Molecular characterization of Human Norovirus strains circulating among children with acute Gastroenteritis in KPK, Pakistan

A thesis submitted in partial fulfilment of the requirement for the degree of Masters of Science in Healthcare Biotechnology

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2024

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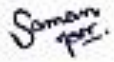

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This thesis is dedicated to

My Parents,

For their unwavering support, love, and constant belief in me

My Siblings,

For being the pillars of strength and encouragement throughout this journey

And Myself,

For the countless hours of hard work, dedication, and unyielding commitment

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List of Abbreviations

AGE	Acute Gastroenteritis
NoV	Norovirus
RV	Rotavirus
AstV	Human astrovirus
SV	Sapovirus
NS1	Non Structural Protein 1
NV	Norovirus
VP	Viral Protein
ORF	Open Reading frame
GGI	Genogroup I
DALYs	Disability-Adjusted Life Years
ASDR	Age-Standardized Death Rate
CDC	Centers for Disease Control and Prevention
RdRp	RNA-dependent RNA polymerase
KTH	Kyber Teaching Hospital
STH	Saidu Teaching Hospital
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
AVL	Viral Lysis Buffer

AW Wash Buffer

cDNA Complementary DNA

EIA Enzyme Immuno Assay

ELISA Enzyme Linked Immuno Sorbent Assay

MEGA Molecular Evolutionary Genetic Analysis

NIH National Institute of Health

WHO World Health Organization

ORS Oral Rehydration Solution

PBS Phosphate buffered saline

PCR Polymerase Chain Reaction

RT-PCR Reverse-Transcriptase Polymerase Chain Reaction

Taq Thermus aquaticus

µl Micro liter

µM Micro molar

UV Ultraviolet

ABSTRACT

Gastroenteritis is a significant public health concern worldwide, causing over 70 million fatalities each year and accounting for 10% of juvenile mortality, making it the second leading cause of death globally. Norovirus infections are common in low-resource countries and have a substantial impact on severe childhood gastroenteritis, leading towards 1.1 million cases of hospitalizations and 218,000 fatalities among children under 5 annually. No data is available on the prevalence and genetic diversity of NoV in KPK region of Pakistan, where NoV can be the possible cause of having high death rate among pediatric population. A total of 803 fecal samples collected from children under the age of 5 years admitted at Saidu Teaching Hospital, Swat (STH) and Khyber Teaching Hospital (KTH) were screened for NoV on the basis of VP1 gene by using one-step conventional RT PCR. The amplified products were separated using Gel Electrophoresis and the positive samples were cycle sequenced. Sequencher was used to edit the raw data obtained from genetic analyzer and FASTA sequences were retrieved which were then analyzed using BLAST followed by construction of phylogenetic tree on MEGA software. This research demonstrated that noroviruses have a substantial role in the episodes of childhood diarrhea, with a 15.69% positivity rate (n=126) among the samples collected from KPK region. Our study revealed that GGII strains were more prevalent with 57.94% (n=73) positive cases as compared to GGI with 42.06% (n=53) positive samples. Among genogroup I, GGI.3 was found to be the most prevalent genotype while GGII.2 was the most prevalent genotype among genogroup II. Gender based analysis demonstrated that NoV infections were more common in females as compared to males. The largest percentage of NoV was found among children less than 2 years. The study's results provide optimism for shaping public health policies to reduce the effects of norovirus-induced gastroenteritis, despite obstacles in resource-constrained regions worldwide.

INTRODUCTION

Gastroenteritis poses a significant public health threat with an estimated 10% pediatric mortality rate, equating to 70 million deaths annually worldwide, thereby ranking it as the second most prominent cause of mortality on a worldwide scale [1] [2][3]. In low resource nations, norovirus infections rank as the second leading cause of severe gastroenteritis among children. These infections result in approximately 1.1 million cases of hospitalizations and 218,000 deaths annually among children under the age of 5 [4]. Human noroviruses (hNoVs), belong to the Caliciviridae family and are categorized under the Norovirus genus, cause both sporadic instances and outbreaks of acute gastroenteritis (AGE) [5]. Currently, noroviruses are categorized into two recently proposed genogroups and ten established genogroups (GI-GX) based on the phylogenetic clustering of complete VP1 amino acid sequences. The genogroups of NoV exhibit diversity with the capsid gene's amino acid sequence varies up to 60% [6]

Norovirus infection has been found to be linked to a range of disorders, such as necrotizing enterocolitis in neonates, recurrent diarrhea in those with compromised immune systems, and postinfectious irritable bowel syndrome.

Like many water-borne infections the spread of norovirus is deeply correlated with poor sanitation and socioeconomic deprivation mainly due to the fecal-oral route of transmission. The disease can also be transmitted via direct person-to-person contact. While the Infection is typically self-resolving, causing acute gastroenteritis with symptoms such as nausea, vomiting, diarrhea, and abdominal cramps. Most cases resolve within a few days without specific medical treatment. However, certain groups are at a higher risk of experiencing more severe outcomes and complications. These high-risk groups include young children, the elderly, and individuals with weakened immune systems. In these populations, norovirus infection may lead to more prolonged and severe symptoms, potentially resulting in dehydration and requiring medical

attention. Risk factors increasing the susceptibility encompass deographics, environment, and seasonality, with minors facing a higher risk. Although treatment options like rehydration (orally or intravenously), appropriate diet choices, zinc supplements, antibiotics may be used for treatment in the short-term but long-term solutions include promoting hygiene practices, ensuring access to clean water, and proper toilet systems to decrease fecal contamination and education of mothers about early identification of acute diarrhea so that high risk groups can be saved form severe outcomes of the disease [7]. Since 2016, the World Health Organization (WHO) has recognized the pressing need for a norovirus vaccine. However, developing an effective vaccine has been challenging due to several factors.

Pakistan, with a population of around 240 million, faces challenges with limited access to healthcare services, resulting in child mortality rate of 87 per 1,000 live births and an estimated 465,000 deaths per year among children under the age of five. Diarrhea-related mortality, especially in combination with pneumonia, is notably high, causing approximately 74,209 deaths.

Despite the significant impact of norovirus in Pakistan, there is a lack of data on its prevalence and genetic diversity. Limited studies have focused on the demographic aspects of NoV in hospitalized children with AGE. This study addresses the immediate need for a comprehensive understanding of NoV in the KPK pediatric population and contributes significantly to broader norovirus research.

By offering insights into genetic evolution, transmission dynamics, and potential intervention strategies, the study provides valuable information. Despite global challenges in addressing infectious diseases, particularly in resource-limited areas, the findings offer hope for guiding public health policies to mitigate the impact of NoV-related gastroenteritis.

LITERATURE REVIEW

2.1 Gastroenteritis

Gastroenteritis poses a significant public health threat with an estimated 10% pediatric mortality rate, equating to an estimated 70 million deaths annually worldwide, thereby ranking it as the second leading cause of death globally [1] [2] [3] . This disease is correlated with poor sanitation and socioeconomic deprivation. Gastroenteritis is transmitted by fecal-oral route, consumption of contaminated food or water, and direct person-to-person contact. This is the primary route for obtaining this infection, rendering it a major cause for outbreaks caused by water-borne pathogens.

The years between 1990 to 2010 marked a significant 51**%** decrease in disability-adjusted life years (DALYs) caused by diarrheal diseases, and a 49% decrease in age-standardized death rate (ASDR). However, despite these improvements, diarrheal diseases still rank as the **fourth** most significant contributor of DALYs and the seventh most common cause of mortality worldwide. Consequently, there are an estimated 1.45 million fatalities per year [8]. Earlier research indicated that diarrheal infections ranked as the second most common cause of death among children aged 3 to 5. They accounted for 78% of the total mortality rate caused by diarrhea in children residing in the countries of Africa, Eastern Mediterranean and Southeast Asia.

Viruses are the predominant causative agents of acute gastroenteritis (AGE), responsible for over 60% of cases in children, with bacteria and parasites accounting for 8-10%. **Rotavirus** and **noroviruses** are the predominant viral agents responsible for over 58% of all instances of diarrhea in the United States. Following them are enteric Adenoviruses, Astroviruses, and Sapovirus [9].

Norovirus has surpassed rotavirus as the leading cause of AGE in children in countries with developed economies who have established a rotavirus vaccine program. According to the U.S. Centers for Disease Control and Prevention (CDC), over 60% of acute gastroenteritis cases in the United States are attributed to norovirus on an annual basis. [10].

2.2 Epidemiology of NoV in Pakistan

Pakistan is a highly populated nation that has inadequate availability of effective healthcare services. Based on estimates from the WHO, Pakistan's population is around 240 million. NoV infections are the second largest cause of severe pediatric gastroenteritis in low-income countries, causing 1.1 million hospitalizations and 218,000 deaths in children under age 5. [11] The death rate attributed to diarrhea is significantly high, with an estimated number of approximately 74,209 deaths, especially when it occurs in combination with pneumonia.

Pakistan currently has a dearth of data pertaining to the prevalence and genetic diversity of NoV. To date, there is a scarcity of research that details the demographic characteristics of norovirus in pediatric patients admitted to hospitals with severe gastroenteritis in Pakistan. In 2004, a research group conducted a hospital-based study to investigate the genetic basis of noroviruses. The study aimed to gather epidemiological data on human astrovirus (AstV), norovirus (NoV), and sapovirus (SV). Out of the 517 samples tested, 51 tested positive for NoV. The analysis of NoV GI sequences revealed that they exhibited a clustering pattern, indicating the presence of three unique GI genotypes. The majority of the NoV sequences (66.7%) were found to be associated with NoV GI genotype 7 (NoV GI/7), which is characterized by the Chiba virus cluster. The NOR89JB viral cluster, consisting of three NoV sequences, accounted for 25% of the total. One NoV sequence, accounting for 8.3% of the total, was categorized as NoV GI/6, which is commonly referred to as the Musgrove virus cluster. The classification of all 39 NoV GII sequences indicated the presence of five unique

GII genotypes. A total of sixteen NoV sequences, accounting for 41.0% of the samples, exhibited similarity to genotype 1, commonly referred to as the Lordsdale virus.

No further data was made available after this study till 2015 where NoV was tested in four largest cities (Karachi, Rawalpindi, Lahore, and Peshawar) in Pakistan where 16.1% samples were reported positive for NoV.

The results of the sequence analysis revealed the presence of 14 distinct NoV genotypes, consisting of four genotypes associated with gastrointestinal (GI) infections (GI.3, GI.5, GI.7, GI.8) and ten genotypes associated with gastrointestinal infections (GII.2, GII.3, GII.4, GII.5, GII.6, GII.7, GII.9, GII.13, GII.16, and GII.21). The GI.7 and GII.4 genotypes were the most common, accounting for 12.2% of the infections. Prior studies have shown that hospitalized children with AGE in Pakistan exhibit the presence of many norovirus genotypes, and there is no obvious preponderance of GII.4 viruses [11].

Despite this knowledge the genetic profile of NoV form other regions remains missing especially the KPK region of Pakistan which is known for its distinct cultural, environmental, and socioeconomic factors, offers an interesting context for studying NoV.

The objective of this study is to address significant knowledge gaps regarding the epidemiology, genetic variation, and clinical significance of NoV infections among children in KPK. Through the utilization of advanced molecular approaches such as polymerase chain reaction (PCR) and sequencing, our goal is to identify the accurate genotypes and variations of NoV that are widespread in this community. This will provide insights into the molecular attributes that might impact the seriousness of gastroenteritis.

This study not only fulfills the immediate requirement for a more detailed comprehension of NoV in the KPK pediatric population but also makes a valuable contribution to the wider field of norovirus research. It provides valuable insights into the genetic evolution, transmission

dynamics, and potential strategies for focused interventions. Despite the difficulties faced by the worldwide health community in dealing with infectious diseases, especially in areas with little resources, the results of this study offer hope for guiding public health policies to reduce the effects of NoV-related gastroenteritis.

2.3Human NoroVirus (hNoV)

Noroviruses were formerly referred to as Norwalk or Norwalk-like viruses. Their name was derived from the first Norwalk strain, which precipitated an epidemic of gastroenteritis in a school located in Norwalk, Ohio in 1968 [12].Noroviruses are characterized by their small, round shape, ranging 28–35 nm in size.

2.3.1 Classification

Human noroviruses (hNoVs), belong to the Caliciviridae family and are categorized under the Norovirus genus, cause both sporadic instances and outbreaks of acute gastroenteritis (AGE). At present, noroviruses are classified into two newly proposed genogroups and ten established genogroups (GI-GX) according to the phylogenetic clustering of complete VP1 amino acid sequences. Human infection is recognized for viruses classified under the GI, GII, GIV, GVIII, and GIX (previously GII.15) families. Genotypes comprise additional subdivisions within each genogroup: nine in GI, twenty-six in GII, two in GIV, one in GVIII, and one in GIX. Furthermore, a minimum of fourteen GI polymerase (GI. P) types and thirty-seven GII.P types have been identified within GI and GII via the clustering of nucleotide sequences in the polymerase region.

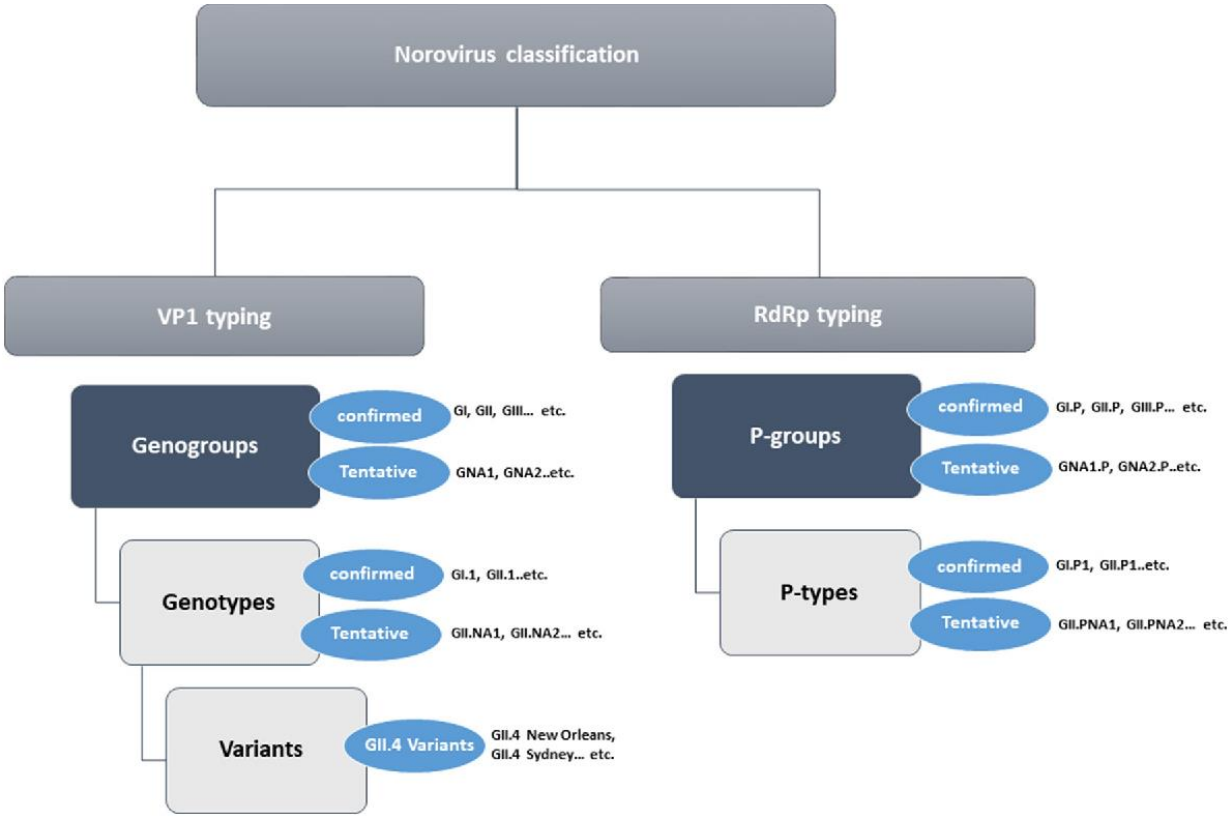


Figure 1.1: Classification of Norovirus

NoV classification into genogroups, genotypes, variants, P-groups, and P-types. Currently, the representation of tentative genogroups, genotypes, P-groups, and P-types is limited to a single sequence or several non-identical sequences originating from a single geographic area. Consequently, these sequences are referred to as non-assigned (NA). [13]

2.3.2 Structure and Genome of NoV

The Human norovirus (NoV) is a single-stranded positive sense RNA virus with a genome length ranging from 7.5 to 7.7 kilobases. It is connected to a non-structural protein at its 5' end known as viral protein genome-linked (VPg). Furthermore, it exhibits polyadenylation at the 3' terminus. There are three open reading frames (ORF) in the NoV genome.

- The **ORF1** gene produces a polyprotein weighing 200 kDa , consisting of six Non-Structural proteins. The NS proteins, namely p48, VPg, p22, nucleoside-triphosphatase (NTPase), protease, and the RNA-dependent RNA polymerase (RdRp), are arranged in sequential order starting from the 5′ end (26–28).

- The **ORF2** is responsible for encoding the capsid's primary structural protein, known as VP1.
- The ORF3 is responsible for encoding the secondary structural protein of the capsid, known as VP2 [14].

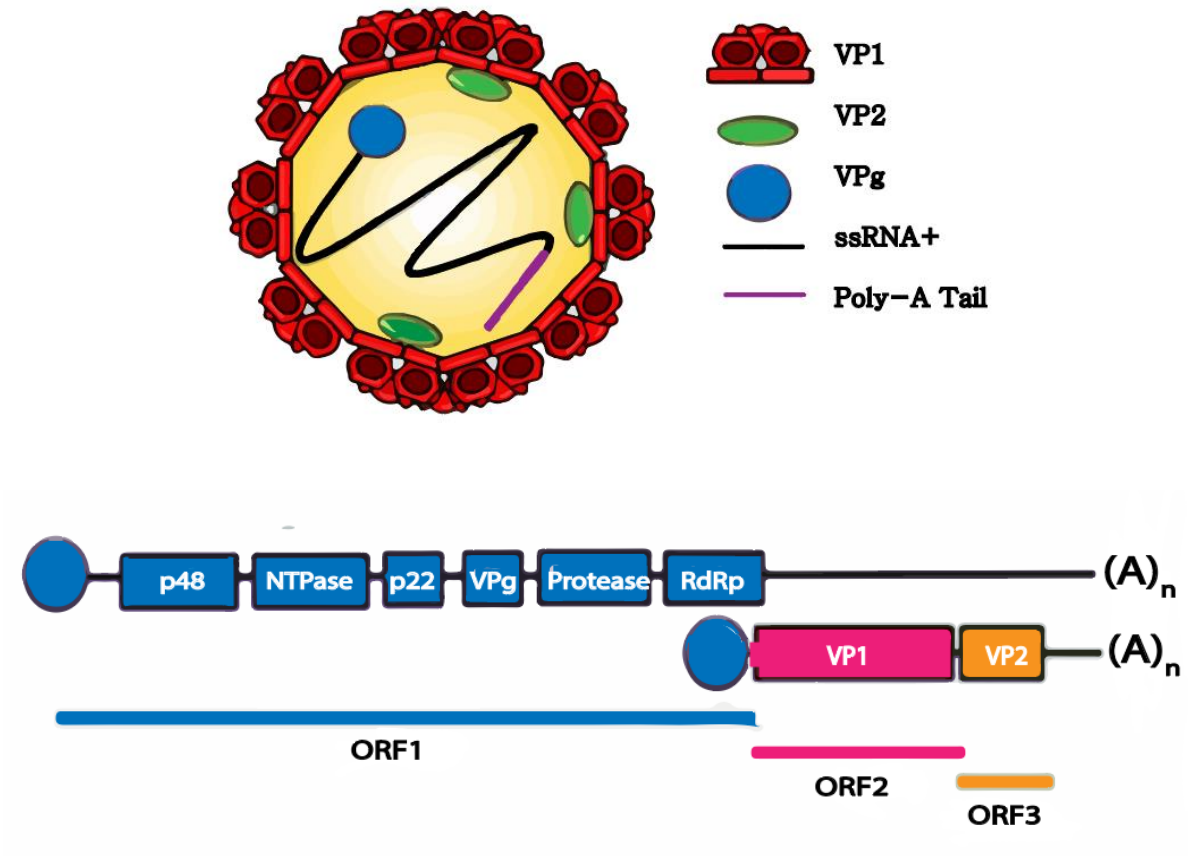


Figure 1.2: The molecular composition of human norovirus (NoV)

Illustration depicting the 90-dimer protein surface of the primary structural protein VP1 in the capsid of the NoV virion. VP2 is present within the viral capsid, with a range of 1 to 8 per virion. The RNA genome exhibits NS VPg protein covalently attached to the 5′ end and exhibits a poly-adenine tail at its 3′ end (ssRNA+). The ORF1 gene is responsible for encoding a set of non-structural proteins, namely p48, NTPase, p22, VPg, and RdRp. ORF2 encodes the major structural protein VP1, while ORF3 encodes the minor structural protein VP2. The subgenomic RNA associated with VPg, is displayed beneath the ORFs, which encodes VP1 and VP2. VPg refers to a circular structure that is linked to both genomic and subgenomic RNAs.[15]

Table 1.1: The established functions of NoV structural and non-structural proteins

Proteins	**Function**
NS 1 and NS 2	• Regulate the function of the viral RdRp. • Modify various immunological systems. • Act as a stimulant of cellular secretory pathways.
NS 3	• Identical function as helicase • Hydrolysis and assembly of NTPs. • It facilitates the in vitro RNA synthesis by the RdRp.
NS 4	• It imitates a signal that is used to export COPII-coated vesicles from the RER. • Inhibits the vesicle's fusion with the Golgi Apparatus. • Regulates disintegration of the Golgi Apparatus. • The Golgi Apparatus-dependent secretion of proteins exhibits antagonistic action.
NS 5	• Coordinate with initiation factors • Regulate the production of viral proteins.
NS 6	• Involved in the processing the viral polyprotein.
NS7	• Responsible for the translation of viral RNA.
VP 1	• Structural protein which is responsible for the viral binding to HBGA. • Involved in Immune escape.
VP 2	• Responsible for viral RNA translation. • Structural protein which is involved in the stability of the viral capsid.

2.3.3 Clinical Features

NoV induces AGE in individuals of all age groups. The disease usually starts after a period of 12-48 hours of incubation and is marked by sudden onset of diarrhea without blood, vomiting, sickness, and abdominal pain. Certain individuals may exclusively encounter symptoms of vomiting or diarrhea. The presence of mild fever and fatigue can also be indicative of an infection, leading to the common usage of the term "**stomach flu**" to characterize the condition. However, it is important to note that there is no biological connection to influenza. While symptoms might be intense, they usually improve on their own within 1 to 3 days in individuals who are otherwise in good health.

Nevertheless, it is possible for individuals, especially younger children, elderly individuals, and hospitalized patients, to experience longer durations of illness ranging between 4 to 6 days.

Norovirus infection has been reported to be associated with **necrotizing enterocolitis** in newborns, persistent diarrhea in individuals with compromised immune systems, and postinfectious irritable bowel syndrome. However, further data from analytic studies is necessary to establish a definitive causal relationship between norovirus infection and these conditions.

Norovirus is mostly excreted via fecal matter, however it may also be detected in the vomit of infected individuals. However, it is uncertain if only the existence of the virus suggests a potential for transmission. The presence of the virus in feces may be identified for an average duration of 4 weeks after infection. However, the highest amount of viral shedding takes place over a span of 2-5 days after infection, resulting in a viral load of around 100 billion viral copies per gram of feces. However, the lack of a cell culture method or small animal model for human norovirus, it remains uncertain if these viruses can cause infection. Consequently, the duration

of contagiousness in an infected individual following illness is also unknown. Moreover, it is noteworthy that approximately 30% of norovirus infections do not show any symptoms. However, even in these cases, individuals might still shed the virus, but in smaller quantities compared to those who develop symptoms. The precise impact of asymptomatic infection on the spread and occurrences of norovirus outbreaks is yet unknown.

2.3.4 Host Susceptibility Factors to NoV

Risk factors for gastroenteritis include **demographics**, **environment**, and **seasonality**; minors are at a greater risk. Susceptibility is also seen to be increased by measles, immunodeficiencies, and malnutrition (e.g., vitamin A deficiency). Acute diarrhea is prevalent, whereas persistent diarrhea is observed in 3–19% of cases, accounting for 50% of all fatalities. Entry of NoV requires binding and recognition of **HBGAs**, which are various carbohydrates found on cell membranes. VP1's P domain is comprised of the P2 subdomain, which is essential and varies between norovirus genogroups. Studies of susceptibility indicate that non-secretors devoid of FUT2 gene function exhibit resistance. Individuals who are secretor-positive are at an increased risk of contracting NoV infections. Clinical research involving NoV-infected volunteers establishes a connection between NoV strains and particular HBGAs via binding patterns that vary according to ABO, secretor, and Lewis blood types. NoVs that possess Lewis patterns exclusively bound to Lewis’s antigens and/or H antigen, whereas those that possess A/B binding sequences detect both A/B and H antigens. Cluster formation is induced by comparable binding patterns, which demonstrates the importance of HBGA binding patterns in the evolution of NoV [15].

2.3.5 Treatment/Management

In general, the treatment and care of children with AGE consist of **rehydration** (either orally or intravenously), choosing an appropriate diet, providing zinc supplements, and implementing additional therapies such as probiotics.

Intravenous (IV) rehydration is necessary for infants under 6 months of age, individuals with chronic diseases, premature newborns, those with a fever above 38°C (100.4°F) if they are below the age of 3 months, or between the ages of 3 months and 36 months if the patient's temperature exceeds 39°C (102.2°F). Indications such as persistent vomiting, bloody bowel movements, changes in mental state (consciousness), sunken eyes, and reduced urine production are further warning signs that may prompt the clinician to administer intravenous fluids to the patient.

Antibiotics (Ciprofloxacin, TMP-SMX, erythromycin, and metronidazole) are prescribed under specific circumstances. They have the potential to reduce the severity and duration, and even avoid some consequences such as the spread of infection. are often prescribed antibiotics. Nevertheless, it is advisable to refrain from using antibiotics in cases when the responsible pathogens have been identified as Bacillus cereus, Clostridium, and certain Salmonella species.

The efficacy of **probiotics** in aiding recovery or treating symptoms has not been clearly established through scientific research; investigations have led to inconclusive results. Probiotics are thought to have the potential to prevent diarrhea when the sickness is caused by C. difficile. Antispasmodic medications such as loperamide should not be used in children with signs of dysentery [9].

2.3.6 Prevention

For preventive measures, implementing hygiene practices such as ensuring access to clean water and proper toilet systems can effectively decrease fecal contamination. Additionally,

immunization, especially against RV, significantly reduces both illness and death rates. It is crucial, especially in poor countries, to develop and implement efficient methods of educating mothers about the early identification of acute diarrhea as a potentially hazardous illness and the appropriate actions to take [16].

2.3.7 Vaccines

Since 2016, the World Health Organization (WHO) has acknowledged the urgent need to produce a vaccine for norovirus. Nevertheless, it is quite challenging to develop a widely efficient vaccine, mostly due to a few significant factors.

- NoV exhibits genetic and antigenic diversity, encompassing 5 genogroups (GI, GII, GIV, GVIII, and GIX) that collectively include 35 genotypes.

- Simultaneous detection of several variants of different genotypes is common.

- Even within a single genotype, like the most common GII.4, new variations appear often due to fast evolution.

- Noroviruses have limited ability to replicate in cultured cells, rendering the traditional methods of using live weakened or inactivated vaccines impractical. Therefore, the widely adopted approach for developing vaccines involves employing nonreplicating strategies based on recombinant protein utilizing virus-like particles (VLPs).

- Furthermore, the lack of a standardized **cell culture-based neutralization assay**, a reliable animal model, and dependable immunological correlates of NoV protective immunity provide further challenges in the development of vaccines. Regardless of these limitations,

progress has been made, and four potential vaccines have been reached the clinical trial phase [17].

Norovirus vaccine development primarily targets the main capsid protein, which also serves as the genetic region utilized for genotyping purposes. Hence, it is crucial to prioritize the verification of genotypes such as GII.3, GII.4, and other novel variants by employing advanced technologies for detection. The vaccine candidates must provide protection against a wide range of genotypes and possess the ability to easily adjust to newly developing genotypes. Childhood immunization plays a crucial role in reducing the prevalence of noroviruses, which are a major cause of diarrheal disorders in children and can lead to more severe sickness and even death. If immunization effectively inhibits transmission, it may also decrease illnesses across all age groups. Essentially, given the rapid rate at which noroviruses evolve, it is crucial to implement developing technologies in order to gather more extensive genetic diversity data of NoV from both ambient samples and clinical specimens [18].

2.3.8 Technologies for the Detection and Analysis of Noroviruses

2.3.8.1 Nested Polymerase Chain Reaction (PCR) with Sanger Sequencing

The genotyping of NoV is usually carried out using **nested PCR** to amplify the VP1 region of NoV in complementary DNA (cDNA) samples, utilizing genogroup specific primers to provide a template for Sanger sequencing. The primer pairs used in nested PCR are specifically designed to amplify VP1 sequence, which includes the N-terminal region and the shell region of the capsid. Sanger sequencing is used to analyze the resultant amplicon in order to determine the NoV genotype present in the samples. This approach is extensively utilized in clinical laboratories to track the evolution of norovirus due to its ability to offer sequencing information.

2.3.8.2 Reverse-Transcription Quantitative Polymerase Chain Reaction Assays (RT-qPCR)

The utilization of RT-qPCR tests is prevalent in the field of molecular diagnostics for the identification of norovirus in clinical samples. RT-qPCR presents numerous advantages, such as its capacity to detect norovirus across various types of samples, its notable sensitivity, and its proficiency in identifying genogroups. The RT-qPCR technique has the capability to identify norovirus in various sources such as feces, water, food, and environmental components. This allows for the evaluation of potential transmission patterns.

In addition, the infectious nature of norovirus necessitates a minimum quantity of viral particles ranging from 18 to 1000 copies for infection. RT-qPCR may detect and measure as few as ten copies of NoV, while also providing information on the number of viruses present.

Furthermore, the utilization of unique primer sets in RT-qPCR enables the identification of genogroups, making it a highly efficient technique for the detection of norovirus genogroups. Nevertheless, when comparing the nested PCR with Sanger sequencing technique to RT-qPCR, it becomes evident that the latter demonstrates a diminished resolution and is constrained to providing information solely pertaining to norovirus genogroups.

2.3.8.3 Enzyme Immunoassay (EIA)

The enzyme immunoassay (EIA) is commonly used in clinical practice as the predominant method for rapidly recognizing pathogens. These assays can be utilized in extensive clinical and epidemiological research. Antibodies are necessary for the detection of different norovirus genogroups and/or genotypes in EIAs. Therefore, EIAs rely on the utilization of either polyclonal or monoclonal antibodies to detect and track various virus-like particles (VLPs). Prior research has shown that the accuracy and precision of norovirus EIAs differ depending

on the diagnostic targets. Commercial enzyme immunoassays (EIAs) for norovirus are accessible for the analysis of small quantities of stool collected as samples. Nevertheless, the EIA exhibits a lesser level of sensitivity compared to other tests.

Consequently, it is only appropriate to employ the EIA as an additional test in conjunction with other tests, such as RT-qPCR, in order to enhance the effectiveness of detection.

2.4 Public Health perspective of NoV

Although NoV causes almost 20% of all cases of acute gastroenteritis, it has not garnered as much attention as other infectious organisms. The characterization of the global economic burden of norovirus is crucial for funding agencies, policy makers, public health officials, and manufacturers in order to determine the appropriate allocation of attention and resources towards the advancement of candidate vaccines aimed at preventing and controlling norovirus. The economic burden of NoV has the greatest impact among children aged less than 5 years, whereas older age groups in some places have the highest cost per sickness. The majority of these significant expenses arise from the decline in productivity caused by acute sickness. The economic impact of norovirus gastroenteritis is significant across low, medium, and high-income nations, indicating its significance as a worldwide economic issue.

NoV has garnered comparatively little media coverage and has been subject to fewer programmatic endeavors in comparison to other infectious viruses that impose a significant cost. As an example, there is a lack of comparable alternatives for NoV in initiatives that specifically target rotavirus, such as Global Rotavirus Laboratory Networks, Global Rotavirus Surveillance, and the ROTA Council. Consequently, norovirus may be allocated less financing and financial assistance. An analysis of research conducted in the United Kingdom indicates

that the allocation of funds and focus by policy makers towards norovirus is not commensurate with the extent of its impact on public health.

There are several potential factors contributing to the very limited focus on norovirus. Estimating the illness burden has proven to be difficult, mostly due to the limited availability of diagnostic tools with sufficient sensitivity. Furthermore, there are individuals who tend to link NoV largely with outbreaks occurring in cruise ships, which may be considered as having relatively low health significance, as well as in healthcare facilities [19]

However, it is important to note that out of the approximate 20 million episodes reported annually in the US, only a small proportion (less than 1%) are linked to documented outbreaks[20]

Furthermore, despite the common perception that NoV is only responsible for self-limiting, mild gastroenteritis that seldom necessitates medical intervention, nor does it result in serious illness or mortality, it is estimated that around 70,000 fatalities among children under the age of 5 are attributed to norovirus each year on a global scale [21]

Furthermore, it is worth noting that funding organizations and policy makers with a specific emphasis on low- and middle-income countries (LMICs) may assign a greater level of importance to norovirus in higher income nations, given that these locations are the primary sources of norovirus data [22]

Various methods are being developed to prevent and manage NoV, such as antiviral drugs, disinfectants, and particularly vaccines, it is crucial and essential to comprehend the worldwide economic impact of NoV [23]

Policy makers, funders, public health authorities, and product manufacturers require further economic data in order to ascertain the appropriate placement of NoV on their priority list and to decide the allocation of time, effort, and money. Furthermore, in the absence of further data about the global allocation of the economic burden, there may be insufficient evidence to determine the specific locations and individuals to focus efforts and allocate resources. Up to now, economic research on norovirus has assessed the consequences of various outbreaks in both hospital and community settings, as well as evaluated the cost-effectiveness of a hypothetical vaccine of norovirus. These studies have been conducted in high-income nations such as the United States, Switzerland, and Scotland [24], [25], [26], [27], [28] There has not been a comprehensive global evaluation of the financial impact of norovirus. Therefore, a computer simulation model was constructed in various countries and territories, both within the World Health Organization (WHO) region and on a worldwide scale to evaluate the economic impact of NoV.

2.5 Problem Statement

No data is available on the prevalence and genetic diversity of NoV in KPK region of Pakistan, where NoV can be the possible cause of having high death rate among pediatric population in Pakistan.

2.6 Objectives

The study was undertaken with the subsequent objectives:

- To analyze the prevalence of Norovirus among children <5 years of age with acute gastroenteritis in Khaybar Pakhtunkhwa (KPK).
- To explore the molecular epidemiology and genotype distribution of Human Noroviruses in KPK.

MATERIALS AND METHODS

3.1 Ethics Statement:

The study was approved by Institutional Review Board (IRB) of National University of Science and Technology, Islamabad. The current investigation aimed at the assessment of norovirus gastroenteritis in children from Pakistan was carried out by the endorsement of the concept and research strategy provided by the Internal Assessment Authority of the National Institute of Health, Islamabad, Pakistan. Verbal and written consent was obtained from all the participating individuals.

3.2 Study design:

A hospital-based cross-sectional study was undertaken to investigate the molecular diagnostic and epidemiological aspects of Noroviruses in relation to gastro-enteric illnesses in newborns in Pakistan. From January 2022 to December 2022, fecal samples were collected from two tertiary care institutions, specifically Saidu Teaching Hospital (STH) and Khyber Teaching Hospital (KTH), encompassing children under the age of 5 years.

3.2.1 Study Site

The current study was carried out in Khyber Pakhtunkhwa (KPK), situated in the northwestern region of Rawalpindi, at around 300 kilometers from Islamabad, the capital city of Pakistan. KPK is **fourth** in terms of urbanization and population among the provinces of the country, including an extensive area of 101,741 sq.km. The province has a population of about 30 million, resulting in a population density of over 295 people per square kilometer (295 persons/sq.Km). The region displays a diverse demographic landscape, characterized by a balance between rural and urban areas. Almost 53.2% of the population resides in urban centers, while the rest of the population is spread out in rural areas. This distribution contributes to the socio-economic and cultural diversity within the province.

Figure 3.1: Geographical location of Pakistan. Site of study is encircled here.

3.2.2 Study population:

Children **under the age of five years** who were admitted to the hospitals with acute gastroenteritis throughout January 2022 to December 2022 were included in the current study.

3.2.3 Sample collection:

Samples were collected from children admitted to the hospital with acute gastroenteritis as their primary diagnosis.

Case definition of gastroenteritis

According to the standards set forth by the World Health Organization (WHO), genuine instances of gastroenteritis are characterized by the occurrence of three or more loose or watery stools within a 24-hour timeframe. Nevertheless, this definition fails to encompass the regular excretion of formed or loose feces, as well as the "pasty" feces expelled by infants who are breastfed.

Inclusion criteria

All children aged less than 5 years, admitted to the hospital and having signs of acute gastroenteritis were included in the study. The primary symptoms to be determined were fever (above 38.5ºC or 101.5ºF) persisting for more than 3 days, abdominal discomfort, vomiting, and symptoms of dehydration such as sunken eyes, dry skin, and dry mouth.

Exclusion criteria

Children who were hospitalized for the treatment of diarrhea caused by other non-infectious etiologies such as heavy metal poisoning, stomach discomfort, intolerance to food or pancreatic diseases were excluded.

The research study excluded children over the age of 5 who had symptoms of bloody diarrhea and were suspected of having bacterial or parasite diseases. In addition, individuals with insufficient medical and demographic data were also not included.

3.2.4 Sample processing:

Specimens were obtained from study participants in screw-capped containers and promptly dispatched to the Department of Virology at NIH Islamabad. The processing of fecal samples involved the removal of debris and other impurities present in the stool. For this purpose, 10% weight/volume suspensions of stool samples were created by combining 1-2 grams of sample with 10ml of phosphate-buffered saline (PBS) containing magnesium and calcium chloride in 15ml airtight tubes. The mixture underwent thorough blending using a mechanical shaker (Vortex-Genei II) with the tubes securely sealed for 15 minutes. Following this, centrifugation was carried out at 1500g for 20 minutes at 4ºC. With caution, 1ml of the supernatant was then transferred to a sterilized 1.5ml cryogenic tube within a Class-II biosafety cabinet. These cryotubes were stored at -20ºC until required for further testing. The initial fecal sample tubes were sealed at the top with parafilm tape and stored in a freezer at -80ºC.

3.3 RNA Extraction

The 10% suspension of fecal matter was permitted to reach ambient temperature. The RNA extraction procedure was conducted using the QIAamp viral RNA minikit following the manufacturer's instructions.

The kit is comprised of a lysis buffer known as AVL, an elution buffer and two wash buffers referred to as AW1 and AW2. In addition, the kit comprises minispin columns and 2ml collection tubes that are equipped with a Silica membrane that is charged. The sample was lysed using a lysis buffer, which also led to the inactivation of RNases under conditions of intense denaturation. In order to enhance the effective attachment of viral RNA to the silica membrane within the spin columns, carrier RNA was introduced into the AVL buffer. The experimental procedure involved the combination of 560 microliters of AVL buffer with 200 microliters of a 10% fecal suspension within a tube. The mixture was well blended using pulse vortexing for a duration of 15 seconds, followed by incubation at 30ºC for a period of 15 minutes. Following this, a volume of 560 microliters of pure ethanol (100%) was introduced into each tube, and subsequently subjected to vortexing for a duration of 15 seconds. A sterile 2ml collection tube was used to install the QIAamp small spin column, which was then filled with 630ul of the sample solution. The centrifugation procedure was conducted for a duration of 1 minute, at a rotational speed of 8000 revolutions per minute (rpm). The process of centrifugation led to the acquisition of a filtrate, which was then discarded. The column was subsequently placed using a new 2ml collecting tube. The identical methodology was replicated once more utilizing the remaining 630 microliters of the sample, and the resulting filtrate was once again discarded. The spin column underwent a washing process using 500ul of AW1 at a rotational speed of 8000rpm for a duration of 1 minute. Subsequently, a wash was performed using 500ul of AW2 at a rotational speed of 14000rpm for 1 minute. After removing the filtrate, the RNA elution process was conducted using 60 microliters of elution buffer. The tube was

maintained at ambient temperature for a duration of 1-2 minutes, after which the column was centrifuged at 8000rpm for a period of 1 minute. The RNA that was acquired was thereafter preserved at a temperature of -70ºC for additional investigation.

3.4 Conventional RT-PCR based identification and genotyping of Norovirus.

The Norovirus samples were genotyped using conventional reverse transcription polymerase chain reaction (RT-PCR) using the OneStep RT-PCR kit (QIAGEN, Valencia, CA) utilizing GI and GII oligonucletide primers. Genotyping was done by amplifying C region at the 5'end of VP1 gene [11]

Conventional one step Reverse transcriptase PCR was used to differentiate between genotypes of norovirus in positive samples. The primers GISKF, GISKR, GIISKF, and GIISKR were used to identify different genotypes of Norovirus. These primers target the capsid gene and amplify both GGI and GGII, along with their respective genotypes, by sequencing.

The amplification process was conducted using a thermocycler, namely the Applied Biosystems GeneAmp® ABI 9700 PCR system)

Table 3.1 : Primers used for screening and genotyping of NoV

Primers	Sequence (5'-3-)	Genotype	Target	Size (bp)
GISKF	CTGCCCGAATTYGTAAATGA	GI Norovirus	Capsid	330bp
GISKR	CCAACCCARCCATTRTACA			
SGIISKF	CNTGGGAGGGCGATCGCAA	GII Norovirus	Capsid	344bp
GIISKR	CCRCCNGCATRHCCRTTRTACAT			

To summarize, 5 µl of isolated RNA underwent reverse transcription into complementary DNA (cDNA) for a duration of 30 minutes at a temperature of 42°C. Subsequently, the reverse transcriptase was subjected to heat inactivation at 95°C for 15 minutes. Afterwards, the complementary DNA (cDNA) was amplified using 2 µM of each oligonucleotide primer in a final reaction volume of 50 µl. The PCR experiment consisted of 35 cycles, each conducted under specific thermocycling conditions. These conditions included an initial denaturation at 94°C for 30 seconds, followed by annealing at 50°C for 30 seconds, and an extension step at 72°C for 60 seconds. Next, a last extension phase was conducted at a temperature of 72°C for a duration of 10 minutes.

Table 2.2 The PCR reaction mixture

Reaction Components for QIAGEN One Step RT PCR Kit	
Kit Components	**Volume**
5X PCR Buffer	10
dNTPs (10mM)	2
Forward Primer (20uM)	2
Reverse Primer (20uM)	2
Enzyme Mix	2
RNase Inhibitor	0.25
Nuclease Free Water	26.75
Template	5
Total Volume	**50**

The thermal profile used is provided in the given table. The amplified Norovirus PCR products were separated on a 2% agarose gel containing 0.5ug/ml Ethidium bromide. The gel was then placed under a UV Trans illuminator, namely the Gel-Doc XR system, in order to see the

amplified DNA bands at a greater magnitude. The size of GGI and GGII products was determined by comparing them to a DNA marker. The typical band size for the Norovirus product was 330bp and 344bp for GI and GII respectively.

Table 3.3: Thermal profile used for the conventional PCR reaction

Thermal Profile for PCR		
Stage 1	Reverse Transcription	42°C for 30 min
	Initial PCR Activation	95°C for 15 min
Stage 2 (35 cycles)	Denaturation	94°C for 30 sec
	Annealing	50°C for 30 sec
	Extension	72°C for 1 min
Stage 3	Final Extension	72°C for 10 min
	Holding Temp	4°C for ∞

Purification of PCR products by AxyPrep Mag PCR Clean-up Assay

The PCR products were purified using the AxyPrep Mag PCR Clean-up kit (Qiagen, Part No. 28106) according to the instructions that were provided by the manufacturer. The AxyPrep Mag PCR Clean-up was added to the PCR product at a ratio of 1.8 times the volume of the PCR product. The mixture was then combined in a 1.5ml microcentrifuge tube. The mixture was mixed thoroughly 5 times using a pipette, followed by incubation at room temperature for 5 minutes to achieve maximum recovery. The test solution was placed onto a 96-well having v bottom microtiter plate. The reaction plate was positioned on a 96 well Magnet Plate for 3 minutes. The solution from the reaction plate was removed by aspiration and discarded. The

attached magnetic beads and PCR product were washed twice using 200uL of 70% ethanol for 30 seconds each time. The residual ethanol was evaporated using air drying at room temperature for 5 minutes. The reaction plate was detached from the magnetic plate, and 40 microliters of elution buffer was added and thoroughly mixed using a pipette 5 times. The reaction plate was placed on a 96-well magnetic plate for 1 minute in order to separate the beads from the solution. The eluate was transferred to a new tube for storage and subsequent analysis.

3.5 Cycle Sequencing Reaction

It is a type of PCR that generates single-stranded amplicons using one primer. Additionally, fluorescently tagged dNTPs (BigDye terminators) are utilized in PCR termination procedures to yield PCR fragments of varying sizes. An optical device in an automated DNA sequencing machine examines PCR fragments. To improve the data accuracy and sequence agreement, PCR amplicons were sequenced in both directions.

The ABI PRISM® Big Dye™ Terminator v3.1 was used for cycle sequencing, following instructions. The reaction mixture was made. Cycle sequencing was performed using a kit using 5-20ng of pure DNA and manufacturer guidelines. The 10ul PCR reaction contained 2ul of BigDye slayer reaction mix, 3.2pmol of primer, and deionized water. Tubes were set in a thermal cycler for 25 amplification cycles. Each cycle involved heating samples to 96ºC for 30 seconds, primer tempering for 15 seconds, and DNA strand extension at 60ºC for 4 minutes. The amplified products were purified before further study.

3.6 Big Dye Terminators Removal by AxyPrep MagTM Dye Clean step

This process eliminates the incorporated BigDye terminators, charged molecules, and salts from the cycle sequencing reaction product in order to enhance the quality and intensity of the sequence peaks. AxyPrep MagTM Dye Clean kit (Qiagen, Part No. 28106) was used to purify

the amplified cycle sequence products according to the manufacturer's instructions. Each sample was treated with 10 microliters of AxyPrep Mag DyeClean and 41 microliters of 85% ethanol. The mixture was thoroughly mixed by pipette mixing 7 times and then kept at room temperature for 5 minutes. The solution was placed onto a 96-well V-bottom microtiter plate. The reaction plate was then placed on the Magnetic Plate for 3 minutes and the cleared solution from the reaction plate was aspirated and discarded. The attached magnetic beads and PCR product were washed twice using 100 uL of 85% ethanol for 30 seconds each time. The supernatant was discarded without displacing the beads. The ethanol was fully evaporated by air drying for 10 minutes at room temperature. The reaction plate was then detached from the magnetic plate, and 40ul of elution buffer was poured. Once again, the sample was left to incubate at room temperature for 5 minutes. The reaction plate was placed on a 96-well magnetic plate and left undisturbed for 3-5 minutes until the solution became transparent. A volume of 35 ul of the transparent sample was transferred onto a fresh plate in preparation for loading onto the detector.

3.7 Editing of sequence data

Sequencher® version 5.4.1 was used to edit the raw data obtained from automated Genetic Analyzer. This program was used to trim sequence data, assemble contigs, and generate FASTA files.

3.8 Analysis using the Basic Local Alignment Search Tool (BLAST)

Online BLAST analysis employing FASTA files allowed quick comparison of genomic sequences. BLAST analysis was done at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The closest matched and reference strain sequences for each genotype were saved in FASTA format. Later, these FASTA files were employed for phylogenetic analysis and evolutionary study.

3.9 Phylogenetic Analysis

The study analyzed viral strains from throughout the world for biological links. These interactions were shown using phylogenetic trees. MEGA (Molecular Evolutionary Genetic Analysis) was used to compare reference and study sequence FASTA files. The MEGA program's default maximum likelihood strategy rebuilt the phylogenetic trees.

3.10 Statistical Analysis

The bootstrap technique was used to assess the statistical significance of the phylogenetic study. Bootstrapping is a resampling technique that includes reconstructing the trees over several rounds, often 100 or 1000, and evaluating if the same nodes are retrieved. Bootstrap values equal to or greater than 70 were considered reliable, indicating a high likelihood of the real clade and serving as a strong statistical support, as proposed by Hillis and Bull (1993).

RESULTS

4.1Sample details:

A total of **803 fecal samples** were collected from children under the age of 5 years, who were hospitalized due to severe diarrhea between January 2022 and December 2022. Subsequently, the samples were sent to the Department of Virology, National Institute of Health, Islamabad, where further laboratory testing was conducted.

4.1.1 Epidemiological characteristics

The results of norovirus testing showed that 15.69% (n=126) of the 803 samples tested positive for norovirus infection.

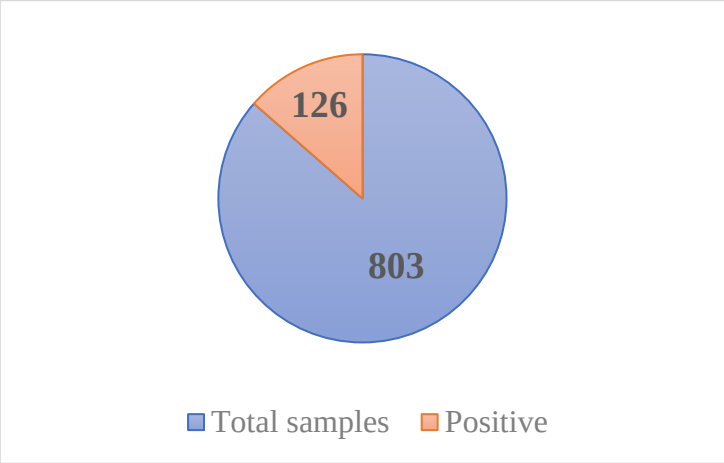


Figure 4.1: Frequency of Norovirus positive samples

4.1.2 Frequency of NoV Genogroups

The presence of norovirus was assessed in two genogroups, GGI and GGII. The findings indicated that 42.06% (n=53) of the norovirus positive samples belonged to GGI, while GGII was more prevalent, accounting for 57.94% (n=73) of the samples.

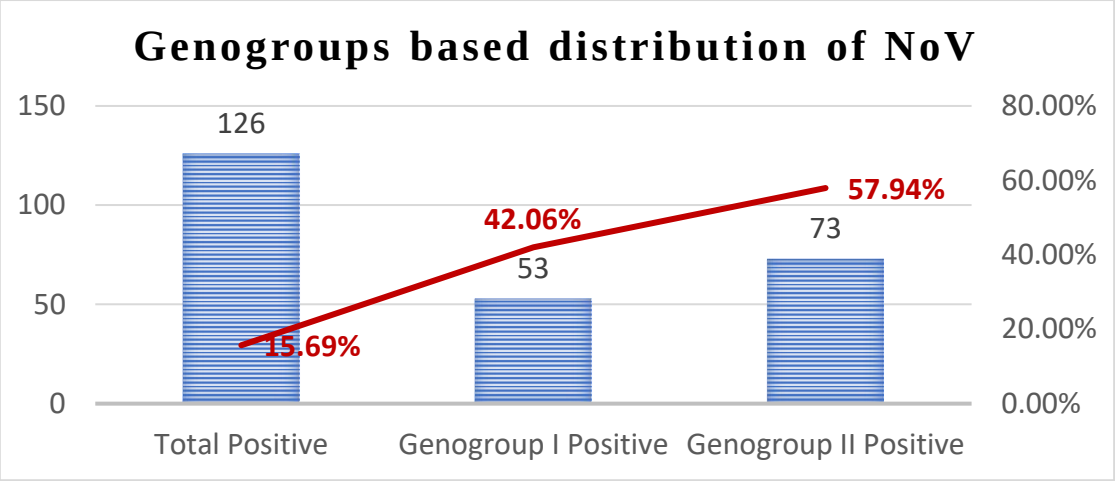


Figure 4.2 : Frequency of NoV positive cases along with its genogroups GGI and GGII represented by blue columns, red line represents the NoV positivity rate

4.1.3 Site wise distribution of NoV samples

Stool samples were obtained from two tertiary care hospitals, Saidu teaching hospital (STH) and Khyber teaching hospital (KTH). A total of 803 stool samples were obtained, with 253 samples sourced from KTH and 550 samples collected from STH.

Among the 253 samples collected from KTH, 34 samples tested positive for NoV. In case of STH, out of the total 550 samples, 92 samples were found to be positive.

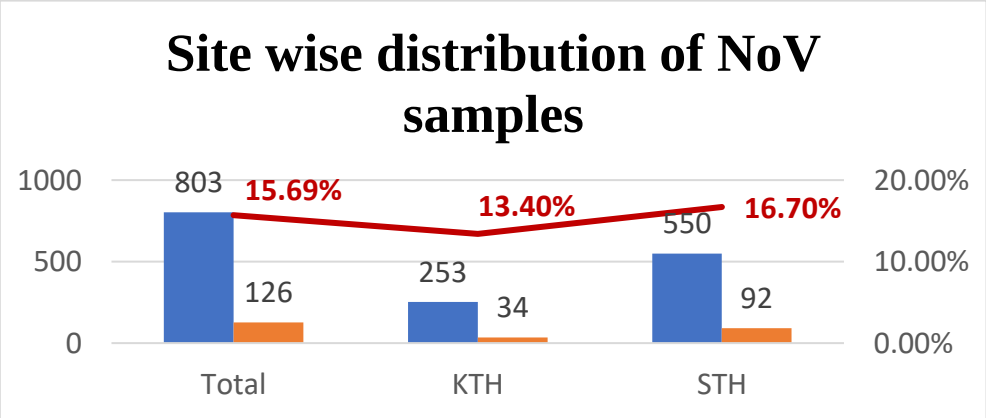


Figure 4.3: Site wise distribution of NoV samples.

4.1.4 Month wise distribution of total, positive and percentage of Norovirus in 2022

Assessment of norovirus investigations indicated that of the 803 samples, 15.69% (n=126) tested positive for norovirus infection. NoV infections were reported throughout the year with a notable percentage occurring in December, accounting for 33.33% of the samples (n=3).

The highest infection rates for NoV positive samples were recorded in the following months: December (33.33%), January (23.44%), February (30.00%), March (16.00%), April (28.79%), May (6.67%), June (25%), July (5.22%), August (12.14%), September (6.58%), October (17.39%), and November (0.00%).

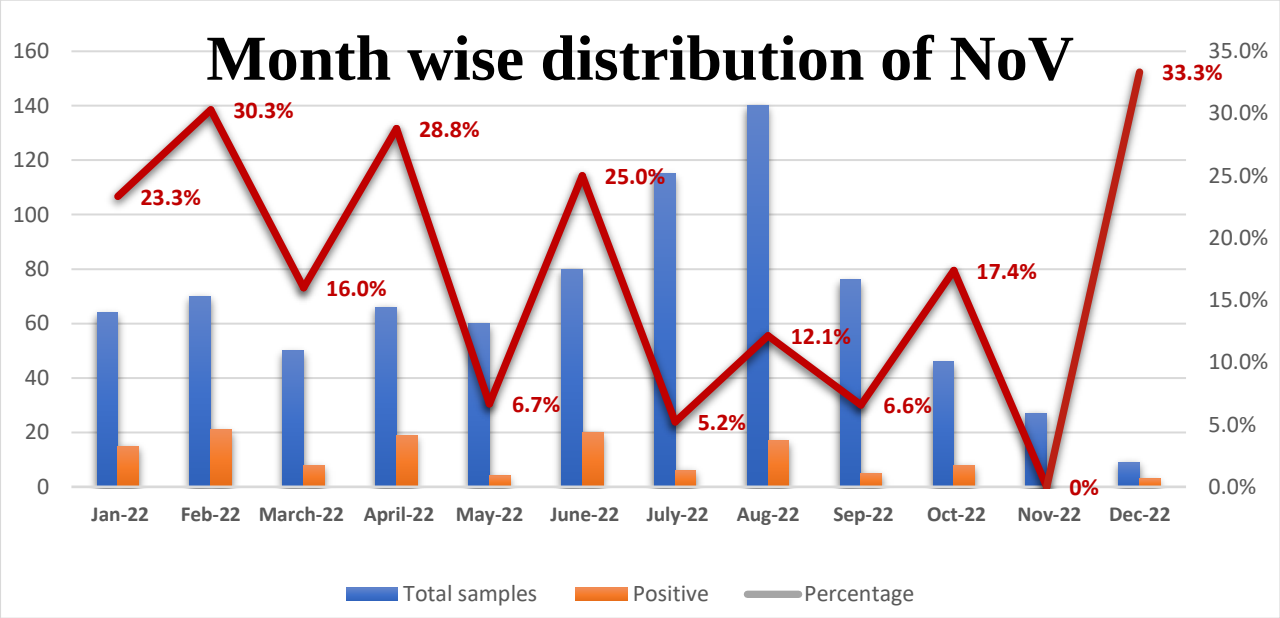


Figure 4.4: Seasonal Trend of NoV (December exhibited the greatest incidence of Norovirus infections in our study)

4.1.5 Gender wise distribution of Norovirus Positive samples

An analysis was conducted to identify gender-based variation and the male-to-female proportion of Norovirus infections among the registered patients by evaluating the gender-based differentiation between positive and negative cases. An examination of gender distribution revealed that among the 803 samples, enteric infections were more prevalent in

men (n=494) compared to females (n=309). However, NoV infections were more common in females, accounting for 16.50% (n=51), while males accounted for 15.18% (n=75), as seen in the Figure below.

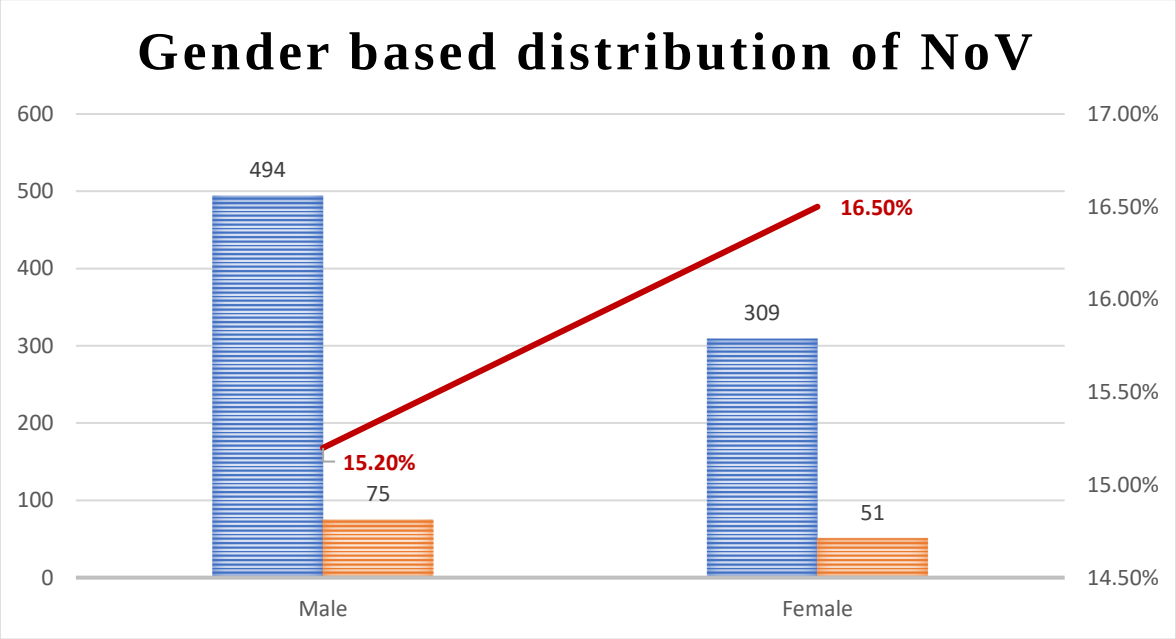


Figure 4.5: Gender wise distribution of NoV positive samples

4.1.6 Age-group based distribution of NoV Positive samples.

An analysis of NoV positive cases based on age groups was conducted to identify the age group most vulnerable to the NoV infection among the hospitalized patients. The children who were part of this research were placed into distinct age groups. For children under 24 months, the groups were formed with an interval of 6 months. For children over 24 months, the groups were formed at an interval of 12 months. The remaining children, owing to a smaller number of participants, were grouped together from 36 to 60 months. Positive cases were detected in all age groups, with the highest occurrence seen among children aged 0-5 months.

The age group with the largest percentage of positive samples (21.74%; n= 25) was 0-5 months, followed by children aged 24-35 months (18.75%; n=48) as seen in the figure.

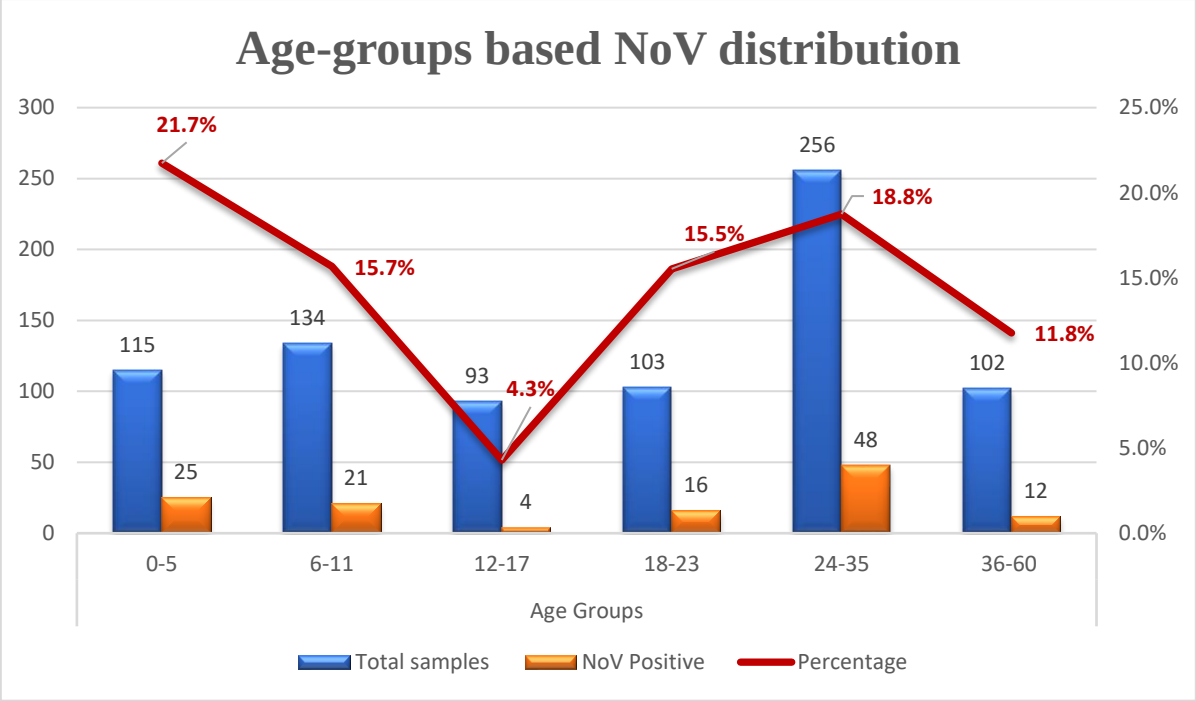


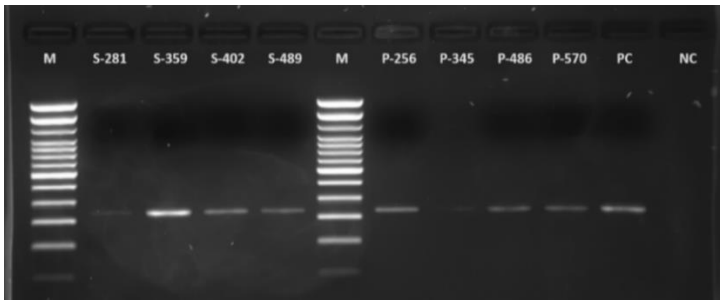
Figure 4.6: Age-groups based NoV distribution.

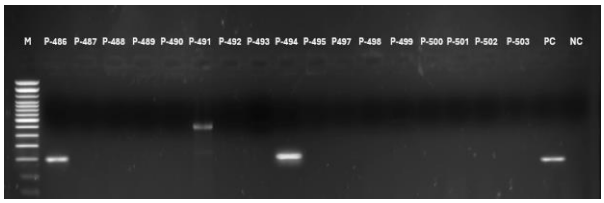
Distribution of Nov infection among various age groups (Blue bars represents numbers of samples tested for each age-group whereas red bars show NoV positive samples and green line represents NoV positivity rate)

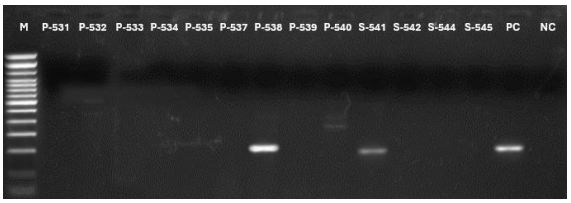
4.2 Molecular analysis of KTH and STH samples for NoV infections

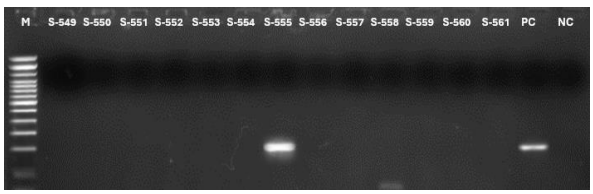
A total of 803 samples collected from STH and KTH were screened from NoV by using one-step conventional RT PCR. The positive samples were sequenced on the basis of VP1 gene. Electrophoresis Gel photographs for conventional PCR have been shown below.

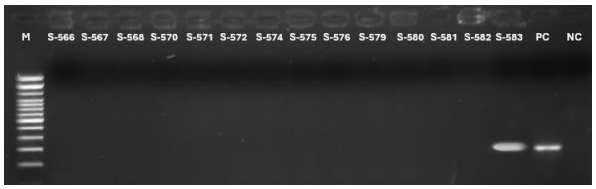
4.2.1 Gel Electrophoresis results of amplified products of VP1 gene of NoV GGI

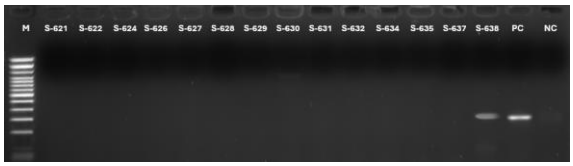


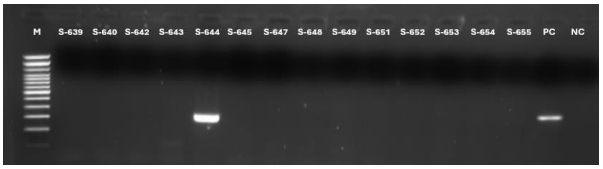


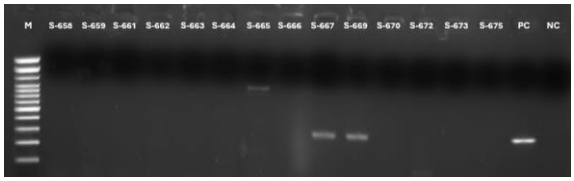


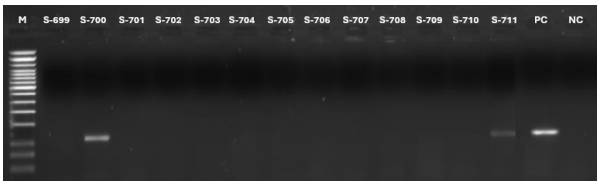


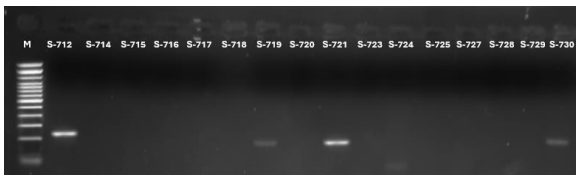


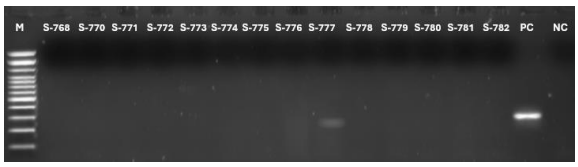


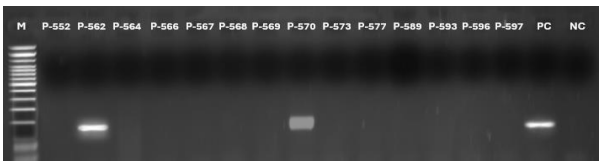












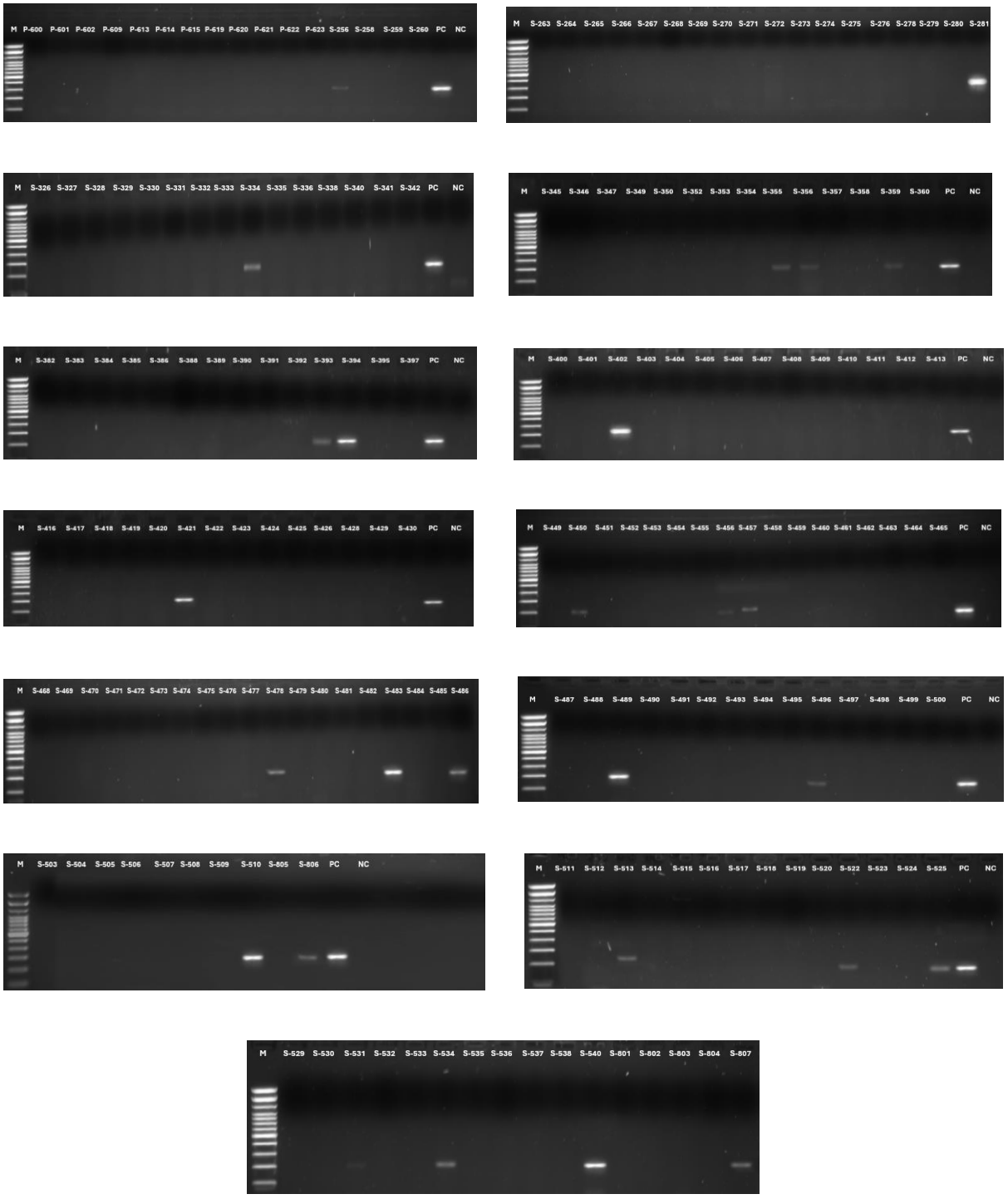
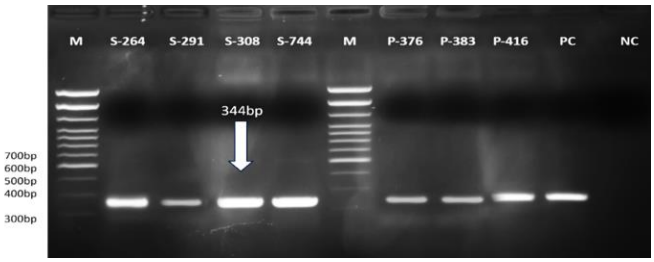
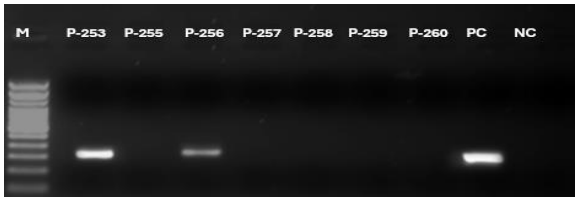
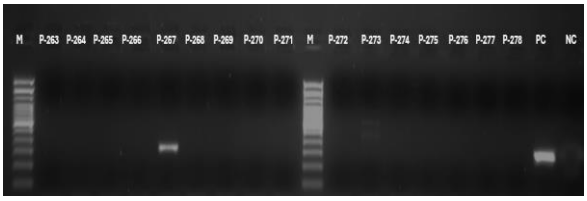


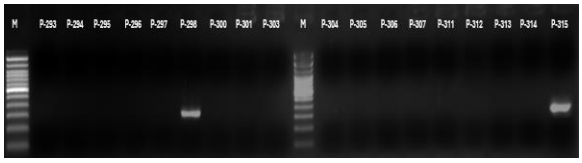
Figure 4.7: Gel Electrophoresis results of amplified products of VP1 gene of NoV (330bp for GGI)

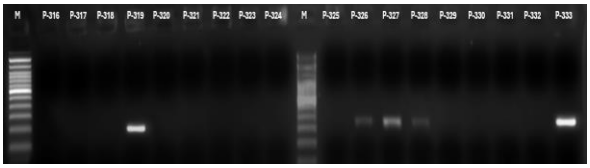
4.2.2 Gel Electrophoresis results of amplified products of VP1 gene of NoV GGII

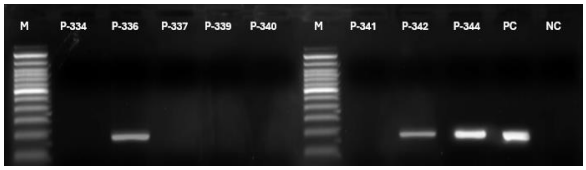


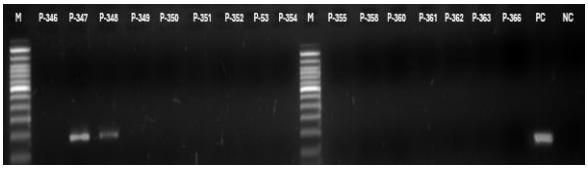


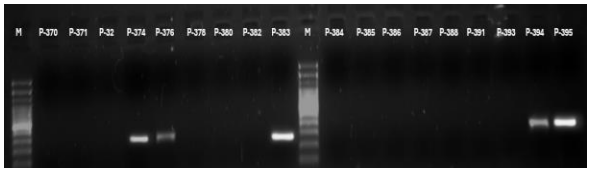


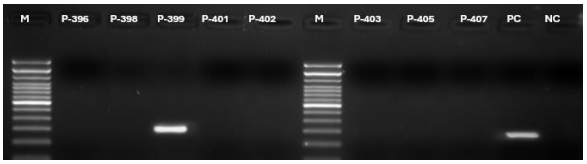


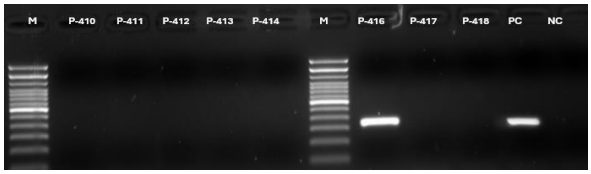


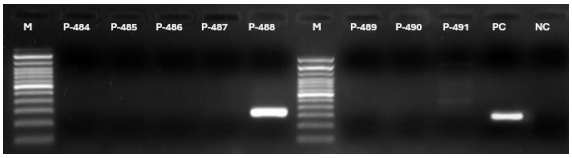


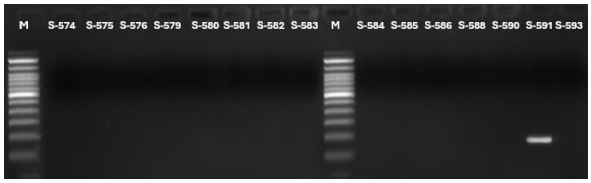


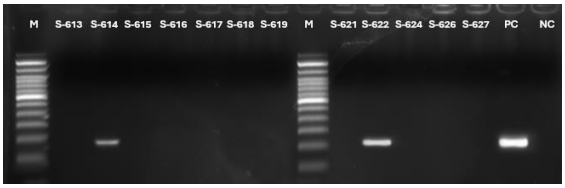












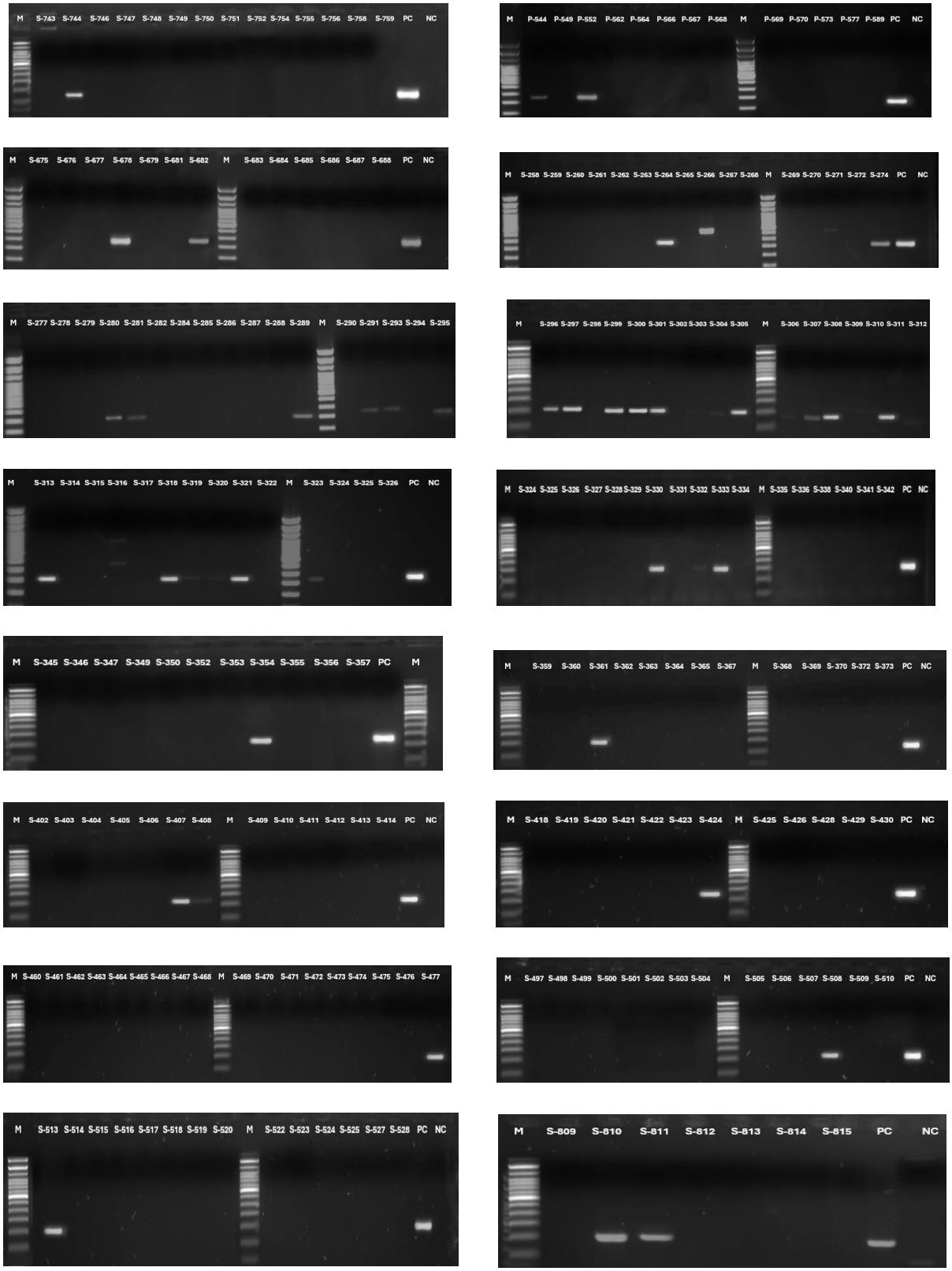


Figure 4.8: Gel Electrophoresis results of amplified products of VP1 gene of NoV (344bp for GGII)

4.3Phylogenetic analysis of human Norovirus strains on the basis of VP1 gene

Phylogenetic analysis of NoV positive samples was conducted to identify the circulating strains of NoV in KPK region of Pakistan. Samples collected from STH and KTH were sequenced based on VP1 genomic region to determine their genotypes. Out of 126 positive samples, 73 were GGII whereas 53 samples were tested positive for NoV GGI.

4.3.1 NoV GGI

42 out of 53 samples were sequenced to demonstrate the NoV genotypes. GI.3 was identified as the Predominant strain among Genogroup I of NoV

Table 4.1 Frequency of NoV GGI Genotypes

Genotype	Number of samples
GI-1	5
GI-3	19
GI-5	3
GI-6	1
GI-7	2
GI-8	12

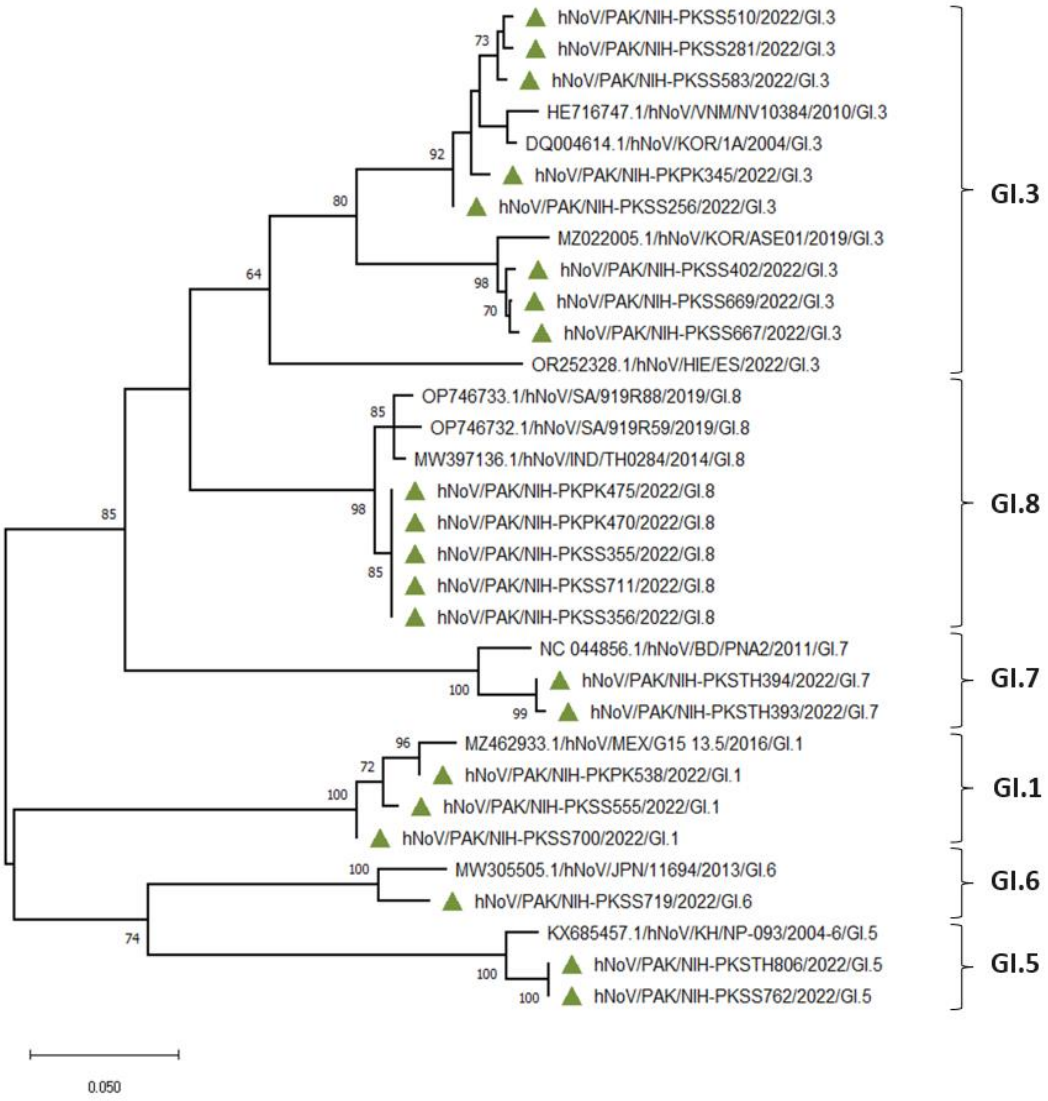


Figure 4.9: Phylogenetic tree of Norovirus VP1 gene

Genotyping of Norovirus GI strains. Phylogenetic study of GI norovirus strains found in Pakistan between January 2022 and December 2022. The phylogenetic tree was created using the maximum likelihood technique with 1000 bootstrap repetitions in MEGA11. The study identified specific Norovirus strains using unique IDs that correspond to the city and hospital of origin: PKPK for Khyber teaching hospital, KPK, Pakistan and PKSTH stands for Saidu teaching hospital, KPK, Pakistan.

4.3.2 NoV GGII

60 samples were sequenced out of 73 to determine the circulating genotypes in this region. Our findings reported that GII.2 was the most prevalent genotype.

Table 4.2: Frequency of NoV GGII Genotpyes

Genotypes	Number of samples
GII-2	19
GII-3	6
GII-4	5
GII-6	4
GII-9	3
GII-13	6
GII-14	1
GII-17	11
GII-21	5

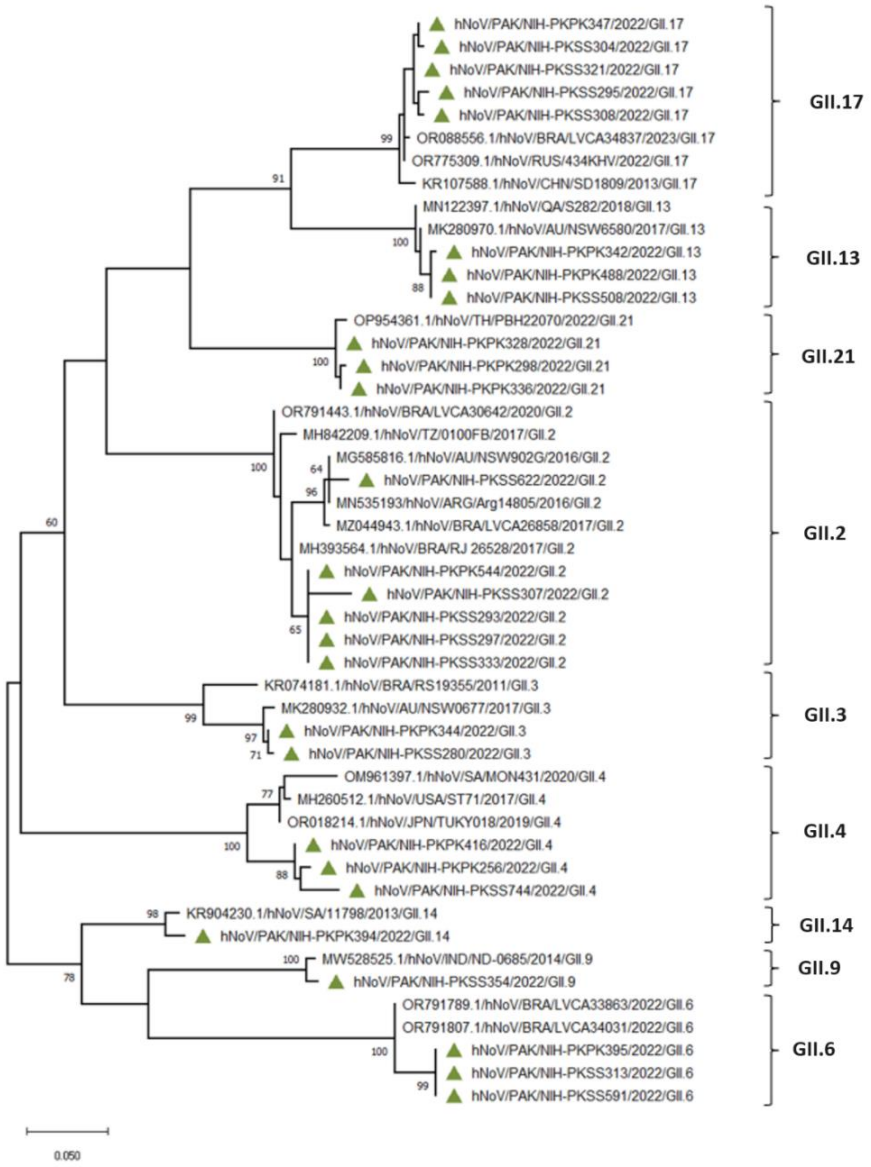


Figure 4.10: Phylogenetic tree of Norovirus VP1 gene

Genotyping of Norovirus GII strains. Phylogenetic study of GII norovirus strains found in Pakistan between January 2022 and December 2022. The phylogenetic tree was created using the maximum likelihood technique with 1000 bootstrap repetitions in MEGA11. The study identified specific Norovirus strains using unique IDs that correspond to the city and hospital of origin: PKPK for Khyber teaching hospital, KPK, Pakistan and PKSTH stands for Saidu teaching hospital, KPK, Pakistan.

DISCUSSION

This study aimed to investigate the molecular diversity and genotype distribution of Norovirus strains present in children with acute gastroenteritis in KTH and STH hospitals in KPK, Pakistan, from January 2022 to December 2022. Limited data is currently accessible pertaining to the prevalence and molecular diversity of norovirus within this geographical area. Diarrhea has a substantial impact on illness and death rates, especially in developing nations. Norovirus ranks as the second most prevalent cause of diarrheal illnesses among children. Necrotizing enterovirus (NoV) exhibits a high level of contagiousness and can be readily transferred through interpersonal contact or by sharing food or utensils that may have been contaminated by vomit particles or fecal matter. According to the Center for Disease Control and Prevention (CDC), individuals who are infected release a substantial number of NoV particles, with just a small quantity being capable of infecting a healthy individual. [29][30].

In developing countries, NoV causes approximately one billion episodes of diarrhea with 2.5 million deaths annually. NoV primarily affects children under the age of 5 years. Contrarily, less than 1% of child deaths are associated with diarrhea in developed countries. In Africa and South Asia, loose bowel diseases are responsible for child deaths and account for one out of every 8 children globally [30]. In South Asia, 32% of the annual 7.6 million fatalities in children under 5 years of age have been reported [31]. Pakistan has been targeted as one of the Asian countries having a remarkable number of child deaths under the age of 5 years caused by diarrhea, based on global health survey which was conducted in Asian countries during 2012-2013. Currently, very less research studies have been

reported in Pakistan to explore and quantify the diarrheal disease burden caused by NoV[11].

As diarrhea is the most significant health issue in Pakistan, this study was conducted for the molecular detection and genotyping of Norovirus.
In this research, out of 803 samples, 15.69% (n=126) samples were tested positive for NoV. These findings show close proximity with earlier studies on the NoV, which reported 16.1% and 14% positive cases in Pakistan [11] [32]. Various factors affect the rate of NoV infections including study population, age of patients, duration of study, diagnostic techniques used and the geographical location of the research (temperate or tropical)

In current study, NoV Positive cases were reported throughout the year with the highest episodes observed during winters with 33.33% NoV positivity rate in December.

Similar findings were reported by Dey et al. (2009), where 917 samples were taken from children with acute gastroenteritis in Bangladesh. NoV episodes have been observed in winters, More data regarding location and seasonality could be really informative for the determination of factors predicting the NoV seasonality [33]. Children under the age of 24 months were grouped with an interval of 6 months while children over 24 months were placed in groups with an interval of 12 months. The remaining children were grouped together from 36-60 months due to a smaller number of participants. Positive cases were detected in all age groups, with the highest occurrence seen among children aged 0-5 months with 21.74% followed by the age group 24-35 months with 18.75% positive cases. Similar findings were reported in 2013, where higher infection incidences were observed during first 6

months [34]. Gender based analysis demonstrated that gastroenteritis was reported more in males (n=494) as compared to females (n=309), while NoV infections were more prevalent in females accounting for 16.50% (n=51) than males with 15.18% (n=75) positive cases. Similar findings were reported in 2023 [32]. While other studies have shown no gender-based differences in infection rates.

Our study revealed that GGII strains were more prevalent with 57.94% (n=73) positive cases as compared to GGI with 42.06% (n=53) positive samples. This finding aligns with the NoV prevalence reported in previous research conducted in 2015 with GGII 74.41% and GGI 25.58% [4]. Another study conducted in India exhibited the higher GGII infection rate among NoV infected patients as compared to NoV GGI. [35]. In Bangladesh, Rahman et al reported 66.1% GGII positivity rate according to the study conducted in 2013 [36].

Although NoV GGII is globally recognized as the most prevalent strain, the variation in the prevailing rates among all the studies conducted might be attributed to differences in the study designs, duration, and the geographical location of the research. The main goal of this study was the genotyping of NoV, and a spectrum of genotypes were observed after molecular sequencing.

Phylogenetic analysis was used to identify the genetic diversity of NoV strains circulating in the KPK region of Pakistan. The findings reported that strains were associated with GII-2, GII.3, GII.4, GII.6, GII.9, GII.13, GII.14, GII.17, and GII.21. While in case of NoV Genogroup I GI,1, GI.3, GI.5, GI.6, GI.7, and GI.8 were observed when these sequences were compared with the reference sequences from National Center for Biotechnology Information (NCBI) utilizing the maximum likelihood approach.

In this study, NoV GGII.2 was the most prevalent genotype among genogroup II. In contrast, previous studies have reported that NoV GGII.4 was the most prevalent strain. Our findings regarding Genogroup I demonstrated the most significant strain was NoV GGI.3 while earlier research have reported GGI.7 as the most prevalent genotype of NoV genogroup I. The shift in viral dynamics could be due to a higher biological fitness, larger viral shedding in infected patients, a wider range of receptor specificity and a rapid mutation frequency. [37], [38], [39]

Switching human receptors and changing antigenicity owing to genetic drift might prevent short-term herd immunity during large pandemics. [40], [41], [42]

Our findings demonstrate the emergence of GG1.3 and GGII.2 variants and indicate that specific variants of NoV are selected from a pool of co-circulating NoV strains.

In summary, NoV had a significant influence on children's diarrhea in KPK, Pakistan, in community and hospital settings. The peak of NoV-induced diarrheal events was related with GGI.3 and GGII.2 genotype variations, replacing the multiple genotypes circulating before the surge. The GGI.3 and GGII.2 genotypes shed more viruses than other genotypes in children, which may explain their high incidence in person-to-person transmissions.

The most common norovirus strain in Pakistan in the globe is GII.4. This investigation revealed fewer norovirus GII.4 samples than GGII.2. These findings demonstrate the genetic variety of circulating norovirus GII genotypes in Pakistan during the epidemic and sporadic cases and stress the need for community-wide molecular monitoring.

NoV detection is not a routine diagnostic practice in Pakistan, it is not reported leading towards underestimation of the NoV incidences. In addition to climate, the high incidence of viral gastroenteritis is linked to increased birth rates and

inadequate hygienic conditions during flooding and heavy rainfall. Population density and high birth rates contribute to increased rates of transmission and prolonged circulation of virus throughout the year.

This study provides substantial and reliable data that might help in improving the technical capabilities of national public health agencies in establishing advanced molecular diagnostics techniques at the major hospitals in Pakistan.

In conclusion, the prevalence of viral gastroenteritis, particularly NoV, is notably high among the indigenous Pakistani population. Large scale molecular epidemiology research should be conducted in more regions of Pakistan. Although this study may not provide a comprehensive overview of the NoV disease burden in the country, it gives a helpful insight for public health officials to assess and establish well-informed immunization policies.

CONCLUSION AND FUTURE PROSPECTS

This research concludes that noroviruses have a substantial role in the episodes of childhood diarrhea, with a 15.69% positivity rate among the samples collected from KPK region. Our study revealed that GGII strains were more prevalent with 57.94% (n=73) positive cases as compared to GGI with 42.06% (n=53) positive samples. Among Genogroup I, GGI.3 was found to be the most prevalent genotype as opposed to the previously demonstrated GGI.7 genotype showing a significant shift in viral dynamics. In the case of Genogroup II, GGII.2 was the most prevalent genotype among genogroup II as opposed to the previously reported GGII.4 genotype showing a shift in viral dynamics. This shift in viral dynamics could be due to a higher biological fitness, a wider range of receptor specificity and a rapid mutation frequency. This study provides a diverse influence on public health measures. Strain-specific results are crucial for the development of targeted vaccines which eventually lead towards more effective prevention. Development of a flexible epidemiological monitoring system is crucial in order to effectively manage the emerging strains.

This study serves as evidence of the significance of local knowledge and also makes a vital addition to the worldwide understanding of Norovirus epidemiology. The information reported here will serve as a solid platform for future research, global partnerships, and evidence-based treatments aimed at effectively reducing Norovirus-related illnesses in children.

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Molecular characterization of Human Norovirus strains circulating among children with acute Gastroenteritis in KPK, Pakistan

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2024

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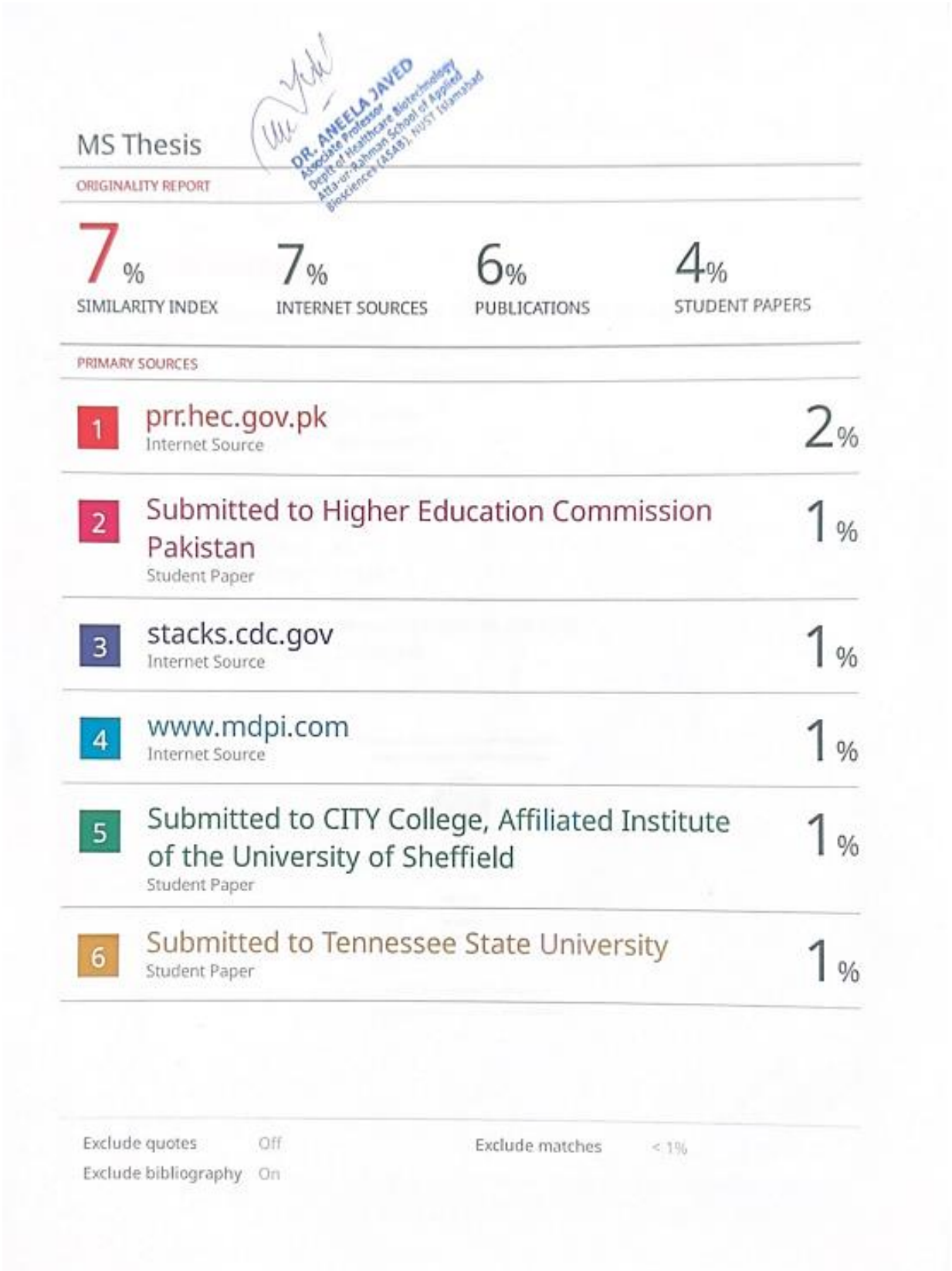

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