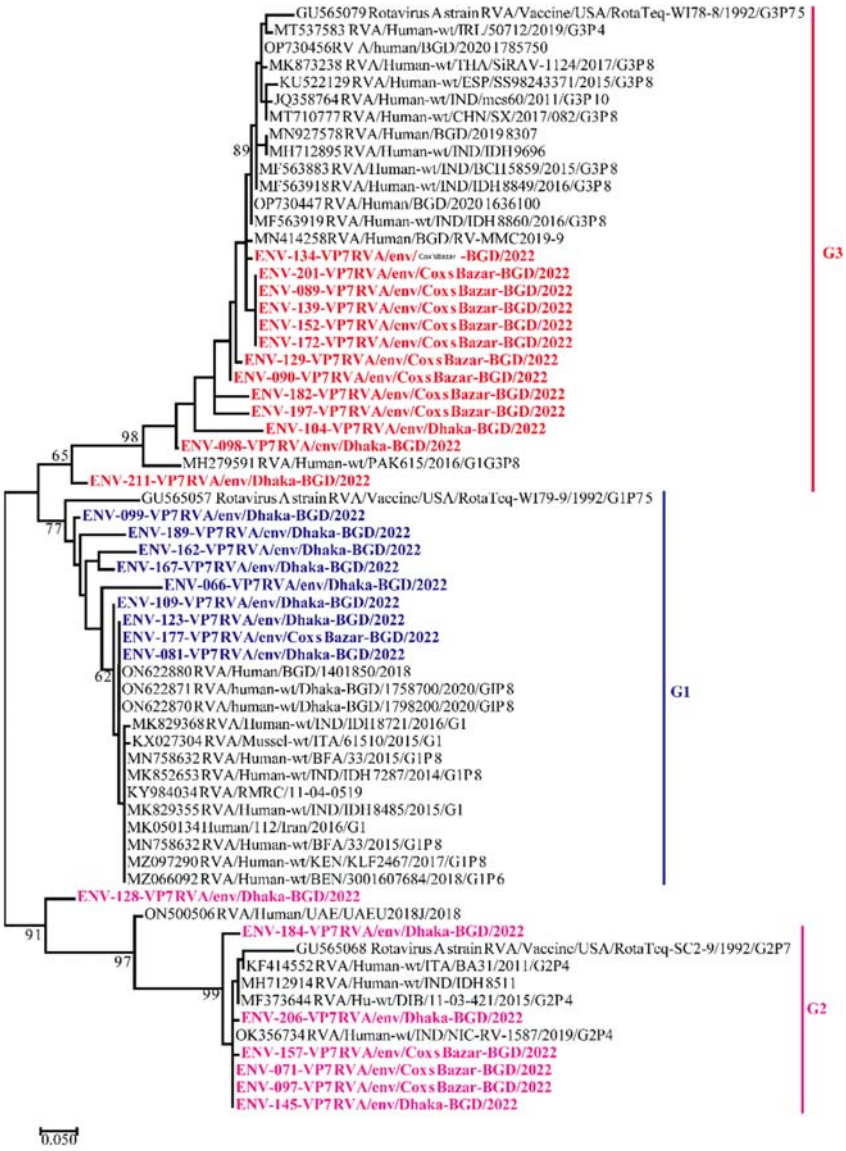


Figure 4.12: Phylogenetic tree of the VP7 genome segments of Dhaka and Cox's Bazar isolates rotavirus sequences. Phylogenetic tree based on nucleotide sequence of the G1-encoding gene for ENV-099, ENV-189, ENV-162, ENV-066, ENV-109, ENV-123, ENV-081 and ENV-177 (Blue marked), G2 encoding gene for ENV-128, ENV-184, ENV-206, ENV-157, ENV-071, ENV-097, and ENV-145 (Pink marked) and G3 encoding gene for ENV-134, ENV-201, ENV-089, ENV-139, ENV-152, ENV-172, ENV129, ENV090, and ENV-182.

4.5.2 Phylogenic tree for VP4 genome segments:

Figure 4.13: Phylogenetic tree of the VP4 genome segments of Dhaka and Cox's Bazar isolates rotavirus sequences. Phylogenetic tree based on nucleotide sequence of the P8-encoding gene for ENV-090, ENV-082, ENV-184, ENV-167, ENV-098, ENV-211, ENV-099, ENV-109, ENV-139, ENV-172, ENV-089, ENV-145, ENV-081, ENV-184, ENV-167, ENV-098, ENV-211, ENV-099, ENV-109, ENV-145, ENV-104 ENV-104. ENV-090, ENV-139, ENV-172, and ENV-089. (Pink marked) and P4-encoding gene for only ENV-097 (Blue marked).

Chapter-5: Discussion:

Rotavirus is a well-known waterborne virus responsible for numerous outbreaks of viral gastroenteritis globally. People who regularly use water sources are more susceptible to rotavirus infection. When that water is discharged as wastewater, traces of rotavirus can be detected therein. For that, various types of environmental samples, such as drainage systems, surface water, and septic tank effluent can be collected for rotavirus detection. This helps to estimate the level of rotavirus activity and identify the most prevalent serotypes in a region. Dhaka and Cox's Bazar being the major cities in Bangladesh, attract people from diverse backgrounds, increasing the chances of encountering different rotavirus genotypes in these areas.

The restricted gene family hypothesis put out by RNA hybridization, confirmed that the four G/P combinations accounted for more than 80% of all global strains: P[4] strains held G2 specificity, and P[8] strains held G1, G2, or G4 specificity (Gentsch *et al.*, 1996). But on the contrary, recent observations challenge earlier findings, revealing a more higher and complex diversity of rotaviruses in nature. These findings have expanded our understanding of how rotaviruses evolve, involving techniques like the introduction of animal viruses into the human population, chromosomal rearrangements, and sequential point mutations or changes.

Since 1978, icddr,b has been conducting a diarrhea surveillance program, later expanding to another rural area in 2010. Rotavirus was identified as the most common cause of infantile diarrhea in 1978 and continues to be a significant contributor to child mortality. Rotavirus-related deaths range from 5,700 to 13,400 annually, now accounting for 80% of diarrhea-related deaths in Bangladesh. Until the mid-1990s, over 90% of human rotavirus infections were caused by four main genotype combinations: G1P[8], G2P[8], G3, and G4P[8] (Matthijnssens et al., 2008b).

The predominant rotavirus strains identified include G1P[8], G2P[8], G2P[4], and G3P[8], along with isolated G1, G2, and P[8] strains in countries like India, Pakistan, and Bangladesh, while Mediterranean region countries exhibit the prevalence of frequent strains like G9P[4] and G4P[8]. Single G1, G2, and G3 strains as well as the dominant G1P[8], G2P[8, G2P[4], and G3P[8] strains were found in this investigation.

In this investigation/study/research work, rotavirus strains were characterized by genotyping VP7 and VP4 genes using molecular sequencing approach. Results showed G1 as the most

prevalent in Dhaka (53%) and G3 in Cox's Bazar (72%). Other G types in Dhaka included 27% G2 and 20% G3, while Cox's Bazar had 7% other G1 and 21% G2. So, it has been vividly manifested that although genotype G3 could ensure its highest prevalence in Cox's Bazar, its prevalence in Dhaka is exorbitantly low. In case of combined genotypes, G1P[8] dominated in Dhaka, while Cox's Bazar had G3P[8], with the unique strain G2P[4]. This G2P[4] strain may be possibly linked due to the presence of Rohingya population in Cox's Bazar, as the wastewater was collected from Rohingya camps. Some limitations in sample conditions may also have affected strain detection.

For the phylogenetic analysis of genotype G3, we obtained the nucleotide sequences of related strains from GenBank and compared them to the sequences we had obtained from the wastewater samples from Dhaka and Cox's Bazar (ENV-134, ENV-201, ENV-089, ENV-139, ENV-152, ENV-172, ENV129, ENV090, and ENV-182). Phylogenetic analysis revealed that these strains, along with others from Bangladesh, India, China, Spain, Ireland, and Pakistan, all fell within the G3 genotype, which is also present in of the rotavirus vaccines, RotaTeq that carries the G3 sequence. The vaccination reference strain likewise clusters with them in the same cluster.

Similarly, for genotype G1, sequences retrieved from ENV-099, ENV-189, ENV-162, ENV-066, ENV-109, ENV-123, ENV-081, ENV-177 and sequences from Bangladesh, India, Italy, Burkina Faso, and Benin, were grouped together along with the RotaTeq vaccine, due to its G1 genotype sequence similarity.

However, for G2, sequences from Dhaka and Cox's Bazar (ENV-128, ENV-184, ENV-206, ENV-157, ENV-071, ENV-097, and ENV-145) did not match any from Bangladesh but formed a cluster with strains from the Middle East, Arab, Dubai, India, and Italy.

For Phylogenetic analysis of genotype P8, we obtained the nucleotide sequences of related strains from GenBank and compared them to our recovered sequences from ENV-090, ENV-139, ENV-172, and ENV-089 that were collected from Cox's Bazar, while ENV-081, ENV-184, ENV-167, ENV-098, ENV-211, ENV-099, ENV-109, ENV-145, and ENV-104 originated from Dhaka. These strains, along with P8 strains found in various locations such as Bangladesh, India, Dubai, Thailand, Ethiopia, China, and Kenya, collectively constitute the P8 subgroup.

Regarding P4, a single strain (ENV-097) was identified which was from Cox's Bazar. In contrast, sequences obtained from GenBank originated from Dubai and India, and all of them form the P4 cluster.

Moreover, the bootstrap values of the two phylogeny trees were near 100, which indicates that there was not much of mutational discrepancy among the reference sequences. The presence of vaccine strain also had higher bootstrap value, which implies that those vaccines have the potential to effectively work against them in the perspective of prevention.

Two renowned Rotavirus vaccines, RotaTeq and Rotarix, target different strains. RotaTeq covers G1, G2, G3, G4, and G9 strains, while Rotarix specifically targets G1P[8]. Rotarix successfully halted 87-91% of severe rotavirus diarrhea in a trial conducted in Latin America, which included both full homotypic G1P[8] strains and partially heterotypic G3P[8], G4P[8], and G9P[8] strains. Fully heterotypic G2P[4] strains were rather rare (Parashar, 2016). In older persons G2P[4] prevalence were increased in Austria when both vaccines were used (Bibera et al., 2020). Rotarix was first made available in Brazil in 2006. The prevalent Genotype was G1P[8], but in the ten years following the release of Rotarix, its prevalence sharply declined (Bibera et al., 2020). So, It can be inferred that if Rotarix can be be employed for immunizing the vulnerable people of Dhaka and Cox's Bazar, such decline in the prevalence of G1P[8] could be observed.

G2P[4] was the predominate genotype in the post-vaccination interval, reaching approximately 75% prevalence in 2016/2017, according to another observation conducted in the UK when Rotarix was used to prevent rotavirus. G2P[4] prevalence in studied strains fell to 14.9% in 2017–2018, while G3P[8] and G9P[4] were more common. We can therefore conclude that G2P[4] remains non-prevalent during the G1P[8] dominance phase. However, after receiving the Rotarix vaccine, G1P[8] starts/started to decline. In countries with national rotavirus vaccination programs, there has been a decrease in overall rotavirus strain prevalence. This decrease has not been linked to changes in strain distribution or the emergence of a dominant genotype, according to a 2017 report from the European Centre for Disease Prevention and Control. Older age groups tend to have more G2P[4] genotypes, potentially acting as a source of infection for younger individuals who are ineligible for vaccination. persistent rotavirus activity in older children and the elderly five to six years following rotavirus vaccination for all.

Given the genetic diversity and dynamic evolution of RV strains, which can be shed in high amounts by infected individuals, these vaccines face challenges as they consist of fixed genetic components. If the vaccination strains can produce immunity against the broad range of changing rotaviruses to prevent the emergence of new vaccine-resistant strains, that would be

a significant molecular achievement. To determine the necessity for reformulation of vaccines, it's crucial to assess vaccine effectiveness against these strains and evaluate how large-scale vaccination programs affect their prevalence and epidemiological characteristics.

Chapter-6: Conclusion:

In conclusion, this study demonstrates the prevalence of prominent and distinct rotavirus strains in Dhaka and Cox's Bazar. It also emphasizes the rapid rotavirus transmission throughout the localities along with varying frequencies of different genotypes. However, relevant studies in a broader canvas are prerequisite for precise characterization of the dominant circulating strains in this geographical region which can support targeted vaccine development. Besides, molecular characterization and phylogenetic evaluation of regional sequences can hold promising potential for clarifying their association with globally circulating strains. We hope this study will help the policy makers to take on effective wastewater monitoring programs to enable the early identification and evaluation of concerning RV strains. As a consequence, it can be expected that improvised medical preparedness and response strategies would be able to lessen the likelihood and impact of rotavirus-related outbreaks.

Reference:

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