COLLEGE OF HEALTH SCIENCES

SCHOOL OF BIOMEDICINE AND LABORATORY SCIENCES

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

Molecular Epidemiology and Genotypic Characterization of Rotavirus Among Children Under Five Years Age with Acute Diarrheal Illness in Addis Ababa, Ethiopia

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Addis Ababa, Ethiopia

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LIST OF ABBREVIATIONS AND ACRONYMS

CDC	Centers for Disease Control and Prevention
cDNA	Copy Deoxyribonucleic Acid
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
dsRNA	Double Stranded Ribonucleic Acid
EDHS	Ethiopian Demographic and Health Survey
EIA	Enzyme Immunoassay
GARV	Group A Rotavirus
HT	Hydroxy-tryptamine
MOH	Ministry of Health
NIHR	National Institute for Health and Care Research
NSP	Non-Structural Protein
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase-PCR
RCWG	Rotavirus Classification Working Group
RNA	Ribonucleic Acid
RV	Rotavirus
RVA	Rotavirus A
TAC	TaqMan Array Card
VP	Viral Protein
WHO	World Health Organization

ABSTRACT

Introduction: In children under five years**,** diarrhea is the second most common cause of mortality worldwide. Various enteric pathogens are responsible for diarrhea, including viruses, bacteria, and parasites. Among the viral etiologies, rotavirus is the leading cause of diarrheal illness in children. The rotavirus-associated diarrheal illness is highest in sub-Saharan Africa, South Asia, and Southeast Asian countries. Rotavirus A is categorized in to several G and P genotypes based on VP7 and VP4, respectively. The most common genotypes associated with human infection globally are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8].

Objectives: To determine molecular epidemiology and identify circulating genotypes of Rotavirus among children under five years with acute diarrheal illness in selected health facilities in Addis Ababa, Ethiopia.

Methods: A cross-sectional study design was conducted in selected health facilities in Addis Ababa, Ethiopia, from September 2023 to January 2025. A total of 200 children with acute diarrheal illness were recruited using a convenience sampling technique. After obtaining consent, caregivers were interviewed with a structured, pre-validated questionnaire for sociodemographic status, and 5 mL of stool specimens were collected from the study participants. The stools were tested for Rotavirus using the TaqMan Array Card -Real Time Polymerase Chain Reaction, including genotype identification. Data was entered into Epidata version 4.7 and analyzed using SPSS version 25 software. The results were presented using tables and interpreted using chi-square and odds ratio.

Results: Of the 200 stool specimens collected from the children, rotavirus was detected in 45 (22.5%) samples. Thirteen G-P genotype combinations have been identified, G1P[4], G2P[4], G3P[8], G8P[4], G9[P4], G9P[8], G2P[6], G12P[8], G10P[8], G4P[8], G1P[8], G8P[8], and G8P[6] are the circulating genotypes among the population. Out of these, three new genotypes, G8P[4], G10P[8], and G4P[8] were detected for the first time in Ethiopia. Factors associated with rotavirus diarrhea were analyzed. Generally, only two factors (vomiting and vaccination status) were statistically significantly associated with rotavirus positivity; while other child-related associated factors were not significantly correlated with rotavirus infection.

Conclusion: This study provides an important insight into the updated prevalence, circulating genotypes and the emergence of new genotypes of rotavirus among the population. These findings underline that despite the presence of the Rotarix vaccine in the country, Rotavirus remains a significant etiologic agent for acute gastroenteritis. Therefore, continuous Rotavirus surveillance and implementation of prevention and control strategies are crucial to reduce the burden of Rotavirus-related diarrheal illness.

Keywords: Rotavirus, Genotypic characterization, Molecular epidemiology, Acute diarrheal illness, Ethiopia

CHAPTER ONE: INTRODUCTION

1.1. Background

In children under five years age, diarrhea has been identified as one of the main causes of morbidity and mortality, primarily in low and middle-income countries (Omolajaiye *et al*., 2020). Diarrhea is the second common cause of mortality among this age group worldwide, accounting for 1 in 9 child deaths (CDC, 2013) . Various enteric pathogens are responsible for diarrhea including viruses, such as Rotavirus, Norovirus, Adenovirus, and Astrovirus (Najafi *et al*., 2013), enteric bacteria, such as *Escherichia coli*, *Campylobacter*, *Shigella*, or *Salmonella* spp. (Mero *et al*., 2021), and parasites, such as *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* spp. (Saeed *et al*., 2015)

Rotavirus, a non-enveloped RNA virus that possesses eleven segments of double-stranded RNA (dsRNA), which belongs to member of the Reoviridae family, is 70–75 nm in size (Digwo *et al*., 2023). The eleven segments of the double-stranded RNA genome encode six structural viral proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and six non-structural viral proteins (NSP1-NSP6) (Koki Ndombo, 2017). The infectious particle of rotavirus consists of three concentric protein layers. The outer capsid's constituent viral proteins, VP7 and VP4, contain neutralization-specific epitopes. The inner layer is surrounded by the VP6-containing inner capsid. The latter is made up of the enzymatic replication complexes (VP1 and VP3), VP2 (scaffolding protein) (Greenberg & Estes, 2009; Li *et al*., 2009). The VP6 (inner capsid protein) is used to classify Rotavirus in to nine serogroups, i.e. A-J. In human, most gastrointestinal infections are due to Rotavirus group A (Boni-Cisse *et al*., 2018).

The Rotavirus A is further categorized in to several G and P genotypes based on outer layer proteins, VP7 and VP4, respectively (Nokes *et al*., 2010). So far, about 42 G and 58 P Rotavirus genotypes have been reported from different sources (Ojobor *et al*., 2020). Of these, 12 G and 15 P types have been recovered from human being (Jia, 2017). The most common genotypes associated with human infection globally are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] (Jia, 2017; Nokes *et al*., 2010). In Ethiopia, G12P[8], G3P[6], G1P[8], and G3P[8] are the predominant Rotavirus A genotypes (Damtie *et al*., 2020).

Currently, there are four WHO prequalified Rotavirus vaccines for human use; a monovalent Rotarix (G1P[8]), a pentavalent RotaTeq (G1, G2, G3, G4 and P[8]), a pentavalent Rotasil (G1, G2, G3, G4 and G9), and a monovalent Rotavac (G9P[11]) (Rota Council, 2019). In Ethiopia, the Rotarix vaccine was introduced and included in 2013 to its Expanded Program on Immunization. The vaccine is administered 4 weeks apart, at 6th weeks and 10th weeks of age (MOH, 2021).

1.2. Statement of the Problem

Globally, diarrheal infections cause an estimated 1.3 million deaths annually where majority of the deaths occur in low and middle-income countries. About a quarter of deaths among children in Southeast Asia and Africa are attributed to acute diarrheal illness (Shrestha *et al*., 2019).

Rotavirus was principal cause of diarrheal infections mortality worldwide, being responsible for over 258,000,000 illnesses and 129,000 deaths in children under the age of five, out of which 104,733 deaths occurred in Sub-Saharan Africa in 2016 (Troeger *et al*., 2018). In the same year, total deaths due to rotavirus infection in Ethiopia were 6817 (Tate *et al*., 2016).

According to the World Health Organization's estimate, Rotavirus infection is responsible for 8,700 deaths each year among children under five years of age in Ethiopia (Damtie *et al*., 2020). On the other hand, the prevalence of rotavirus infection among children with acute diarrheal illness in Ethiopia varied significantly in different regions, as low as 8.04% (Feleke *et al*., 2018) in a community-based study in Amhara regional state and as high as 43.63% in a health facility-based study in Oromia regional state (Ramos *et al*., 2015).

Rotavirus vaccines have shown significant efficacy in reducing severe diarrhea and hospitalizations in developed countries relative to developing countries. In Europe, Rotarix prevented 90.4% of severe rotavirus infections; in Africa, however, this percentage was just 61.2% (Madhi *et al*., 2016a; Vesikari *et al*., 2007). In industrialized nations like the United States and Finland, RotaTeq has an efficacy rate of 83 to 100% in treating acute gastroenteritis; in developing nations, it is just 30 to 74% (Patel *et al*., 2009; Vesikari *et al*., 2006). This indicates the vaccine efficacy in developing countries are significantly very low in reducing severe diarrhea and hospitalization.

In addition, genotyping data collected after the vaccination was introduced, identified genotype recombinants that were previously unknown. In Ethiopia, the predominant strains before vaccine introduction were G1P[8], G3P[6], G12P[8], and G2P[6] (Abebe *et al*., 2014; Yassin *et al*., 2012), while in the post-vaccine era G2P[4], G3P[8], and G9P[8] genotypes evolved as a common cause of illness in different parts of the country (Abebe *et al*., 2018; Gelaw *et al*., 2018). However, there is lack of up-to-date information regarding the prevalence and distribution of Rotavirus genotypes in Addis Ababa, Ethiopia. Understanding the burden and distribution of Rotavirus genotypes is crucial as certain types may have differing levels of virulence, host susceptibility, and response to vaccine. In addition, genotypic characterization of Rotavirus is important to understand circulating genotypes and emergence of new strains.

1.3. Significance of the Present Study

Rotavirus is recognized as a significant cause of morbidity and deaths among children under the age of five years. The problem is especially pronounced in the low and middle-income countries, such as Ethiopia. Therefore, studying Rotavirus burden and identifying the circulating genotypes among Ethiopian children will help to understand the gravity of the problem and inform policy makers to implement appropriate prevention and control strategies. Ultimately, it will contribute to the reduction of Rotavirus morbidity and mortality.

Genotypic characterization of Rotavirus is important for identifying emerging genotypes that might circulate among young children. By identifying the circulating genotypes among the young children in Addis Ababa, Ethiopia, this study delivered crucial data on the predominant genotypes of Rotavirus.

There was limited updated data on the burden and circulating genotypes of Rotavirus among young children in Addis Ababa, Ethiopia (Abebe et al., 2018). The current study was filled these research gaps by providing essential data on the prevalence and circulating genotypes of Rotavirus among young children.

Generally, this study is significant to inform policy makers and targeted interventions, consequently reducing the Rotavirus-associated illnesses and deaths among young children in Ethiopia.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Virological Characteristics of Rotavirus

Rotaviruses are non-enveloped viruses with icosahedral nucleocapsid symmetry that belong to the *Reoviridae* family and the Rotavirus genus. They are approximately 70 nm in size and consist of 11 segments of double-stranded RNA. As mentioned earlier, the virus has three protein layers: an outer layer, an inner layer, and an internal core surrounding the 11 segments of dsRNA (Bányai *et al*., 2018).

The genetic diversity resulting from interspecies reassortment complicates efforts to control rotavirus infections through vaccines (Matthijnssens & Van Ranst, 2012). Evidence from molecular epidemiology studies indicated that Rotavirus interspecies transmission could occur between human and animals, with certain animal rotavirus strains identified from humans and vice versa. Usually, this happens when people are in close contact with animals, giving the virus a chance to switch hosts. Once inter-species transmission occurs, the segmented genome of rotavirus facilitates reassortment, in which gene segments from different strains combine to produce novel variants (Malik *et al*., 2020). For instance, Rotavirus A genotype G10[P11] is a predominant strain in cattle. Even though G10 was initially a bovine Rotavirus, recently it has been reported in human in combination with P[8] human strain and forming G10P[8] genotype. This genotype combination is a rare human genotype which result from reassortment between bovine G10 and human P[8] types (Esona *et al*., 2011; Matsushima *et al*., 2012).

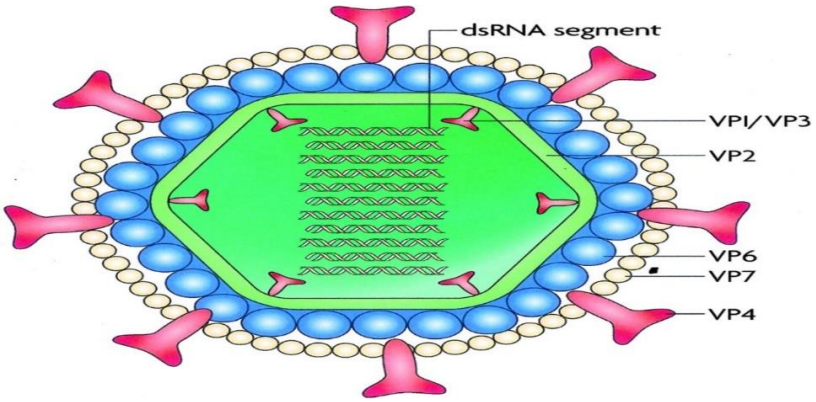

Figure 2.1: Schematic representation of Rotavirus virion (Adapted from Li *et al*., 2009)

The viral genome encodes six structural (VP1-VP4, VP6, and VP7) and six non-structural (NSP1- NSP6) proteins (Figure 1). Rotavirus VP7 and VP4 are used in binary classification schemes to distinguish rotavirus into genotypes G (glycoprotein) and P (protease-sensitive), in that order. According to various surveillance investigations, 42 G-types and 58 P-types have been identified to date worldwide on both humans and animals (Ojobor *et al*., 2020). The most common VP7 genotypes in humans are G1, G2, G3, G4, and G9 worldwide. The most common VP4 strains are P[4], P[6], and P[8], while the majority of circulating rotavirus strains are composed of G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] combinations (Li *et al*., 2009).

Rotavirus genomic diversity is generated through multiple mechanisms, including interspecies transmission, genomic reassortment during mixed infections, successive point mutations, and genomic rearrangements within gene segments (Nokes *et al*., 2010).

2.2. Modes of Transmission, Pathogenesis, Pathology and Clinical Presentations

Rotavirus mainly transmitted through the fecal-oral route and can be transmitted to others through contaminated hands, objects, food, or water. Once ingested, the virus targets and infects the cells that line the small intestine and enterocytes, leading to the development of diarrhea (Lundgren & Svensson, 2001).

Rotavirus-induced diarrhea is non-inflammatory and comprises both malabsorptive and secretory processes. Malabsorption arises from virus-mediated devastation of absorptive enterocytes, downregulation of the expression of absorptive enzymes, and physiologic alterations in tight junctions among enterocytes that lead to paracellular leakage. Raised intracellular Ca2+ concentration is a secretory component of diarrhea associated with rotavirus infection and is crucial for stimulating Cl- secretion (Ramig, 2004) (Figure 2).

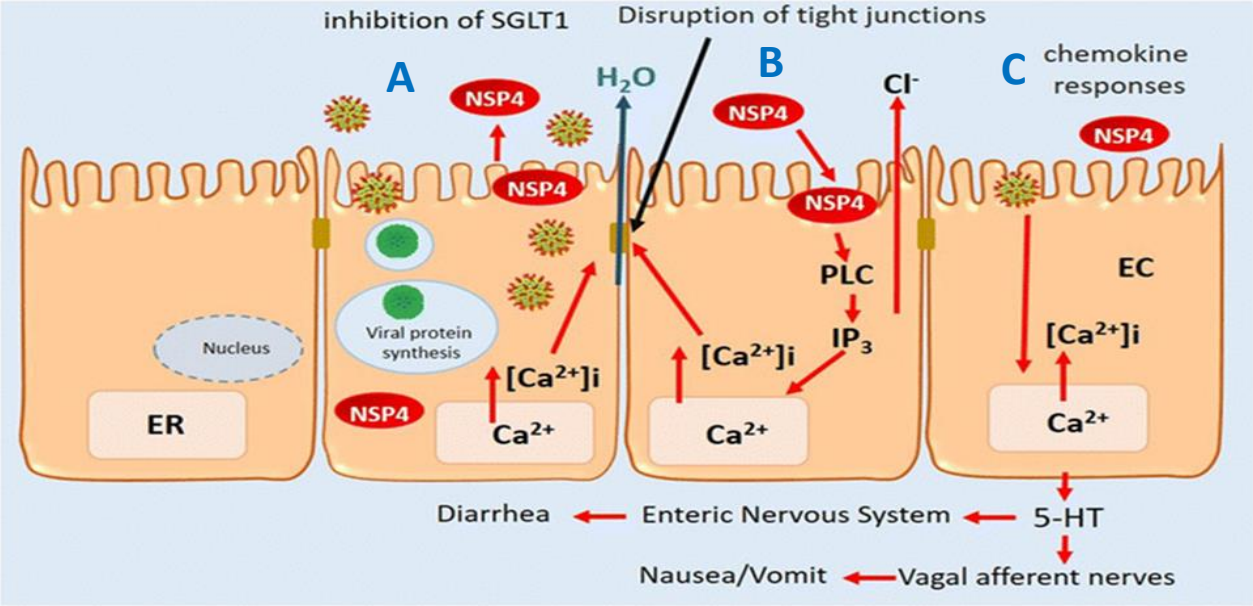

Figure 2.2: Model of Rotavirus infection and pathogenesis (Adapted from Gonzalez *et al*., 2017)

Virus entry, formation of viroplasm and replication, and release of virions and viral proteins such as NSP4 ; **(A)** NSP4 inhibits SGLT1 (Sodium Glucose Co-Transporter 1) in the intestine, which is responsible for the absorption of glucose and sodium ions from the intestinal lumen into the epithelial cells leading to the disruption of nutrient absorption and electrolyte balance in the host intestine. **(B)** Rotavirus NSP4 can disrupt tight junctions through a PLC (Phospholipase C) dependent pathway, leading to the activation of chloride secretion. The activation of chloride secretion contributes to the pathophysiology of rotavirus infection by promoting fluid and electrolyte loss (Vlasova AN, Amimo JO, 2017). **(C)** Increased intracellular calcium levels mediated by NSP4 can cause enteroendocrine cells to secrete 5-HT, which can then stimulate enteric neurons and ultimately increase intestinal motility. Additionally, NSP4-dependent 5-HT release can also activate vagal nerves that project to regions of the brain associated with nausea and vomiting (Crawford *et al*., 2018).

The incubation period of Rotavirus infection is nearly two days. Rotavirus replicates in the cytoplasm of mature enterocytes lining the tips of intestinal villi, preventing the uptake of nutrients and causing severe diarrhea that can be fatal if left untreated (Crawford *et al*., 2018).

The clinical presentations of rotavirus infection include sub-clinical sickness, watery diarrhea for short period, and recurrent, copious diarrhea with vomiting and fever, which may lead to electrolyte imbalance, dehydration, shock, and even death (Arguello *et al*., 2017).

Frequent watery stools appear one or two days after the rapid onset of fever and vomiting that characterize rotavirus infection. Majority of symptoms normally go away in three to seven days, while vomiting usually only stays of one or two days (Bányai *et al*., 2018). Children can acquire Rotavirus infection recurrently for the reason that both immunization and natural infection don't provide complete immunity to protect from upcoming infections (Arguello *et al*., 2017). The first Rotavirus infection causes the most severe symptoms. Rotavirus released in feces in large number and transmit through fecal-oral route (Dennehy, 2013).

2.3. Prevalence of Rotavirus among Children under Five Years

Rotavirus infection was accountable for 128,500 deaths among children under five years in 2016; thus, 28.8% of the deaths from diarrhea were due to rotavirus. The rotavirus-related death rate was highest in low and middle-income countries. Around 81% of deaths from rotavirus infection among children under five occurred in sub-Saharan Africa (Troeger *et al*., 2018).

Asia

According to Rotavirus surveillance done in Southeast Asia to determine the epidemiology of RV and circulating genotype from 2008–2018, 40.78% of diarrheal diseases in children were due to Rotavirus infection (Lestari *et al*., 2020). Another sentinel surveillance in Southeast Asia, Myanmar, showed an overall prevalence of the Rotavirus among young children was 49.9% (Win *et al*., 2017).

Some hospital-based cross-sectional studies done in South Asia, Pakistan, to determine the epidemiology of Rotavirus among children under five years, the prevalence of Rotavirus was 26.4% (Sadiq *et al*., 2019). Another cross-sectional study done in Bangladesh, presented that the prevalence of Rotavirus among children under five years of age was 24.4% (Dey *et al*., 2020).

Europe

A multicentered hospital-based surveillance conducted in different European countries to determine the prevalence of Rotavirus among children demonstrated diverse burden. In France (57.6%, out of 300 enrolled children), Germany (54.6%, out of 494 enrolled children), Italy (29.8, out of 246 enrolled children), Spain (33%, out of 192 enrolled children), and United Kingdom (38.2%, out of 390 enrolled children). The overall prevalence of Rotavirus was 43.4% (Forster *et al*., 2009).

Another study conducted in Ukraine to among children under five years of age demonstrated a prevalence of 20.7% (Soloviov *et al*., 2022). A Similar study done in Italy among children under five years of age with acute diarrheal illness revealed that 34.9% were positives for Rotavirus (Zuccotti *et al*., 2010).

America

A study carried out in Colombia to assess the prevalence of Rotavirus and its circulating genotypes among children with acute gastroenteritis showed a prevalence of 30.5% (Martinez-Gutierrez *et al*., 2019). Another comparable study conducted in the United States of America to determine the prevalence of enteric viral pathogens among young children, presented prevalence of Rotavirus as 6.8% (Rodriguez-Baez *et al*., 2002).

Africa

Studies carried out in different southern parts of African countries showed varied prevalence of Rotavirus. A hospital-based surveillance conducted in Zambia among young children presented 32% prevalence of Rotavirus (Simwaka *et al*., 2018). Another cross-sectional study conducted in the country revealed that the prevalence of Rotavirus was 36% (Simwaka *et al*., 2021). Additionally, the pooled national prevalence of Rotavirus among children under the age of five in South Africa was 23% (Omatola *et al*., 2021).

Diverse studies were conducted in different Western African countries to demonstrate the prevalence of Rotavirus among children under five years of age. A cross-sectional study done in Cote D'Ivoire among 684 enrolled children with acute diarrheal illness, 27.1% were positive for Rotavirus (Boni-Cisse *et al*., 2018).

Similar studies done in Nigeria showed various prevalence rates of Rotavirus in children ranging from 13.8% (Junaid *et al*., 2011) to 56 % (Tagbo *et al*., 2014).

As mentioned in studies carried out in some Central African countries to assess the prevalence of Rotavirus and circulating genotypes among young children, the prevalence of rotavirus was 55% in admitted children with acute diarrheal illness and 21% in control children in Gabon (Manouana *et al*., 2021). A comparable study conducted in the Democratic Republic of Congo showed the prevalence of Rotavirus among young children was 46.4% (Mayindou *et al*., 2016).

Studies done in various Eastern African countries to determine the burden of Rotavirus infection under five years of age demonstrated a prevalence of 25% in Malawi (Bennett *et al*., 2018). As stated in two cross-sectional studies carried out in Kenya, the prevalence of Rotavirus among under five years of age was 31.5% in 2012 (Agutu *et al*., 2017) and 14.5% in 2017 (Nzisa, 2017).

Ethiopia

A previous cross-sectional study from Gondar, Northwest Ethiopia found Rotavirus prevalence of 25% among 450 enrolled children under the age of five. The study also reported that children younger than six months had lower positivity rate compared to other age groups (Gelaw *et al*., 2018). Another study in Wagera district (Northwest Ethiopia) reported Rotavirus in 18% of young children with diarrhea and 3.3% without diarrhea (Feleke *et al*., 2018). Furthermore, a study carried out in 2009 in Hawassa, Southern Ethiopia among under five years of age children with acute diarrheal illness found a Rotavirus prevalence was 22% (Yassin *et al*., 2012).

A previous study in Addis Ababa, Ethiopia found that Rotavirus infection was responsible for 7.7% of children deaths (Abebe *et al.,* 2018). In addition, similar study done in the Amhara region, Ethiopia revealed that Rotavirus infection was responsible for 6.4% of all deaths among children under five years (Feleke *et al*., 2018). A hospital-based surveillance was done in Addis Ababa, Ethiopia from 2007 to 2012 to determine the burden of Rotavirus infection. Rotavirus was detected in 21% of 1841 enrolled children (Abebe *et al*., 2014).

2.4. Genotypes of Rotavirus Circulating among Children Younger than Five Years

For human Rotaviruses, the most common genotypes are G1, G2, G3, G4, and G9 coupled with P[4], P[6], or P[8]. Currently, G9 and G12 genotypes have been progressively identified as significant genotypes in several nations (Prasetyo *et al*., 2022).

Asia

As stated in different studies conducted in Nepal at various times, the predominant G and P combinations were G1P [8], G12P[6], and G12P[8] in 2003 (Uchida *et al*., 2006), while G12P[6], G1P[8] , G9P[8] and G9P[4] were the predominant genotypes in 2014 (Dhital *et al*., 2017) and G12P[6], G1P[8], G3P[8], and G1P[6] were the predominant genotypes in 2017 (Shrestha *et al*., 2019).

According to a study conducted in Bangladesh to demonstrate genotypes of Rotavirus, the predominant genotype combinations were G1P[8], G2P[4], G9P[4], G1P[6], G9P[8], G9P[6] and G11P[25] (Dey *et al*., 2020).

In Myanmar, the predominant Rotavirus genotype combinations were G1P[8] and G12P[8] in 2009. G12P[8] was identified from 2009 to 2012. However, G2P[4] became the most prevalent genotype in 2012 and 2013. In 2013 and 2014 G1P[8] was the predominant genotype. The majority of Rotavirus genotypes in 2015 encompassed G9P[8] and G3P[8]. Although G9P[8] evolved in the country in 2011, it was the predominant genotype in 2014 and 2015 (Lestari *et al*., 2020).

Europe

As stated in a study done in various hospitals in different European countries, circulating genotypes of Rotavirus were diversified among the countries. In United Kingdom, France, and Germany the predominant genotype was G1P[8]. In Spain the prevalent genotype of Rotavirus was G9P[8]. Whereas G4P[4] and G1P[8] were the most circulating genotypes in Italy (Forster *et al*., 2009).

Africa

As mentioned in a systematic review done in South Africa, G1P[8], G2P[4], G3P[8], and G1P[6] were the predominant strains before introduction of vaccine in the country. After introduction of the vaccine, more genotype diversity were observed; where G9P[8], G2P[4], G12P[8], and G1P[8] were the most prevalent genotypes (Omatola *et al*., 2021).

As indicated in a hospital-based surveillance done in Zambia, G1P[8], G2P[4] and G2P[6] were the most common circulating genotypes in 2012. In the next year the predominant genotypes were G2P[4] and G2P[6]. In 2014, G1P[8], G2P[4], and G2P[6] were the prevalent genotypes. As stated in the study, G2P[4] and G2P[6] continued to circulate in the country from 2012 to 2015 (Simwaka *et al*., 2018).

As mentioned in a study conducted in Nigeria, the predominant genotypes of Rotavirus among children under five years of age were G1P[4], G2P[4], G1P[6], G1P[8], G2P[6], G2P[9], and G9P[6] (Maina *et al*., 2022).

Studies conducted in Kenya in different years showed different circulating genotypes of Rotavirus. From 2002 to 2004 the predominant genotype combinations were G1P[8], G9P[8], G8P[4], G8P[6] and G8P[8] (Nokes *et al*., 2010). In 2012, the most predominant rotavirus strains in the country were G3P [4], G12P [6], G9P [6], and G3P [6] (Agutu *et al*., 2017).

Ethiopia

According to study conducted in Gonder, Northwest Ethiopia, the predominant genotypes of Rotavirus were G3P[8], G2P[4], G9P[8], G12P[8], and G3P[6] (Gelaw *et al*., 2018). A comparable study done in Hawassa, South Ethiopia, presented that the prevalent Rotavirus genotype combinations were G3P[6], G1P[8], G2P[4], G8P[6], G12P[8], and G12P[6] (Yassin *et al*., 2012).

According to a hospital-based surveillance carried out in Addis Ababa, Ethiopia from 2007 to 2012, the most common circulating Rotavirus genotype combinations were G1P[8], G12P[8], and G3P[6] (Abebe *et al*., 2014). Similar surveillance study conducted the city from 2011 to 2016 demonstrated the predominant genotypes of Rotavirus.

Subsequently, the predominant genotypes were G12P[8] in 2011 and 2012, G2P[4] in 2013, G9P[8] in 2014, G3P[6] and G2P[4] in 2015, and G3P[8] in 2016 (Abebe *et al*., 2018).

2.5. Factors Associated with Rotavirus Diarrheal Illness

Several factors associated with Rotavirus diarrheal illness have been reported. In developing nations with limited access to clean water and sanitation, Rotavirus remain a significant cause of diarrheal illness among young children. Poor sanitation and hygiene practices facilitate the transmission of the virus which spread primarily through feco-oral route. Additionally, malnutrition increases susceptibility to Rotavirus infection and aggravates disease severity (Troeger *et al*., 2018).

Environmental and seasonal factors also play significant roles in the epidemiology of rotavirus. According to various studies report, prevalence of Rotavirus peaks maximum during cooler and drier season, possibly due to increased indoor crowding and decreased ventilation, which facilitated person to person transmission (Malik *et al*., 2020). Moreover, relatively low vaccine coverage in low- and middle-income countries remains a vital influencing factors of Rotavirus prevalence and severity of the infection. Vaccination has been shown to significantly reduce the incidence of rotavirus-related diarrhea, highlighting the importance of vaccination in controlling spread of the infection (Burnett *et al*., 2017).

In addition to environmental and nutritional factors, the low coverage of rotavirus vaccination programs in many African countries remains a critical challenge. Although Ethiopia has included the rotavirus vaccine in its national immunization schedule, coverage rates are still suboptimal in rural areas, leaving many children unprotected (Geweniger & Abbas, 2020). Research conducted in Ethiopia has shown that children from households with unsafe drinking water sources and improper waste disposal are at increased risk of contracting rotavirus disease. Moreover, malnutrition, which is prevalent among children in Ethiopia and across Africa, exacerbates the severity of rotavirus-related diarrhea by weakening immune defenses. According to studies conducted in Ethiopia and elsewhere, clinical symptoms like vomiting, fever, and dehydration are significantly associated with Rotavirus diarrheal illness (Hashi *et al*., 2016).

2.6. Laboratory Diagnosis

Rapid detection and diagnosis of enteric pathogens are crucial for early management of diarrheal illness. Additionally, it is important for prevention and control of acute gastroenteritis. Currently several laboratory diagnostic methods are available for the detection and identification of Rotavirus genotypes. These methods include Immunochromatographic test, Electron Microscopy, Enzyme Immunoassays, and Reverse Transcriptase-Polymerase Chain Reaction (Soltan *et al*., 2016).

2.6.1. Electron Microscopy

Electron Microscopy (EM) uses negative staining with phosphotungistic acid for detection of pathogens. Rotavirus can be detected using EM due to distinct morphologic appearance of virus with spikes and three layers. The electron microscopy detection threshold is approximately 10^7 virus particles/ml of stool. The sensitivity and specificity of electron microscopy for rotavirus detection is relatively high, ranging from 70% to 95% and 95% to 100% respectively (Morinet *et al*., 1984; Trépanier *et al*., 1981). However, the technique requires highly skilled professionals, expensive materials and equipment, many trained professionals to reliably detect Rotavirus from abundant stool samples (Goldsmith & Miller, 2009).

2.6.2. Enzyme Immunoassay

Enzyme Immunoassays (EIA) have substituted Electron Microscopy as the diagnostic methods for the detection of Rotavirus. This method is rapid, easy to perform, and sensitive method for the identification of Rotavirus. EIA uses reactive antibodies against VP6 capsid for detecting Rotavirus A (Soltan *et al*., 2016).

Rotavirus antigen is detected using EIA, a colorimetric reaction in a solid-phase sandwich. In addition, the optical density can be measured using an EIA plate reader. Despite having more varied sensitivity and specificity, EIAs are more sensitive than EM tests. Clinical and public health laboratories most frequently use commercially available enzyme immunoassay tests because of their excellent specificity and sensitivity (>95% each) (Bányai *et al*., 2018).

2.6.3. Polymerase Chain Reaction (PCR)

Currently, molecular methods such as Reverse Transcriptase-Polymerase Chain Reaction and real-time quantitative Reverse Transcriptase-Polymerase Chain Reaction have substituted other diagnostic methods with advantages of higher sensitivity and specificity (Soltan *et al*., 2016). It is possible to identify rotavirus in stool samples at much lower concentrations than required for detection by EM and EIA by combining Reverse Transcriptase and Polymerase Chain Reaction assays.

RT-PCR can be used to confirm that RNA extracts include rotavirus (Bányai *et al*., 2018). Reverse Transcriptase Polymerase Chain Reaction can be done singleplex or multiplex conformations in combination with real-time fluorescence capture, gel electrophoresis or probe hybridization. Compared to traditional Reverse Transcriptase Polymerase Chain Reaction assays, real-time qRT-PCR assay has many advantages: high sensitivity and specificity, rapid turnaround time, enhanced output and ability to perform viral load (Logan *et al*., 2006).

2.7. Rotavirus Vaccine

The live-attenuated oral Rotavirus vaccines show significant decrease in severe dehydration due to Rotavirus infection (Folorunso & Sebolai, 2020). WHO announced the first effective vaccine against Rotavirus diarrheal disease in 2006. Following introduction of the vaccine, mortality due to diarrhea was reduced by 16.6%, from 1.88 million to 1.57 million between 2007 and 2017 (Roth *et al*., 2018).

Rotavirus-associated deaths in children under five years were declined progressively 453,000 in 2008 to 129,000 deaths in 2016 (Troeger *et al*., 2018). The cumulative efforts of the four Rotavirus vaccines that are licensed by WHO: RotaTeq, Rotarix, Rotasil, and Rotavac, have made this possible. However, the efficacy and effectiveness of the vaccines vary expressively. The efficacy of RotaTeq and Rotarix ranged from 85% to 98% in high and middle-income countries (Ruiz-Palacios *et al*., 2006; Vesikari *et al*., 2015). While efficacy of the vaccines in low-income countries like in Africa and Asia showed relatively lower performance ranging from 51% to 64% (Madhi *et al*., 2016; Zaman *et al*., 2017).

According to 2016 Ethiopian Demographic Survey (EDHS) report, the national Rotavirus immunization coverage was around 47%. The report showed immunization coverage varies significantly from region to region. The Rotavirus immunization coverage was as low as 14.6% in Afar and as high as 77% in Addis Ababa (Abebe *et al*., 2018; Tamirat and Sisay, 2019). Another report by EDHS in 2019, the national rotavirus full immunization coverage was increased to 52.3%. Similar to 2016 survey report, in 2019 survey significant variation was observed at regional levels, with the highest immunization coverage in Addis Ababa (91.67%), Tigray (70.77%), and Dire Dawa (70.39%). In contrast, low immunization coverage was observed in the Afar (24.1%) and Somali (26.42%) regions (Geweniger and Abbas, 2020).

CHAPTER THREE

3. OBJECTIVES OF THE STUDY

3.1. General Objective

- To determine the molecular epidemiology of Rotavirus and identify circulating genotypes among children under five years with Acute diarrheal illness in Addis Ababa, Ethiopia, from September 2023 to January 2025.

3.2. Specific Objectives

- To determine the prevalence of rotavirus in Addis Ababa
- To identify the circulating genotypes of rotavirus in Addis Ababa
- To assess risk factors associated with rotavirus diarrheal disease

CHAPTER FOUR

4. METHODS AND MATERIALS

4.1. Study Area

The study was conducted in six selected health facilities in Addis Ababa, Ethiopia. Addis Ababa city is the capital of Ethiopia and the headquarters of African Union. Addis Ababa has a latitude and longitude of 9°1′48″N /38°44′24″E, with an elevation of 2,355 meters (7,726 ft) above sea level. The capital city holds 527 square kilometers of area in Ethiopia and a total population of 5.5 million out of which 411,765 are children under five years (Review, 2023).

The study was conducted in Tikur Anbessa Specialized Hospital (TASH), Teklehaimanot Health Centre (THC), Kirkos Health Centre (KHC), Lideta Health Center (LHC), Bethzatha Private Hospital (BPH), and Weyzero Beletshachew Public Health Center (WBPHC), Addis Ababa Ethiopia. TASH is the largest specialized referral hospital in Ethiopia, with over 800 beds. It provides tertiary-level referral diagnoses and treatment for approximately 400,000 patients yearly who referred from all over the country. TASH, Department of Pediatrics and Child Health serves for 12,000 admissions with 150 bed capacity and it is the largest pediatric service in the country serving as a tertiary referral center. THC has annual 29,000 out patients served with 1500 diarrhea among under-five children. KHC has annual out patient service of 27,000 patients with 1800 diarrhea under-five children. THC and KHC were selected because they had the experience with Rotavirus surveillance as sentinel health centers. LHC, BPH, and WBHC are selected because they have a good load of cases of diarrheal illness, and also closer proximity to TASH for sample transport, and storage.

4.2. Study Design and Period

A cross-sectional study was conducted from September 2023 to January 2025.

4.3. Population

4.3.1. Source population

The source population was all children under the age of five in Addis Ababa city, presenting with acute diarrheal illness.

4.3.2. Study population

Children under five years with acute diarrheal illness (defined as 3 or more watery or looser than normal stools per day in less than two weeks) who visit the four health centers, and both hospitals, were the source populations.

4.4. Eligibility

4.4.1. Inclusion criteria

- Children under the age of five with diarrhea as one of the primary symptoms and who visited health facilities,
- Provision of informed and signed consent by parents or guardians.

4.4.2. Exclusion criteria

- Patients whose residence is out of Addis Ababa were excluded

4.5. Sample Size and Sampling Technique

4.5.1. Sample size determination

The minimum sample size is determined using the single population proportion formula. The prevalence of Rotavirus among children under the age of five taken from a previous study is 17.5% (Damtie *et al*., 2024).

$$n = \frac{(Z_{\alpha/2})^2 \times P \times (1-P)}{d^2}$$

n= sample size

Z= Standard normal distribution at 95% CI which is 1.96

P= prevalence from previous study

d= margin of error which is taken as 5%

$$n = \frac{(1.96)^2 \times 0.175 \times (1-0.175)}{0.05^2}$$

$$n = \frac{3.8416 \times 0.1443}{0.0025}$$

n = ~ 222

The original design called for 222 study participants in total. However, 200 participants were enrolled because of the extremely sluggish case flow rate. Children with acute diarrheal illness included: 70 from Weyzero Beletshachew Public Health Center, 40 from Teklahaymanot Health Center, 37 from Bethzatha Private Hospital, 22 from Lideta Health Center, 16 from Kirkos Health Center, and 15 from Tikur Anbessa Specialized Hospital.

4.5.2. Sampling technique

The convenience sampling technique was employed to recruit available and accessible study participants during data collection, until the sample size was achieved.

4.6. Variables of the Study

4.6.1. Dependent variable

- Rotavirus infection status and Rotavirus genotypes

4.6.2. Independent variables

- Demographic characteristics (age, gender, etc.)
- Socioeconomic status (access to clean water, sanitation facilities, hand hygiene practices, etc.)
- Clinical information (vomiting, fever, and episodes of diarrhea)
- Vaccination status of rotavirus vaccine

4.7. Data Collection Techniques and Tools

4.7.1. Demographic and Clinical Data Collection

Data collection was conducted by using a pre-tested (English) and local language (Amharic) translated structured questionnaire after getting written informed consent from the parents or guardians. The interview included information about socio-demographic characteristics of parents/guardians and child, clinical information, and other information was collected and transferred to the questionnaire prepared for the study (see Annex-I, II, III).

The questionnaire was filled out by the attending trained nurses or a supporting healthcare professional.

4.7.2. Stool Sample Collection

Safety procedures were implemented before collection of stool samples by wearing Personal Protective Equipment (PPE) such as gloves, a lab coat, and possibly a face mask and then stool specimens were collected in a clean, leak-proof, screw-capped container. Parents/guardians were instructed to provide approximately 5ml or about 2 tablespoons of stool sample.

The container was labeled properly with a unique participants ID number, the date, and time of collection. The sample was stored at 4°C at the collection sites until it was transported to the Microbiology Laboratory, College of Health Sciences, Addis Ababa University. The samples were kept in an ice box with ice packs during transport and stored at -40°C until molecular analysis was done. Permission was obtained from Ethiopian Food and Drug Authority (EFDA) (Ref no: 02/15/1124/01, Tir 26, 2017) to ship the samples to Kamuzu University of Health Sciences (KUHES), Blantyre, Malawi for TaqMan PCR analysis.

4.8. Molecular Investigations

The Laboratory analysis was done at the Kamuzu University of Health Sciences (KUHES), Blantyre, Malawi.

4.8.1. Polymerase Chain Reaction (PCR) Analysis

Nucleic acid extraction

Before the RT-PCR assay, Rotavirus group A dsRNA was extracted from stool suspensions using the Viral RNA Mini-Kit (Qiagen, Hilden, Germany). A volume of 200µl fecal sample underwent a lysate preparation process that included mechanical disruption (bead beating), removal of inhibitors, purification, and elution of RNA using spin columns in the QIAmp Fast RNA Stool Mini Kit. Extrinsic controls PhHV (Phocine Herpesvirus) and MS2-Bacteriophages, were added to each sample during the lysate preparation to evaluate extraction and amplification efficiency. The extracted total nucleic acid (TNA) is then stored at -80°C for further testing. To rule out contamination during the extraction process, a blank is also processed through the complete protocol for each batch of extractions.

TaqMan RT-PCR Assay

The TaqMan Array Card (TAC) is a real-time PCR format. The TAC can detect multiple enteric pathogens and resistance genes on a single card for 8 specimens at a time has designed by University of Virginia. The enteric targets include viruses, bacteria, protozoa, helminths, and resistance genes (Table 3.1).

Table 3.1: Pathogens and Antibiotic resistance Markers can be detected by TaqMan array cards

Port	
L	R
Adenovirus 40/41	Astrovirus & Sapovirus V
Norovirus GI & GII	Sapovirus I/II/IV
Rotavirus	Aeromonas & Plesiomonas
Campylobacter jejuni&coli	C. upsaliensis & C. infans
H. pylori	Salmonella
Shigella/EIEC (ipaH)	V. cholerae
S. flexneri 1a & 1b & 7 (gtrI&gtrC)	S. flexneri 2a & 2b & X (gtrII&gtrX)
S. flexneri 3a & 3b & 5a & 5b (oac&gtrV)	S. flexneri 6 & 4a & 4b (wzx6&gtrIV)
S. flexneri opt & Shigella (ipaH3)	S. sonnei & S. flexneri non6
EPECT_bfpA & eae	EAEC_aaiC & aatA
ETEC_STh & STp	ETEC_LT
ETEC_CFA/I & CS1	ETEC_CS2 & CS3
ETEC_CS5 & CS6	STEC_stx1 & stx2
18S	Bacterial 16S
MS2 & PhHV	MS2 & PhHV
Cryptosporidium	Giardia
Cyclospora & Isospora	E. histolytica & E. bieneusi
ermB & mphA	mefA & mphB
ermA & ermC	msrA & msrD
MCR1 & MCR2	Shigella/E.coli gyrA 83
OXA-48	OXA-1 & OXA-9
CTX-M1 & CTX-M8-M25	CTX-M2-M74 & CTX-M9
SHV & SHV238-240SE-SK	TEM104E & 104K
TEM164R & 164S-C	TEM238G & 238S

The TaqMan Array Card features two ports, designated as Left and Right, each containing 24 wells. These wells are specifically assigned to detect the aforementioned pathogens and resistance genes, enabling targeted multiplex real-time PCR analysis.

Master mix for 8 samples was prepared by mixing 425µL of Ag-Path-ID 2X RT-PCR buffer and 34µL of Ag-Path-ID Enzyme mix. 54µL of the master mix were added into each of eight 1.5mL microcentrifuge tubes. To each tube, 20µL of total nucleic acid extract from stool specimens were added then supplemented with 26µL nuclease-free water. Mixed gently, then centrifuged the tubes to eliminate air bubbles from the mixtures. The TAC cards were allowed to reach room temperature in the original packaging. After mixing properly, 100µL of PCR reaction mix loaded into the fill port of the cards and centrifuged two times at 1,200 rpm for 1 min. The card was sealed, the loading ports were removed, and then the card was inserted into a ViiA7 instrument (Quant Studio Real-Time PCR, Life Technologies). The Ag-Path-ID One-Step RT-PCR kit was used, and the cycling conditions were as follows: 45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min (see the details in Annex V).

Rotavirus dsRNA was reverse transcribed to complementary DNA (cDNA) using random primers (Invitrogen, Paisley, UK) and reverse transcriptase enzyme (Superscript III MMLV-RT, Invitrogen) (Iturriza-Gomara *et al*., 1999). The cDNA was used to assign G and P genotypes (see annex V).

Genotyping of Rotavirus A

Rotavirus positive A samples were genotyped based on cDNA to assign G genotype (G1, G2, G3, G4, G8, G9, G10, G12, eG3) and P genotype (P[4], P[6], P[8], P[11]) using a multiplex reverse-transcription polymerase chain reaction (RT-PCR) assay using oligonucleotide primers **F:** ACCATCTWCACRTRACCCTCTATGAG, and **R:** GGTCACATAACGCCCCTATAGC(Liu *et al*., 2013).

Primers used for genotyping (Table 3.2)

Table 3.2: Primers used for genotyping of Rotavirus A

Gene/ Genot-ype	Primer name	Primer details (positions)	Product size (bp)	References
G1	9T1-1	TCTTGTCAAAGCAAATAATG (nt 176 to 195)	158	(Das et al., 1994)
G2	9T1-2	GTFAGAAATGAYTTCTCCACT (nt 262 to 281)	244	(Das et al., 1994)
G3	9T-3P	GTCCAGTTIGCAGTGTTAGC (nt 484 to 503)	466	(Das et al., 1994)
eG3	9T-3P	TCTTGTCAAAGCAAATAATG (nt 176-195)	158	(Das et al., 1994)
G4	9T-4	GGGTCGATGGAAAATTCT (nt 423 to 440)	403	(Das et al., 1994)
G8	HT8	CGGTTCCGGATTAGACAC (nt 273-256)	274	(Gouvea et al., 1994a)
G9	9T-9B	TATAAAGTCCATFGCAC (nt 131 to 147)	110	(Das et al., 1994)
G10	ET10	TTCAGCCGTTGCGACTTC (nt 714-697)	715	(Gouvea et al., 1994b)
G12	12T-1	GTTGGAGGTGGAGGAGTT (nt 640-760)	700	(Gentsch et al., 1992)
P[4]	2T-1	CTATTGTTAGAGGTTAGAGTC (nt 474 to 494)	483	(Gentsch et al., 1992)
P[6]	3T-1	TGTTGATTAGTTGGATTCAA (nt 259 to 278)	423	(Gentsch et al., 1992)
P[8]	1T-1	TCTACTTGGATAACGTGC (nt 339 to 356)	345	(Gentsch et al., 1992)
P[11]	pB223	GGAACGTATTCTAATCCGGTG (nt 574-594)	314	(Gouvea et al., 1994a)

Quant Studio Real-Time PCR Software v1.3 was used to run the qPCR experiment and to automatically analyze the data and return Ct (cycle threshold) values. The Enteric shiny app (http://shiny.uvaenterics.org:3838/TAC/) was used to predict the correct Ct value without the need of manual adjustment of a baseline. Specimens were considered positive if the amplification curve crossed the threshold line with a Ct value< 35, when the extraction blank was negative (Ct ≥ 35) for this target.

When the extraction blank amplifies for a certain target with Ct < 35, any sample result with Ct < 35 for that target is interpreted as invalid (see annex v).

4.9. Data Quality Assurance

Data quality was maintained by conducting a pretest to check the clarity and comprehensibility of the questionnaires. Data collectors received training on filling out questionnaires, sample collection protocols, sample handling, and stored. All data and samples collected from study participants were labeled with a unique identifier.

Any confusing or ambiguous items were refined before initiating the full-scale data collection, ensuring that respondents understand and interpret the questions correctly. Moreover, data cleaning procedures were applied to remove outliers, missing data, or inconsistencies, and handle missing data appropriately. Additionally, adequate stool samples were collected in stool cup and labeled with the participant's identification code.

Both during the sample collection and analysis, an aseptic technique was followed. Additionally, standard operating procedures (SOPs) were followed for the sample collection, storage, shipment, nucleic acid extraction, master-mix preparation, loading TAC card, PCR analysis, and the TaqMan Array Card output data analysis. Additionally, both endogenous and extrinsic controls were run along with the samples as per the manufacturer's instructions. Likewise, blanks were prepared for each batch of the extraction and run along with the samples

A proper extraction kit (Viral RNA Mini-Kit) was used in this study, and quality controls were included to monitor the efficiency of the extraction process. The biosafety cabinet was cleaned with 70% alcohol before and after nucleic acid extraction and master mix preparation to prevent contamination. Each step of the TaqMan Array Card procedures: nucleic acid extraction, master mix preparation, card loading, and the PCR analysis was done in separate rooms with distinct personal protective equipment in each room, which helped avoid cross-contamination. The data were entered and analyzed using appropriate software. The results were interpreted cautiously, documented precisely, reported clearly and briefly.

4.10. Ethical Considerations

This study is part of the main ongoing project entitled “NIHR Global Health Research Group on Gastrointestinal Infections: Facilitating the Introduction and Evaluation of Vaccines for Enteric Diseases in Children in Eastern and Southern sub-Saharan Africa”.

Ethical approval was obtained from CHS-Institutional Research Ethics Review Committee (IRERC, Protocol no. 030/23/Pedi, July 6, 2024), MOH-National Research Ethics Review Board (NRERB, Ref no. 17/152/356/24, September 17, 2024) with Material Transfer Agreement (MTA) approval and Central University Research Ethics Committee, University of Liverpool, UK.

For the M. Sc project ethical approval was also obtained from Department Research Ethics Review Committee (DRERC/004/2024, March 20, 2024) of Department of Microbiology, Immunology, and Parasitology, College of Health Sciences, Addis Ababa University.

A patient information sheet was prepared and informed consent was obtained from parents/ guardians before data was collected (see Annex I and II). Confidentiality of the data was maintained throughout the study using a unique code number.

4.11. Data Processing and Analysis

For Laboratory result data: Quant Studio Real-Time PCR Software v1.3 and Shiny App were used to automatically analyze the data and returning Ct (cycle threshold) values. Ct value less than 35 was considered as a positive.

For associated factors: data was cleared, encoded for computer entrance, and entered into Epi-data version 4.7 computer program. After computer entrance, data was exported to and analyzed using SPSS version 26. The result was presented using tables, and graphs and was interpreted using the Chi-square and odds ratio. The chi-square test was used to check the association between dependent and independent variables. P-value <0.05 was considered statistically significant.

4.12. Project management

Data cleaning and cross-verification procedures were conducted to ensure the accuracy and completeness of the dataset. The questionnaire was reviewed to identify and retrieve any missing information.

Throughout the study period, all laboratory and clinical data were systematically documented using appropriate records and securely archived on digital media such as CDs, flash cards, and email attachments.

4.13. Operational Definitions

Diarrheal illness: three or more loose or watery stools per day or a definite decrease in consistency and increase in frequency based upon an individual baseline as noted by a caregiver.

Children: 0 to 60 months children with acute diarrhea

Caregiver or Guardian: a mother or father or anyone else responsible for keeping care of the child close.

4.14. Funding source

The M.Sc. project is part of the project entitled" NIHR Global Health Research Group on Gastrointestinal Infections: Facilitating the Introduction and Evaluation of Vaccines for Enteric Diseases in Children in Eastern and Southern sub-Saharan Africa. " The project is a collaborative research effort between Addis Ababa University, Ethiopia, and the University of Liverpool, UK. It was funded by the NIHR (NIHR133066) using UK aid from the UK Government to support global health research. All expenses for this M.Sc. project were covered by the funds obtained from the project and Addis Ababa University.

CHAPTER FIVE

5. RESULTS

5.1. Socio-Demographic Characteristics of the Study Participants

During the study period from September 2023 to January 2025, a total of 200 children under five years old experiencing acute diarrheal illness were participated in the selected health facilities in Addis Ababa as shown in the attached Table 4.1.

Table 4.1: Distribution of children investigated for diarrheal illness in selected health facilities in Addis Ababa

Study sites	Number of children investigated
Tikur Anbessa Specialized Hospital (TASH)	15
Teklehaimanot Health Centre (THC)	40
Kirkos Health Centre (KHC)	16
Lideta Health Center (LHC)	22
Bethzatha Private Hospital (BPH)	37
Weyzero Beletshachew Public Health center (WBPHC)	70
Total	200

Among all the study participants, 108 (54%) were males. The age distribution of the diarrheic children revealed the highest number of participants, 54 (27%) in the 48-60 months age group. Among the 200 children, 61 (30.5%) attend daycare or school, while only 21 (10.5%) were exclusively breastfed as outlined in (Table 4.2).

Table 4.2: Socio-Demographic Status of the Study Participants.

Variables	Category	Frequency	Percent
Sex	Male	108	54
	Female	92	46
Age in months	<6	5	2.5
	6 to <12	11	5.5
	12 to < 24	31	15.5
	24 to <36	50	25

	36 to <48	49	24.5
	48 to <60	54	27
Day care visit	Yes	61	30.5
	No	139	69.5
Breastfeeding status	Exclusive breastfeeding	21	10.5
	Non-exclusive breastfeeding	75	37.5
	Not applicable	104	52

5.2. Clinical Findings of the Study Participants

In terms of diarrhea consistency, 122 (61%) children had watery stools, 67 (33.5%) had loose stools, and 11 (5.5%) had bloody stools. Additionally, 84 (42%) of the children experienced vomiting alongside diarrhea, while 12 (6%) of the study participants were admitted to health facilities. Most of the children (90%) have been vaccinated against Rotavirus (Table 4.3).

Table 4.3: Clinical Features of the Study Participants.

Variables	Category	Frequency	Percent
Consistency of diarrhea	Loose	67	33.5
	Watery	122	61
	Bloody	11	5.5
Vomiting	Yes	84	42
	No	116	58
Vaccination status	Yes	180	90
	No	20	10
Hospitalization	Yes	12	6
	No	188	94
Fever	Yes	71	35.5
	No	129	64.5
Dehydration	Severe dehydration	4	2
	Moderate dehydration	32	16
	No dehydration	164	82

5.3. Socio-Demographic Characteristics of Caregivers (Parents/Guardians) of the Study Participants

Most caregivers, 118 (59%), were housewives. The majority of caregivers, 129 (64.5%), have additional children under 5 years old in the household. Regarding their education level, 71 (35.5%) have completed greater than secondary school. Regarding their drinking water sources, 89 (44.5%) drink tap water without any treatment, 48 (24%) drink water after treatment, and 63 (31.5%) drink bottled water. According to the caregivers response, 99 (49.5%) of them use communal toilets, 96 (48%) use private toilets, and the remaining 5 (2.5%) utilize open field defecation. Concerning their solid waste disposal, 175 (87.5%) collect their waste in a bag, while 25 (12.5) of them dispose in the main compound as outlined in the attached (Table 4.4)

Table 4.4: Socio-Demographic Characteristics of Caregivers of Study Participants.

Variables	Category	Frequency	Percent
Number of children in home (< 5-years of age)	1	71	35.5
	2	89	44.5
	3	38	19
	4	2	1
Education level	None educated	13	6.5
	Less than primary	13	6.5
	Primary school only	52	26
	Secondary school	51	25.5
	Greater than secondary school	71	35.5
Occupation of parents/Guardians	Not employed	18	9
	Housewife	118	59
	Business (self-employed)	26	13
	Business (Other employed)	23	11.5
	Professional (e.g. Teacher, Health care worker)	13	6.5
	Casual laborer	2	1
Source of water	Tap water without treatment	89	44.5
	Treated water	48	24
	Bottled water	63	31.5
Toilet	Private	96	48
	Communal	99	49.5
	Open field	5	2.5
Waste disposal	In the main compound	25	12.5

	Collected in a bag	175	87.5

5.4. Prevalence of Rotavirus

Out of sssthe 200 diarrheic stool samples investigated, rotavirus was detected in 45 (22.5%) using TaqMan Array Card Real-Time PCR assay (Table 4.5) and the detection rate of rotavirus across selected health facilities are shown in (Table 4.6).

Table 4.5: Detection rate of Rotavirus among children with acute diarrheal illness.

Rotavirus	Frequency	Percent
Positive	45	22.5
Negative	155	77.5
Total	200	100

Table 4.6: Detection Rate of Rotavirus Among Children with Acute Diarrheal Illness.

Study sites and number of children investigated	**Frequency**	**Percent**
Tikur Anbessa Specialized Hospital (n=15)	4	8.9
Teklehaimanot Health Centre (n=40)	13	28.9
Kirkos Health Centre (n=16)	4	8.9
Lideta Health Center (LHC) (n=22)	2	4.4
Bethzatha Private Hospital (n=37)	15	33.3
Weyzero Beletshachew Public Health Center (n=70)	7	15.6
Total = 200	45	100

5.5. Genotypic Characterizations of Rotavirus

All the 45 rotavirus positive samples were genotyped based on VP7 (G genotypes) and VP4 (P genotypes). Of the 45 positive samples, 40 (88.9%) were genotyped based on VP7 and 39 (86.7%) were genotyped based on VP4. The circulating G genotypes identified were G1, G2, G3, G4, G8, G9, G10, and G12 whereas the P genotypes included P[4], P[6], and P[8].

G2 was the prevalent G type, identified in 10 (22.2%), while G1 in 9 (20%) and G8 in 6 (13.3%.) The proportions of circulating P-types were P[4] 18 (46.2%), P[8] 15 (38.5%), and P[6] 6 (15.3%).

In this study, thirteen G-P genotype combinations have been identified. G1P[4], G2P[4], G3P[8], G8P[4], G9[P4], G9P[8], G2P[6], G12P[8], G10P[8], G4P[8] and G1P[8] are the predominant genotypes identified (Table 4.7). Out of these, three new genotypes, G8P[4], G10P[8], and G4P[8] were detected for the first time in Ethiopia.

Table 4.7: Distribution of Rotavirus Genotypes in Children with Acute Diarrheal Illness.

Genotypes	Frequency	Percent
G1P[4]	7	15.6
G2P[4]	5	11.1
G3P[8]	3	6.7
G8P[4]	3	6.7
G9P[4]	2	4.4
G9P[8]	2	4.4
G2P[6]	2	4.4
G12P[8]	2	4.4
G10P[8]	2	4.4
G1P[8]	2	4.4
G4P[8]	2	4.4
G8P[8]	1	2.2
G8P[6]	1	2.2
P[6]	2	4.4
G8	1	2.2
Mixed G	5	11.1
Mixed P	3	6.7
Total	**45**	**100%**

5.6. Rotavirus and its association with the study participants sociodemographic status

In this study, the positivity rate of rotavirus was higher in females than males (26.1% vs. 19.4%). Rotavirus was detected at a higher proportion (54.5%) among children age 6-12 months. However, both sex and age are not significantly associated with rotavirus-induced diarrhea ($p>0.05$) (Table 4.8).

The positivity rate of rotavirus was higher among daycare attendants as compared to non-daycare attendants (34.4% vs. 17.3%). However, this study found no significant correlation between daycare/school attendance and rotavirus diarrhea ($p>0.05$).

The positivity rate of rotavirus was highest among children with bloody diarrhea [27.3% (3/11)] compared to children with watery diarrhea [26.2% (32/122)] and having loose stools [14.9% (10/67)]. The consistency of diarrhea was not significantly associated with rotavirus diarrhea.

In the present study, children with vomiting had a higher positivity rate for rotavirus than those without vomiting (46.4% vs. 5.2%). This study found a significant association between vomiting and rotavirus-induced diarrhea ($p<0.05$), with an AOR of 22.638 (7.411,69.152) for children with vomiting.

The rate of rotavirus detection was higher (55%) among children who were not vaccinated against rotavirus compared to vaccinated children (18.9%). This study reported a significant association between rotavirus vaccination status and rotavirus infection ($p<0.05$), with an adjusted odds ratio (AOR) of 13.864 (2.835,67.809) for non-vaccinated children.

Although the rotavirus detection rate was slightly higher among non-hospitalized children (22.9%) relative to hospitalized children (16.7%), no statistically significant correlation between rotavirus infection and hospitalization found in this study.

In present study, the detection proportion of rotavirus was slightly higher among exclusively breastfed children (28.6%) compared to non-breastfed children (23.2%) and non-exclusively breastfed children (20%). However, there is no significant association between rotavirus infection and breastfeeding status.

Generally, only two factors (vomiting and vaccination status) were significantly associated with rotavirus; while other associated factors were not significantly correlated with rotavirus infection (Table 4.8).

Table 4.8: Rotavirus Infection and factors associated to children with acute diarrheal illness

Variables	Category	Rotavirus positive no. (%)	Rotavirus negative no. (%)	COR (95% CI)	AOR (95% CI)	P-value
Sex	Male	21 (19.4)	87 (80.6)	0.684 (0.351,1.331)	0.799 (.315,2.031)	.638
	Female	24 (26.1)	68 (73.9)	1	1	
Age in months	<6	1 (20)	4 (80)	1	1	
	6 to <12	6 (54.5)	5 (45.5)	4.8 (.397,58.013)	6.149 (.102,369.618)	.385
	12 to < 24	7 (22.6)	24 (77.4)	1.167 (.112,12.202)	1.362 (.030,61.220)	.874
	24 to <36	5 (10)	45 (90)	0.444 (.041,4.793)	0.384 (.008,17.712)	.625
	36 to <48	8 (16.3)	41 (83.7)	0.78 (.077,7.931)	.891 (.021,38.672)	.952
	48 to <60	18 (33.3)	36 (66.7)	2 (.208,19.227)	2.052 (.041,102.581)	.719
Day care visit	Yes	21 (34.4)	40 (65.6)	2.516 (1.265,5.002)	1.539 (.436,5.429)	.503
	No	24 (17.3)	115 (82.7)	1	1	
Consistency of diarrhea	Loose	10 (14.9)	57 (85.1)	1	1	
	Watery	32 (26.2)	90 (73.8)	2.027 (.926,4.438)	1.369 (.430,4.365)	.595
	Bloody	3 (27.3)	8 (72.7)	2.137 (.483,9.459)	1.793 (.153,21.032)	.642
Vomiting	Yes	39 (46.4)	45 (53.6)	15.889(6.289,40.143)	22.638 (7.411,69.152) *	.000
	No	6 (5.2)	110 (94.8)	1	1	
Vaccination status	Yes	34 (18.9)	146 (81.1)	1	1	
	No	11 (55)	9 (45)	5.248 (2.016,13.663)	13.864 (2.835,67.809) *	.001
Hospitalization	Yes	2 (16.7)	10 (83.3)	0.674 (.142,3.196)	0.428 (.058,3.146)	.404
	No	43 (22.9)	145 (77.1)	1	1	
Breastfeeding status	Exclusive breastfeeding	6 (28.6)	15 (71.4)	1	1	

	Non-exclusive breastfeeding	15 (20)	60 (80)	0.625 (.207,1.883)	2.399 (.416,13.834)	.328
	Unknown	1 (20)	4 (80)	0.625 (.057,6.801)	1.115 (.059,21.016)	.942
	Not applicable	23 (23.2)	76 (76.8)	0.757 (.263,2.174)	1.536 (.248,9.495)	.644

1=indicate reference group * Significant association at P-value <0.05

5.7. Rotavirus and its association with children's caregivers/guardians

Rotavirus and its association with risk factors related to children's caregivers or guardians is outlined in the attached Table 4.9. The positivity rate of rotavirus was highest (28.2%) among children whose caregivers' or guardians' education level is higher than secondary school. However, this study found no significant association ($p>0.05$) between the guardians' education level and rotavirus infection.

In this study, the positivity rate of rotavirus was highest (53.8%) among children whose parents or guardians had professional occupations (teachers, healthcare workers, etc.) and lowest (16.9%) among children whose parents or guardians were housewives. However, this study found no significant association ($p<0.05$) between rotavirus and parents' occupations.

In terms of sources of drinking water, the detection rate of Rotavirus infection was highest (31.3%) among users of treated tap water, compared to users of untreated tap water (21.3%) and bottled water users (17.5%). However, this study found no significant association ($p>0.005$) between Rotavirus and drinking water sources.

Although there is no significant association ($p>0.05$) between rotavirus and the toilet usage of the children's parents or guardians, the detection rate of rotavirus was highest (28.1%) among private toilet users, compared to open field users (20%) and communal toilet users (17.2%).

The detection rate of Rotavirus was highest (28%) among children whose parents or guardians disposed of solid waste in the main compound, compared to those who collected solid waste in a bag (21.7%). However, no significant association between rotavirus and the method of solid waste disposal.

Table 4.9: Factors Associated to Children's Caregivers/Guardians Related to Rotavirus Positivity

Variables	Category	Positive no. (%)	Negative no. (%)	COR (95% CI)	AOR (95% CI)	P-value
Education level	None/illiterate	3 (23.1)	10 (76.9)	0.765 (.191-3.071)	0.985 (.190-5.095)	.986
	Less than primary	2 (15.4)	11 (84.6)	0.464 (.094-2.280)	0.662 (.105-4.166)	.661
	Primary school only	9 (17.3)	43(82.7)	0.534 (.220-1.293)	0.912 (.318-2.620)	.864
	Some secondary school	11(21.6)	40 (78.4)	0.701 (.301-1.631)	1.09 (.403-2.950)	.865
	secondary school or greater	20 (28.2)	51 (71.8)	1	1	
Occupation of parents/ Guardians	Not employed	4 (22.2)	14 (77.8)	1	1	
	Housewife	20 (16.9)	98 (83.1)	0.714 (.213-2.397)	0.748 (.213-2.629)	.650
	Self-employed	6 (23.1)	20 (76.9)	1.05 (.249-4.422)	0.968 (.218-4.312)	.966
	Other employer	7 (30.4)	16 (69.6)	1.531 (.369-6.351)	1.402 (.296-6.627)	.670
	Professional	7 (53.8)	6 (46.2)	4.083 (.861-19.371)	3.547 (.643-19.577)	.146
	Casual laborer	1 (50)	1 (50)	3.5 (.177-69.339)	4.266 (.190-95.608)	.360
Source of water	Tap water without treatment	19 (21.3)	70 (78.7)	1	1	
	Treated water	15 (31.3)	33 (68.7)	1.675 (.757-3.703)	1.105 (.420-2.901)	.840
	Bottled water	11 (17.5)	52 (82.5)	0.779 (.342-1.778)	0.595 (.235-1.510)	.274
Toilet	Private	27 (28.1)	69 (71.9)	1	1	
	Communal	17 (17.2)	82 (82.8)	0.53 (.267-1.052)	0.56 (.258-1.215)	.143
	Open field	1 (20)	4 (80)	0.639 (.068-5.977)	0.567 (.048-6.690)	.652
Waste disposal	In main compound	7 (28)	18 (78)	1.402 (.545-3.604)	1.877 (.633-5.571)	.257
	Collected in bag	38 (21.7)	137 (78.3)	1	1	

1= Indicate reference group

CHAPTER SIX: DISCUSSIONS

In this study, 200 children under five years with acute diarrheal illness were enrolled. Among the study participants, males were predominant (54%) in this study. This finding aligns with previous studies carried out in Ethiopia that demonstrated males were predominant among children with acute diarrheal illness compared to females (Damtie *et al*., 2024; Yassin *et al*., 2012).

In the current study, the major clinical presentations were vomiting (35.5%) and fever (42%), in addition to diarrhea. These symptoms are in line with those described in other studies done in Ethiopia, where vomiting and fever were common symptoms observed in diarrheal rotavirus infection (Damtie *et al*., 2024).

This study found Rotavirus as an important etiologic agent of acute diarrheal illness. In this study, the prevalence of Rotavirus was 22.5%. The prevalence is a little higher than in prior studies conducted in Ethiopia after the introduction of the Rotavirus vaccine, in Wagera district a prevalence of Rotavirus was 18% among children under five years with acute diarrheal illness using EIA (Feleke *et al*., 2018). Similarly, a multi-centered cross-sectional study conducted among children under five years using RT-PCR in the Amhara region found a prevalence of 17.5% (Damtie *et al*., 2024).

On the other hand, the prevalence of rotavirus in this study agrees with studies conducted in Ethiopia before the introduction of the rotavirus vaccine, in Addis Ababa among children under five years with acute diarrheal illness found a prevalence of 21% (Abebe *et al*., 2014) and 22% in young children with acute diarrheal illness in Hawassa using EIA for virus detection (Yassin *et al*., 2012). However, the prevalence of rotavirus in this study is lower than that reported in a cross-sectional study from Gondar in the post-vaccination era 25%. This study included children under five years with acute diarrheal illness, using RT-PCR laboratory method for the detection and characterization of Rotavirus (Gelaw *et al*., 2018). Variations in the prevalence rates could be attributed to the differences in geographic distribution, laboratory methods, and vaccination status.

The prevalence of rotavirus in this study is in line with studies conducted during post vaccination era in different countries such as in Ukraine (20.7%) (Soloviov *et al*., 2022), Bangladesh (24%) (Dey *et al*., 2020), and Nepal (22.9%) (Dhital *et al*., 2017).

In contrast, the prevalence of rotavirus reported in this study was lower than the findings of other studies conducted in the post-vaccination era in different developing countries, such as a cross-sectional study conducted in Lusaka, Zambia among young vaccinated children; detected a Rotavirus in 36%, out of 424 of enrolled participants using EIA (Simwaka *et al*., 2021), a molecular surveillance conducted among Gabonese young children have shown 55% prevalence of Rotavirus infection out 244 enrolled children (Manouana *et al*., 2021). Similarly, a cross-sectional study done in Nampula, Mozambique found a 34.9 % prevalence of Rotavirus out of 614 enrolled young children using EIA (Chissaque *et al*., 2021). Other comparable studies among the same population using EIA described prevalence of Rotavirus as follows: 36% in Zimbabwe (Mukaratirwa *et al*., 2018), and 30.8 % in Nigeria (Maina *et al*., 2022).

The prevalence of Rotavirus in present study is also lower than the findings of studies carried out in developed nations, such as 30.5% of participants were positive for Rotavirus among 95 enrolled children in Colombia (Martinez-Gutierrez *et al*., 2019) and 37.2% of children under five years were Rotavirus positive in Malaysia (Amit *et al*., 2023). This differences in prevalence of Rotavirus across the countries could be attributable to the methods used, sample size, geographic variations.

In contrast, the prevalence in this study is higher than in other studies conducted during the post-vaccination period in developing and developed countries: a cross-sectional study done in India found 18.8% of Rotavirus positive out of 794 enrolled children by using EIA (Chaudhary *et al*., 2021). Another comparable study carried out in Indonesia among children under five years detected Rotavirus in 8.9% of the participant using EIA (Prasetyo *et al*., 2022). Similarly, out of 365 enrolled young children with acute diarrheal illness in Nairobi, Kenya, in 14.5% Rotavirus was identified using EIA (Nzisa, 2017). The prevalence differences among the countries might be due to the Laboratory methods used, differences in countries' prevention and control strategies, and lifestyles.

Among 45 Rotavirus A-positive samples, eight distinct G-types (G1, G2, G3, G4, G8, G9, G10, and G12) were identified in this study with G2 being the most prevalent (22.2%), followed by G1 (20%), G8 (13.3%), and other unusual G-types like G10. Even though G10 from human samples was reported for the first time in Ethiopia, it was identified from bovines in a multi-centered study conducted in the Amhara region, Ethiopia (Damtie *et al*., 2024). It is important to do whole genome sequencing to check nucleotide similarities between human G10 and bovine G10.

The G genotypes finding of this study contradict with previous studies in Ethiopia, where the predominant G genotypes were G3, G12 and G1 (Damtie *et al*., 2020). These variations could be due to emergence of new strain, differences on targeting genotypes or various factors not explored in this study. On the other hand, G types finding in this study agrees with results from different countries, such as in South Africa (Mwangi *et al*., 2022), Brazil (Gurgel *et al*., 2007), South Korea (Thanh *et al*., 2018), and Kenya (Gikonyo *et al*., 2020), where G2 was the predominant genotype after the introduction of the rotavirus vaccine.

In this study, among 45 Rotavirus A positive samples, three different P genotypes: P[4], P[6], and P[8] were identified, with P[4] being the predominant genotype (46.2%), followed by P[8] (38.5%), and P[6] (15.3%). The predominant P genotype finding in this study contradicts previous studies in the country, where the predominant P genotype was P[8] during pre-vaccination era (Damtie *et al*., 2020). The predominant P-type shift from P[8] in pre-vaccination era to P[4] in post-vaccination could be due to the effect of Rotarix vaccine the country has introduced. On the other hand, the predominant P genotypes of rotavirus in this study align with findings from studies in other countries (Abdel-Haq *et al*., 2003; Maina *et al*., 2022).

In this study diversity of Rotavirus genotype combinations were detected, G1P[4] (15.6%), G2P[4] (11.1%), G3P[8] (6.7%), G8P[4] (6.7%), G9P[4] (4.4%), G9P[8] (4.4%), G2P[6] (4.4%), G12P[8] (4.4%), G10P[8] (4.4%), G1P[8] (4.4%) and G4P[8] (4.4%).

The predominant circulating Rotavirus genotype combinations in Addis Ababa, Ethiopia during pre-vaccination era were G1P[8], G12P[8], and G3P[6]. However, following the introduction of Rotarix vaccine, genotypes like G9P[8], G3P[6], G2P[4], and G3P[8] had emerged as the predominant strains in the city. As demonstrated in a previous systematic review and meta-analysis study done in Ethiopia, the predominant circulating genotypes were G12P[8] (15.4%), G3P[8] (12.9%), G2P[4] (11.8%), and G9P[8] (6.8%) (Damtie *et al*., 2020). The predominant circulating genotypes showed a shift to G1P[4], G2P[4] and G3P[8] in this study. These findings presented a diversity of rotavirus genotypes, notifying the complexity of rotavirus genotypes circulating in the country. Similar findings were reported in the USA (41%) (Abdel-Haq *et al*., 2003), India (20%) (Raorane *et al*., 2020), Nigeria (15%) (Maina *et al*., 2022), and Indonesia (12.5%) (Wahyuni *et al*., 2021). In addition to human samples, G1P[4] and G1P[8] were the predominant genotypes of rotavirus identified from the Nile River in Gaza, Egypt (Rizk and Allayeh, 2020). This shows G1P[4] is a significant genotype detected in both human and environmental samples. Therefore, the genotypes in both sample types need to undergo whole-genome sequencing to determine the genetic closeness.

Three new genotypes, G8P[4], G10P[8], and G4P[8] were detected for the first time in Ethiopia. Even though G8P[4] has been reported for the first time in Ethiopia in this study, it has been identified in different African countries, Europe, and America. In Rwanda, Kenya, and Uganda, G8P[4] underwent whole genome sequencing and had the highest nucleotide similarities in these countries (Mwangi *et al*., 2023). Although the genotype was repeatedly identified in Africa, it was also reported in other continents, like America (Weinberg *et al*., 2012) and Europe (Ianiro *et al*., 2014).

G10P[8] was another uncommon Rotavirus genotype that was identified in this study. This genotype combination was a rare human genotype which result from reassortment between bovine G10 and human P[8] types. Although G10P[8] is a rare human genotype and has not been identified in Ethiopia before, it was reported in human samples in West Africa (Esona *et al*., 2011), Turkey (Durmaz *et al*., 2014), and Vietnam (Matsushima *et al*., 2012), suggesting that G10P[8] has a wider geographical distribution, probably in regions with close human-animal interactions or where transmission of zoonotic infection could occur.

The identification of such uncommon genotype underscores the diversity and evolution of Rotavirus genotypes, highlighting the importance of continuous surveillance to monitor emerging strains that could influence vaccine efficacy.

The other genotype that was reported for the first time in Ethiopia by this study was G4P[8]. However, this genotype has been identified as a common circulating Rotavirus genotype in Qatar (Mathew *et al*., 2023), Turkey (Öncesi *et al*., 2024), and Italy (Ianiro *et al*., 2015). The discovery of G4P[8] in Ethiopia suggests this strain may be spreading to new areas. Keeping an eye on these emerging genotypes is important because they could affect how well current vaccines work and influence our efforts to control rotavirus. This finding highlights the need for ongoing monitoring to better understand how these viruses are changing worldwide and within our own communities.

In this study, children with vomiting had higher positivity rate of rotavirus infection than those without vomiting (46.4% vs. 5.2%). The present study found a statistically significant association between vomiting and rotavirus related diarrhea ($p<0.05$). This finding agrees with other studies carried out in Ethiopia (Damtie *et al*., 2024; Feleke *et al*., 2018) and elsewhere (Rerksuppaphol & Rerksuppaphol, 2011).

Regarding vaccination status, the positivity rate of rotavirus was higher (55%) among unvaccinated children against rotavirus compared to vaccinated children (18.9%). This study found a statistically significant association between rotavirus vaccination status and rotavirus positivity ($p<0.05$). This finding is consistent with the findings of other studies conducted elsewhere (Patel *et al*., 2009).

Strengths of the study

This study used an advanced molecular technique, TaqMan Array Card (TAC), for the detection and genotype identification of Rotavirus. The technique is sensitive and specific method that can detect multiple enteric pathogens (bacteria, viruses, and parasites responsible for diarrheal illness) at the same time, accurately. Hence, this approach can be an advanced-level diagnostic tool for future research on enteric pathogens.

Conclusion

This study provides an important insight into the updated prevalence and circulating genotypes of Rotavirus among the population. These findings underline that despite the presence of the Rotarix vaccine in the country, Rotavirus remains a significant etiologic agent for acute gastroenteritis. Therefore, continuous Rotavirus surveillance and implementation of prevention and control strategies are crucial to reduce the burden of Rotavirus-related diarrheal illness.

Recommendations

Based on the findings of the current study, the following recommendations are made: -

- Future studies should include the whole-genome sequencing to have a comprehensive understanding about the genetic diversity and evolutionary dynamics of circulating Rotavirus strains.
- Future studies should investigate animal sources and environmental sampling, including water sources, sewage, and surfaces to identify reservoirs and transmission pathways of rotavirus.
- Given the diversity of circulating rotavirus genotypes, policymakers should evaluate the potential benefits of introducing supplementary vaccines alongside Rotarix.

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ANNEXES

ANNEX I: INFORMATION SHEET FOR PARENTS/GUARDIANS (ENGLISH VERSION)

Title of the Research Project: Molecular epidemiology and Genotypic Characterization of Rotavirus among children under age five with acute diarrheal illness in Addis Ababa, Ethiopia

Name of the Organization: Addis Ababa University, College of Health Sciences, School of Biomedicine and Laboratory Sciences, Department of Microbiology, Immunology and Parasitology.

Introduction

Your child is invited to participate as a study participant in research conducted by MSc candidate, from Addis Ababa University. Your child's participation is voluntary by obtaining a stool specimen. The research teams will include one principal investigator and three advisors; from Addis Ababa University, the Medical Microbiology unit, and Pediatrics Department. Please take as much time as you need to read or listen to the information sheet.

Purpose of the Research Project: We are researching to determine molecular epidemiology and genotypic characterizations of Rotavirus among children under the age of five with acute diarrheal illness in Addis Ababa, Ethiopia

Participation We are asking you and others to voluntarily participate in this study. Your child is expected to give a stool sample.

Procedures and the expected participation

We are asking you and others to voluntarily participate in this study. If you are willing to participate, you need to understand the purpose of the study and give your consent. Not only this but also specimens collected from your child will be used for the research purpose. You are requested to give your consent to the sample collector. After consent, a sample will be collected by Nurse professionals. Moreover, there will be a face-to-face interview for additional questions which will take about 10 minutes.

Risks: There is no risk that your child may acquire by participating in this study

Potential benefits to subjects and/or to the society

You will not receive any payment for participation in this research study as compensation.

The result of the study will be beneficial for identifying the genotype of Rotavirus circulating in children under age five in Addis Ababa, Ethiopia. The study will help healthcare professionals, health bureaus, the Ministry of Health, and policymakers in the country to increase vaccine coverage and design appropriate infection prevention mechanisms.

Hence, you are indirectly benefiting other patients and society in this respect.

Confidentiality All the data obtained will be kept strictly confidential by using only code numbers that are filled in by the investigators and locking the data.

Right to refuse and withdraw from the Study

Since participation in this study is entirely voluntary, you can refuse to participate in this study at any time. Your refusal will not affect you and the services you want. You may withdraw at any time and place without consequences of any kind.

Contact information

If you have any questions or complaints concerning the study you can ask them at the following address**:**

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Thank you for your Participation and Cooperation!

የመረጃና ውል ቅፅ ለወላጅ ወይም ለአሳዳጊ (Information Sheet Amharic version for Parents/Guardians)

የጥናቱ ርዕስ: በአዲስ አበባ ከአምስት አመት በታች በሆኑ ህጻናት ላይ የሮታቫይረስ ስርጭት እና የዘረመል ባህሪይ ልየታ

የድርጅቱ ስም: አዲስ አበባ ዩኒቨርሲቲ፣ የጤና ሳይንስ ኮሌጅ፣ የማይክሮባዮሎጂ ኢሚኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል

መግቢያ: የአዲስ አበባ ዩኒቨርሲቲ የሁለተኛ ዲግሪ እጩ በሚያደርገው ጥናት ላይ ልጅዎ የጥናት ተሳታፊ በመሆን እንዲሳተፍ/ እንድትሳተፍ ተጋብዟል/ተጋብዘለች። የልጅዎ ተሳትፎ በፈቃደኝነት ነው። የምርምር ቡድኖቹ አንድ ዋና መርማሪ እና ሦስት አማካሪዎችን ያካትታሉ፣ ከአዲስ አበባ ዩኒቨርሲቲ የሕክምና ማይክሮባዮሎጂ ክፍል። እባክዎን በመረጃ ወረቀቱ ላይ ለማንበብ ወይም ለማዳመጥ የሚፈልጉትን ያህል ጊዜ ይውሰዱ።

የጥናቱ ዓላማ: በአዲስ አበባ ከአምስት ዓመት በታች የሆኑ ልጆች የሮታቫይረስ ስርጭት እና የዘር ሐረግ ባህሪ ልየታ ምርምር እያደረግን ነው።

ተሳትፎ: ልጅዎ እና ሌሎች በዚህ ጥናት ውስጥ በፈቃደኝነት እንዲሳተፉ እንጠይቃለን። ልጅዎ የሰገራ ናሙና መስጠት ይጠበቅበታል።

ሂደቶች እና የሚጠበቀው ተሳትፎ: ልጅዎ እና ሌሎች በዚህ ጥናት ውስጥ በፈቃደኝነት እንዲሳተፉ እንጠይቃለን። ለልጅዎ መሳተፍ ፍቃደኛ ከሆኑ የጥናቱን አላማ ተረድተው ፍቃድዎን መስጠት አለብዎት። ይህ ብቻ ሳይሆን ከልጅዎ የሚሰበሰቡ ናሙና ለምርምር ዓላማው ጥቅም ላይ ይውላሉ። ለምርመራ የሰገራ ናሙና መስጠት ይጠበቅበታል። ለናሙና ሰብሳቢው ፈቃድዎን እንዲሰጡ ተጠይቀዋል። ከስምምነት በኋላ፣ ናሙናው በነርስ ባለሙያዎች ይሰበሰባል። በተጨማሪም፣ 10 ደቂቃ ያህል የሚወስድ ተጨማሪ ቃለ መጠይቅ ይኖራል።

ጉዳቶች: ሊያገኟት የሚችል ጉዳት የለም።

ለተሳታፊ ወይም ለህብረተሰቡ ሊሆኑ የሚችሉ ጥቅሞች:

በዚህ ጥናት ለመሳተፍዎ እንደ ማካካሻ ምንም አይነት ክፍያ አያገኙም። በዚህ ምርምር መካፈላችሁ ልጃችሁን በቀጥታ ይጠቅሙታል። ከዚህ ጥናት የተገኙ ውጤቶች የጤና ሰራተኞችን እና ፖሊሲ አውጪዎችን ለማሳወቅ ጥቅም ላይ ይውላል፤ ይህም ከሮታቫይረስ ኢንፌክሽን ጋር በተያያዘ ከ5 ዓመት በታች የሆኑ ህፃናትን ክትባት ለማሻሻል ስልቶችን ለማመቻቸት ነው።

ሚስጥራዊነት: ሁሉም የተገኘው መረጃ በተመራማሪዎች የተሞላ እና መረጃውን በመቆለፍ ኮድ ቁጥሮችን ብቻ በመጠቀም ሚስጥራዊ ይሆናል።

ከጥናቱ የመውጣት እና የመቃወም መብት:

ይህ ምርምር ሙሉ በሙሉ በፈቃደኝነት ለይ የተመረኮዘ ነው ፤ እናም በዚህ ጥናት ውስጥ መሳተፍም እምቢ ማለትም ይችላሉ። ልጅዎ እና ለልጅዎ የሚፈልጉትን አገልግሎቶች አይጎዳውም። ሌላ ማንኛውም ተጽዕኖ ሳይኖር ከዚህ ጥናት መውጣት ይችላሉ።

ለተጨማሪ መረጃ፦

ጥናቱን በተመለከተ ማንኛውም አይነት ጥያቄ ወይም ቅሬታ ካሎት በሚከተለው አድራሻ መጠየቅ ይችላሉ።

ዋና ተመርማሪ፦ ገመቺስ ደጀኔ (በአዲስ አበባ ዩኒቨርሲቲ በህክምና ማይክሮባዮሎጂ ዘርፍ ኤም.ኤስ.ሲ እጩ)

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አማካሪዎች፦

1. ዳንኤል አስራት (ኤም.ዲ.፣ ፒኤችዲ፣ ፕሮፌሰር) አማካሪ ሜዲካል ስፔሻሊስት (ሜዲካል ማይክሮባዮሎጂስት) የማይክሮ ባዮሎጂ፣ ኢሚውኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል፣

የባዮሜዲሲን እና ላቦራቶሪ ሳይንስ ትምህርት ቤት፣ የጤና ሳይንስ ኮሌጅ፣ አዲስ አበባ ዩኒቨርሲቲ አዲስ አበባ፣ ኢትዮጵያ

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ተባባሪ አማካሪ

1. ዶ/ር አለም አብርሃ (ፒኤችዲ፣ ረዳት ፕሮፌሰር) የማይክሮ ባዮሎጂ፣ ኢሚውኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል፣ የባዮሜዲሲን እና ላቦራቶሪ ሳይንስ ትምህርት ቤት፣ የጤና ሳይንስ ኮሌጅ፣ አዲስ አበባ ዩኒቨርሲቲ አዲስ አበባ፣ ኢትዮጵያ

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ለተሳትፎ እና ትብብርዎ እናመሰግናለን!

ANNEX II: INFORMED CONSENT FOR PARENTS/ GUARDIANS (ENGLISH VERSION)

Name of the main researcher: Gemechis Dejene, AAU.

Name of the institute: Addis Ababa University

Funded by: Addis Ababa University and Global Health Research Group on Gastrointestinal infection

Reviewed by: _____

Research Title: Molecular Epidemiology and genotypic characterization of Rotavirus among children under age five with acute diarrheal illness in Addis Ababa, Ethiopia

If you agree to take part, please read this form and sign the consent sheets at the end.

I have read, or it was read to me, the information sheet in the language I understand concerning this study and I understand what will be required of me if my child takes part in the study. I am aware of the possible risks and benefits of this study. I know that being in this study is voluntary. I understand that at any time I withdraw my child from this study without giving a reason and without affecting my child care. I know that there is no special payment for participating my child in the study and I agree my child to take part in this study.

I, being the guardian/parent of_____ (Name of child) have understood all that has been read and my questions have been answered satisfactorily and I agree for my child to participate in this research study by giving stool samples.

Parent/Guardian Printed Name: Date (DD-MM-YYYY)

_____ _____

Parent/Guardian Signature

_____

በመረጃ የተደገፈ ስምምነት ለወላጅ ወይም ለአሳዳጊ (Amharic version of Informed consent for parents/guardians)

የዋና ተመራማሪው ስም፡ ገመቺስ ደጀኔ፣ አዲስ አበባ ዩኒቨርሲቲ

የተቋም ስም፡ አዲስ አበባ ዩኒቨርሲቲ

በአዲስ አበባ ዩኒቨርሲቲ እና በግሎባል ሄልዝ ጥናትና ምርምር ቡድን የተደገፈ

የተገመገመው:_____

የምርምር ርዕስ፡ በአዲስ አበባ ከአምስት አመት በታች በሆኑ ህጻናት ላይ የሮታቫይረስ ስርጭት እና የዘረመል ባህሪይ ልየታ

ለመሳተፍ ከተስማሙ፣ እባክዎ ይህን ቅጽ ያንብቡ እና የስምምነት ወረቀቶቹን በመጨረሻ ይፈርሙ።

ይህን ጥናት በሚመለከት በምረዳው ቋንቋ የተዘጋጀውን የመረጃ ወረቀት አንብቤዋለሁ ወይም ተነቦልኛል እና በጥናቱ ውስጥ ልጄን ለማሳተፍ ምን እንደሚያስፈልገኝ ተረድቻለሁ። ይህ ጥናት ሊያስከትል የሚችለውን ጉዳት እና ጥቅሞች አውቃለሁ። በዚህ ጥናት ውስጥ ልጄን ማስገባት በፈቃደኝነት እንደሆነ አውቃለሁ። ምክንያቱን ሳልገልጽ እና የልጄ መደበኛ እንክብካቤ ሳይነካ በማንኛውም ጊዜ ከዚህ ጥናት ልጄን መውጣት እንደምችል ተረድቻለሁ። በጥናቱ ውስጥ ለመሳተፍ የተለየ ክፍያ እንደሌለው አውቃለሁ እናም በዚህ ጥናት ልጄን ለማሳተፍ ተስማምቻለው።

እኔ የ_____ (የልጁ ስም) አሳዳጊ/ወላጅ በመሆኔ የተነበቡትን ሁሉ ተረድቻለሁ እና ለጥያቄዎቼ አጥጋቢ ምላሽ አግኝቻለሁ እናም ልጄ የሰገራ ናሙና በመስጠት በዚህ የምርምር ጥናት ላይ እንዲሳተፍ ተስማምቻለሁ።

የወላጅ / አሳዳጊ ስም	ቀን (ቀን-ወር-ዓመት)
_____	_____

የወላጅ / አሳዳጊ ፊርማ

_____

ANNEX III: QUESTIONNAIRE (ENGLISH VERSION)

ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES, SCHOOL OF BIOMEDICINE AND LABORATORY SCIENCES

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY, AND PARASITOLOGY

In Addis Ababa, we are conducting basic research on the prevalence of Rotavirus among children under five years and the genotypic characteristics. This questionnaire is to assess the demographic characteristics, Clinical information, and socio-economic status of children and caregivers for evaluation of independent variables of Rotavirus among children in Addis Ababa, Ethiopia. Therefore, we humbly request all the requested participants to come forward and fill it honestly. If you need clarification, please ask the coordinator.

Case recording form

Case identification code_____

Date_____

Study site _____

A. SOCIO DEMOGRAPHIC DATA

1. Age: _____ months

2. Sex Male ☐ Female ☐

3. Birth order: 1st ☐ 2nd ☐and above ☐

4. Number of household members/ family size_____

5. Number of children living in the household_____

6. Where does waste disposal take place? (Tick all that apply):

In the main compound ☐In a garbage pit☐ open field ☐

7. Toilet available: No ☐ private ☐ communal ☐ open field ☐

8. Treatment of drinking water: Nothing☐ Boil ☐ bottled water ☐ Others (Specify) _____

9. Does the child attend day care/ school: Yes☐ No ☐

B. CLINICAL DATA

1. What are the presenting symptoms?

1.1. Diarrhea Yes☐ No☐

1.2. Is the diarrhea: loose ☐watery ☐ bloody ☐

1.3. Number of days the child has diarrhea _____

1.4. Episodes of diarrhea in 24 hours _____

1.5. Fever Yes☐ No☐

1.6. Cough Yes☐ No☐

1.7. Difficulty breathing/fast breathing Yes☐ No☐

1.8. Unable to drink Yes☐ No☐

1.9. Vomiting Yes☐ No☐

1.10. Episodes of vomiting in 24 hours _____

1.11. Convulsions Yes☐ No☐

1.12. Lethargy or unconsciousness Yes☐ No☐ others (specify) _____

1.13. Inability to breast feed or to drink Yes☐ No☐

2. Breast feeding status: Exclusive breast feeding ☐ Non-exclusive breast feeding ☐

Unknown☐ not applicable ☐

3. Is the child requiring hospitalization? Yes☐ No☐

4. Physical examination

4.1. Vital signs: HR _____ RR _____ Temperature_____

4.2. Anthropometric Data

Weight_____

Height/ length_____

Head circumference_____

MUAC_____

Weight-for-age (z-score) _____

Height/length –for-age (z-score) _____

Weight-for-height/length (z-score) _____

4.3. Sunken eyes: Yes☐ No☐

4.4. Sunken fontanel: Yes☐ No☐

4.5. Skin pinch goes back: normal ☐ slowly ☐ very slowly ☐

4.6. Signs of shock any of the following: cold skin, prolonged capillary refill, fast weak Pulse. Yes☐ No☐

4.7 Degree of dehydration: No dehydration ☐ some dehydration ☐ Severe Dehydration ☐

4.8. Was your child vaccinated for the Rotavirus? Yes☐ No☐

4.9. How many doses the child took One ☐ Two ☐ Not Known ☐

C. Socio-economic status of Family / Guardian

1. Occupation of Parents:

Business (other employed) ☐ Student ☐ Housewife ☐

Business (self-employed) ☐ Not employed ☐ Farmer ☐

Professional (e.g. Teacher) ☐ Casual laborer ☐ Unknown ☐

Other, please specify_____

D. Laboratory Data

Stool specimen Consistency: Watery ☐Bloody ☐ Mucoid☐ Mixed☐

1. Stool RT-PCR result for rotavirus: Positive/Negative
2. Genotyping analysis result _____

Name of data collector _____

Signature _____ Date _____

መጠይቅ (Questionnaire Amharic Version)

በአዲስ አበባ ከአምስት አመት በታች በሆኑ ህጻናት ላይ የሮታቫይረስ ስርጭት እና የዘረመል ባህሪይ ልየታ ላይ መሰረታዊ ጥናቶችን እያደረግን ነው። ይህ መጠይቅ የሕፃናትን የስነ ሕዝብ አወቃቀር ባህሪያት፣ ክሊኒካዊ መረጃ እና ማህበራዊ-ኢኮኖሚያዊ ሁኔታ ለመገምገም ነው። ስለሆነም ሁሉም የመጠየቁ ተሳታፊዎች ቀርበው በቅንነት እንዲሞሉ በትህትና እንጠይቃለን። አስታዉሱ ፤ ሥም መፃፍ አያስፈልግም። ማብራሪያ ከፈለጉ አስተባባሪውን ይጠይቁ።

የህሙማን መረጃ መመዝገብያ ቅጽ

የህመምተኛው መለያ ቁጥር _____

ቀን _____

የጥናት ቦታ _____

ሀ. ማህበራዊ መረጃ

1. እድሜ፡ _____ ወራት

2. ጸታ፡ ወንድ _____ ሴት _____

3. የህጻኑ ውልደት ቅደም ተከተል፡ 1 ኛ _____ 2ኛ ____እና ከዚያ በላይ ____

4. የቤተሰብ አባላት ብዛት _____

5. በቤት ውስጥ ነዋሪ ህጻናት ቁጥር _____

6. ቆሻሻ ማስወገጃ ስፍራ (ምልክት ያድርጉ)

በዋናው ግቢ ውስጥ _____ በቆሻሻ ጉድጉዋድ _____ በባዶ ሜዳ ላይ _____

7. የመጸዳጃ ቤት መኖር፡ የለም _____ የግል _____ የማህበረሰብ _____ በባዶ ሜዳ ላይ _____

8. የመጠጥ ውሃ አጠቃቀም፡ ከምንጭ ____ ማፍላት _____ የታሸገ ውሃ _____ ሌላ (ይገለጽ) _____

9. ህጻኑ ት/ቤት/መዋእለ ህጻናት ይከታተላል ፡ አዎ _____ የለም _____

ለ. የክሊኒካል መረጃ

1. ህመምኛው የህመም ስሜት

1.1. ተቅማጥ _____ አዎ _____ የለም _____

1.2. የተቅማጡ አይነት፡ ለስላሳ _____ ውሃማ _____ ደም የቀላቀለ _____

1.3. ስንት ቀን አስቀመጠው (አመመው/ማት): _____

1.4. በቀን ስንት ጊዜ ያስቀምጠዋል/ጣታል: _____

1.5. ትኩሳት፡ አዎ _____ የለም _____

1.6. ሳል፡ አዎ _____ የለም _____

1.7. የአተነፋፈስ ችግር/ቶሎ ቶሎ መተንፈስ፡ አዎ _____ የለም _____

1.8. መጠጣት አለመቻል፡ አዎ _____ የለም _____

1.9. ትውከት፡ አዎ _____ የለም _____

1.10. በቀን ስንትጊዜ ያስመልሰዋል: _____

1.11. ማንቀጥቀጥ፡ አዎ _____ የለም _____

1.12. ህሊና መሳት፡ አዎ _____ የለም _____ ሌላ (ይገለጽ) _____

1.13. ጡት መጥባት/መጠጣት አለመቻል፡ አዎ _____ የለም _____

2. የጡት ማጥባት ሁኔታ፡ ጡት ብቻ¨_____ ከጡት ሌላ ተጨማሪ መስጠት _____ አይታወቅም _____ አይመለከትም _____

3 . ህጻኑ ሆስፒታል መተኛት ያስፈልገዋል? አዎ _____ የለም ____

4. የአካል ምርመራ መረጃ

4.1 ዋና ምልክቶች፡ የልብ ምት ፍጥነት ____ የአተነፋፈስ ፍጥነት ____ የሰውነት ሙቀት _____

የአካል ልኬት

ክብደት ____ ቁመት/ርዝመት _____

ክብደት ለ እድሜ (ዚ ስኮር) _____

ቁመት/ርዝመት ለ እድሜ (ዚ ስኮር) ____

ክብደት ለ ቁመት/ርዝመት (ዚ ስኮር) _____

4.3 የአይን መሰርጎድ ፡ አዎ _____ የለም _____

4.4 የርግብግቢት መሰርጎድ ፡ አዎ _____ የለም _____

4.5 የቆዳ መለጠጥ፡ ቶሎ ይመለሳል _____ ይዘገያል _____ በጣም ይዘገያል _____

4.6 ማንኛውም የሾክ ምልክት (የቀዘቀዘ ቆዳ፣ ያጣት ደም ቶሎ አለመመለስ፣ ፈጣንና ደካማ የደም ቡዋንባ ምት)፡ አዎ _____ የለም _____

4.7 የሰውነት ድርቀት ደረጃ፡ ድርቀት የለም _____ መካከለኛ ድርቀት _____ የከፋ (በጣም) ድርቀት _____

4.8. ልጅዎ የሮታ ቫይረስ ክትባት ወስዱዋል/ዳለች? አዎ ወስዱዋል/ዳለች _____ አልወሰደም(ችም)

_____ አይታወቅም _____

4.9. ክትባቱን ስንት ጊዜ ወሰደ/ች? አንድ___ ሁለት ___ ሦስት ___ አይታዎቅም ____

ሐ. የቤተሰብ /አሳዳጊ/ መረጃ

1. የወላጅ መተዳደሪያ ሥራ: የመንግስት ሠራተኛ ___ ተማሪ ___ ገበሬ _____

የቤት እመቤት _____ የግል ሥራ_____ ባለሙያ _____ አይታወቅም _____

ሌሎች(ግለጥ).....

የላቦራቶሪ መረጃ

1. የ RT-PCR ውጤት ለሮታቫይረስ: አለ/ የለም
2. የጂኖታይፕ ትንተና ውጤት : _____

አስተያየቶች

_____

የውሂብ (መረጃ) ሰብሳቢ ስም _____

ፊርማ _____ ቀን _____

ANNEX IV: SAMPLE COLLECTION PROCEDURES

A. MATERIALS REQUIRED

- Sterile, leak-proof, wide-mouth specimen containers with secure lids.
- Personal Protective Equipment (PPE)
- Label stickers
- Labelling markers

B. PROCEDURES

1. Verify patient identity against medical records
2. Obtain informed consent from the parent/guardian
3. Give instruction to caregivers/guardians on how to collect stool samples
4. Collect ~5ml of stool samples
5. Label the sample with participant identification number, study site, date and time of sample collection
6. Store the samples at 4°C at study sites until it is transported to Addis Ababa University, Tikur Anbessa Specialized Hospital, Laboratory.
7. Transport the samples in ice box
8. Store the samples at -40°C until for molecular analysis

ANNEX V: LABORATORY PROCEDURES

I. Stool Nucleic Acid Extraction

1. PURPOSE

This procedure describes a modified extraction procedure to extract both DNA and RNA from fecal specimens using the QIAmp Fast DNA Stool Mini Kit.

2. PRINCIPLE

This procedure is to purify the total nucleic acid from stool specimens for further pathogen detection with qPCR. The fecal sample undergoes a lysate preparation process and includes mechanical disruption (bead beating), removal of inhibitors, purification and elution of DNA and RNA using spin columns in the QIAmp Fast DNA Stool Mini Kit. Extrinsic controls PhHV (Phocine Herpesvirus) and MS2 are added to each sample during the lysate preparation to evaluate extraction and amplification efficiency. The extracted total nucleic acid (TNA) is then stored at -80°C for testing. To rule out contamination during the extraction process, a blank is also processed through the complete protocol for each batch of extractions.

PhHV and MS2: Careful preparation, storage and use of the MS2 and PhHV are essential because detection of these extrinsic controls is necessary to accurately evaluate the pathogen target PCR results. Detection of MS2 in the sample confirms the extraction and amplification of RNA and DNA were successful. When MS2 is not detected in the sample, it is not possible to accept the results of other RNA targets that were not detected, thus negative RNA target results in that sample would be invalid. MS2 and PhHV detection rates of <90% or Cts consistently above 32 for either PhHV or MS2 may indicate a procedural or reagent problem and should be investigated before continuing with extractions.

Extraction Blank: Processing a blank each day/batch of extractions helps to validate sample results in downstream assays. The extraction blank should be positive only for MS2 and PhHV. A pathogen target detected in an extraction blank may indicate a wider contamination problem.

Positive results for that target in any sample that was extracted on that same day or within that batch would be invalid. The lab should address possible sources of the contamination before continuing with extractions.

The sensitivity of PCR assays requires adherence to good laboratory practices to avoid cross-contamination between samples and laboratory contamination.

3. MATERIALS

- QIAamp Fast DNA Stool mini Kit (cat 51604, 50 preps) plus additional collection tubes (~1 per sample, Qiagen, Cat 19201).
- InhibitEX Buffer
- Buffer AL
- Proteniase K
- Buffer AW1
- Buffer AW2
- Buffer ATE
- MS2/PhHV mix
- Pipettes and compatible aerosol filter tips
- 96%-100% Ethanol
- 2ml screw cap tubes, compatible with bead beater (Fisher 72.694.406/NC9950149; Consider batch pre-filling the tubes with beads if possible)
- 2.0 ml microcentrifuge tubes
- Glass beads, acid-washed 212-300 µM (50-70 U.S. sieve) (Sigma G1277-500G)
- 0.5 ml screw cap tubes for TNA storage
- Fecal Sample
- -70 to -80°C freezer
- Bead beater
  - BioSpec Mini-Beadbeater (BioSpec 693, Mini-Beadbeater-8) -Bead disruption at maximum speed for 2 minutes is sufficient.
- Dry bath 70°C and 95°C
- Vortex mixer
- Microcentrifuge

4. PROCEDURES

Procedure Notes:

- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- Change pipet tips between all liquid transfers. The use of aerosol-barrier pipet tips is recommended.
- After all, vortexing steps, to avoid contamination pulse centrifuge the tubes to remove drops from the inside of the lid.
- Pipet the sample into the QIAamp Mini spin column without moistening the rim of the column.
- Avoid touching the QIAamp membrane with the pipet tip.
- Close the QIAamp Mini spin column before placing it in the microcentrifuge.
- Open only one QIAamp Mini spin column at a time.
- For efficient parallel processing of multiple samples, fill a rack with collection tubes

1. Mix InhibitEX Buffer thoroughly by shaking before use. If a precipitate has formed, incubate at 37-70°C for ~15 minutes or until all precipitate has fully dissolved
2. Prepare fresh each day: Add PhHV and MS2 working solutions to InhibitEX buffer: prepare only enough for N+1 samples to be processed that day: 1ml InhibitEX buffer +1ul MS2/PhHV mix is needed per sample (e.g. For 10 samples 11ml InhibitEX+ 11ul MS2/PhHV mix), mix well.
3. If the beads have not been pre-aliquoted to empty tubes: Add : ~370 mg (one Eppendorf tube capful) of Sigma beads to the aliquot of stool, or the flocked swab tip (or blank tube).
4. Add 1 ml of the Inhibit EX/PhHV/MS2 solution into each specimen tube (or blank). Vortex for 1 min.
5. Bead beat at maximum speed for 2-3 minutes (instrument dependent, see section 7) Incubate the suspension for 5 min at 95°C.
6. Vortex for 15 s then centrifuge sample at full speed (approximately 20,000g) for 1 min to pellet the stool particles.

7. Pipet 25 µl proteinase K into a new 2 ml microcentrifuge tube (snap caps acceptable). Pipet 600 µl supernatant from step 8 into the 2 ml microcentrifuge tube containing proteinase K. Note: Do not transfer any solid material, if necessary centrifuge sample again.
8. Add 600 µl Buffer AL. Note: Do not add proteinase K directly to Buffer AL.
9. Vortex for 15 s, mix thoroughly to form a homogeneous solution. Incubate at 70°C for 10 min.
10. Centrifuge briefly to remove drops from the inside of the tube lid.
11. Add 600 µl of ethanol (96–100%) to the lysate, and mix by vortexing
12. Centrifuge briefly to remove drops from the inside of the tube lid.
13. Label QIAamp spin column lid and place in a 2 ml collection tube. Carefully apply 600 µl of the lysate from step 14 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min.
14. Retain QIAamp spin column, place the column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.
15. Repeat 2 more times to use all of the lysate: carefully apply an additional 600 µl of the lysate from step 14 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. retain the column. Discard the collection tube and filtrate. Repeat until all of the lysate (~1800 ul in total) has been through the spin column.
16. Place the spin column in a new collection tube.
17. Open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.
18. Open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Retain QIAamp spin column, discard the collection tube containing the filtrate.
19. To eliminate the chance of possible Buffer AW2 carryover place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with

filtrate. Centrifuge at full speed for 3 min. Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications.

20. **Elute the total nucleic acid:** Transfer the QIAamp spin column into a labeled microcentrifuge tube. Open the QIAamp spin column and pipet 200 µl Buffer ATE directly onto the QIAamp membrane. Close the cap and incubate for 1-3 min at room temperature, then centrifuge at full speed for 1 min to elute the TNA.
21. **Save the filtrate**: this is the TNA. Discard the column.
22. Prepare two tubes for aliquot storage. Pipet 100 µl of the extracted TNA into each tube. Store at -70 to –80°C.

Preparation and Storage of MS2/PhHV mix

MS2 and PhHV are incorporated during extraction as extrinsic controls in order to evaluate the extraction and amplification efficiency of RNA and DNA. This protocol describes the preparation and storage of MS2/PhHV mix.

Material

1. Lyophilized MS2/PhHV mix, store at -80C until use. Reconstitute one vial at a time.
2. STD Diluent, store at -80C

Protocol

1. Quickly spin the tube to bring the pellet to the bottom.
2. Add 50 µl diluent to each vial, mix well.
3. Use 1 µl per sample extraction follow the extraction SOP. Store the leftover at -80C. Don't use the reconstituted mix that has been frozen-thawed more than two times.

II. Enteric TaqMan Array Card

1. PURPOSE

This procedure describes the detection of enteric pathogens by TaqMan Array card (TAC).

2. INTRODUCTION/PRINCIPLE

Enteric infections are caused by a wide variety of pathogens and simultaneous infection with multiple pathogens can be common, thus making comprehensive diagnostic testing challenging. The TaqMan Array Card (TAC) is a 384-well real-time PCR format for TAC-compatible instrument platforms.

The Houpt Lab at the University of Virginia has developed a custom enteric TAC capable of detecting multiple targets on a single card for 8 samples. The enteric TAC targets include viruses, bacteria, fungi, protozoa, and helminths. MS2 and phocine herpes virus (PhHV) are added to the fecal samples during nucleic acid extraction as extrinsic controls to monitor extraction and amplification.

For quality assurance, each lot of enteric TACs undergoes QC at the University of Virginia (UVA) before being sent to the site labs. Site labs run QC as suggested below or as needed for troubleshooting the assays.

2.1. **Positive controls**: TAC Positive Control is a synthetic construct containing all targets in a single tube (plasmid for DNA targets and in vitro transcripts for RNA viruses). These controls should be run once every six months or coinciding with machine calibration, whichever is less. These controls also serve as standard curve material for quantification analyses.

2.2 Negative Control (No Template Control): Master mix with Nuclease Free water. A negative control should be run once every 10 cards.

MATERIALS

2.1. Enteric TaqMan Array Card (custom designed by UVA, manufactured by ThermoFisher)

2.2. Ag-Path-ID One Step RT-PCR Kit (ThermoFisher Cat. Nos. AM1005, 4387424, 4387391)

2.3. Nuclease-Free Water

2.4. Pipettes (2-20µL, 20-200µL, and 100-1000µL) and compatible aerosol filter tips

2.5. 1.5mL Microcentrifuge tubes

2.6. TAC positive controls

2.7. Nucleic acid extracts from stool or extraction blank

3. Equipment

3.1. Life Technologies TAC-compatible instrument platform with TAC block installed (ViiA 7, QuantStudio 7 Flex, or QuantStudio 12K Flex)

3.2. Compatible Sorvall or Heraeus Centrifuge with TAC buckets (see Centrifuge-Compatibility-TAC.pdf, available at https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/PCR/qPCR/pdfs/Centrifuge-Compatibility-TAC.PDF)

3.3. -70 to -80°C freezer

3.4. -20°C freezer

3.5. Vortex

3.6. Microcentrifuge

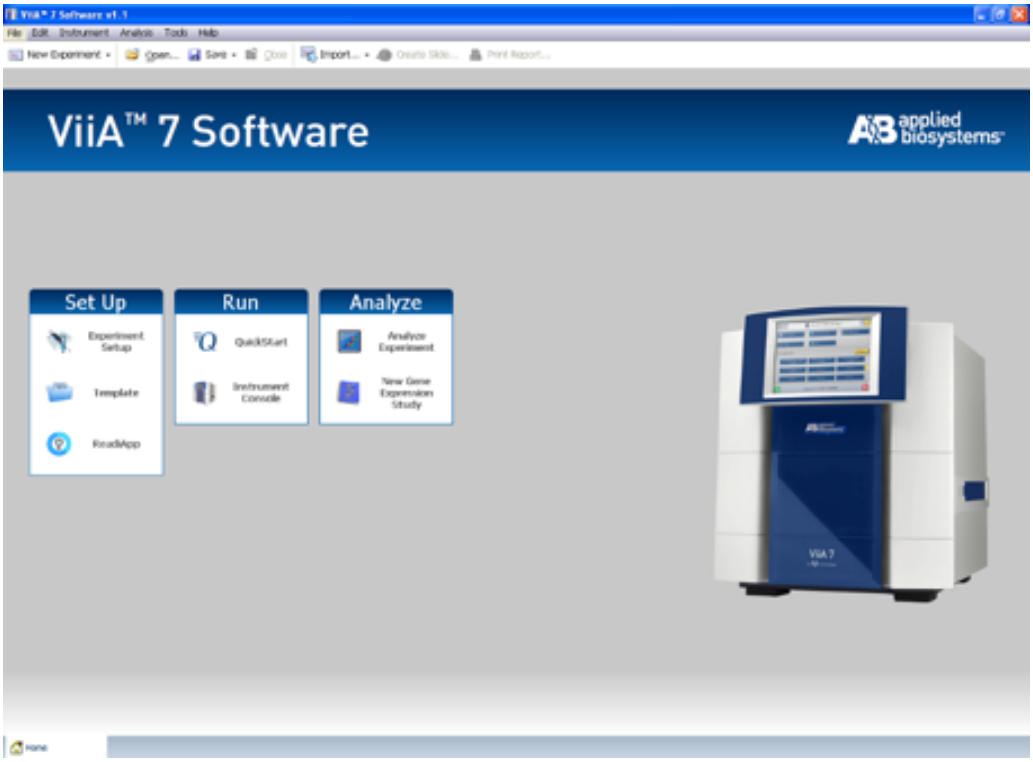
4. PROCEDURES

4.1. Equipment preparation

4.1.1. Ensure that the correct thermocycling block, heated cover, and loading tray are installed.

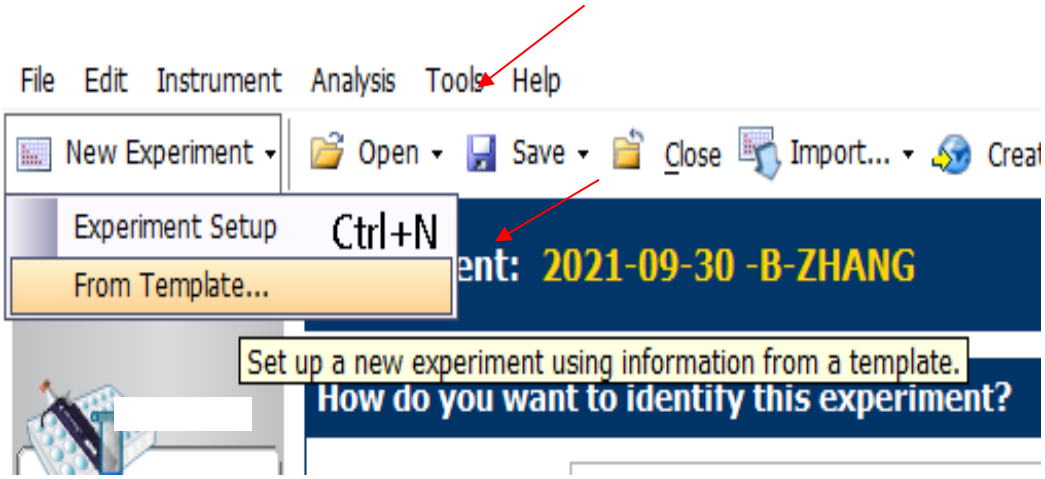
4.1.2. Turn on the instrument and then the computer. Open the ViiA 7 or QuantStudio software programs as appropriate.

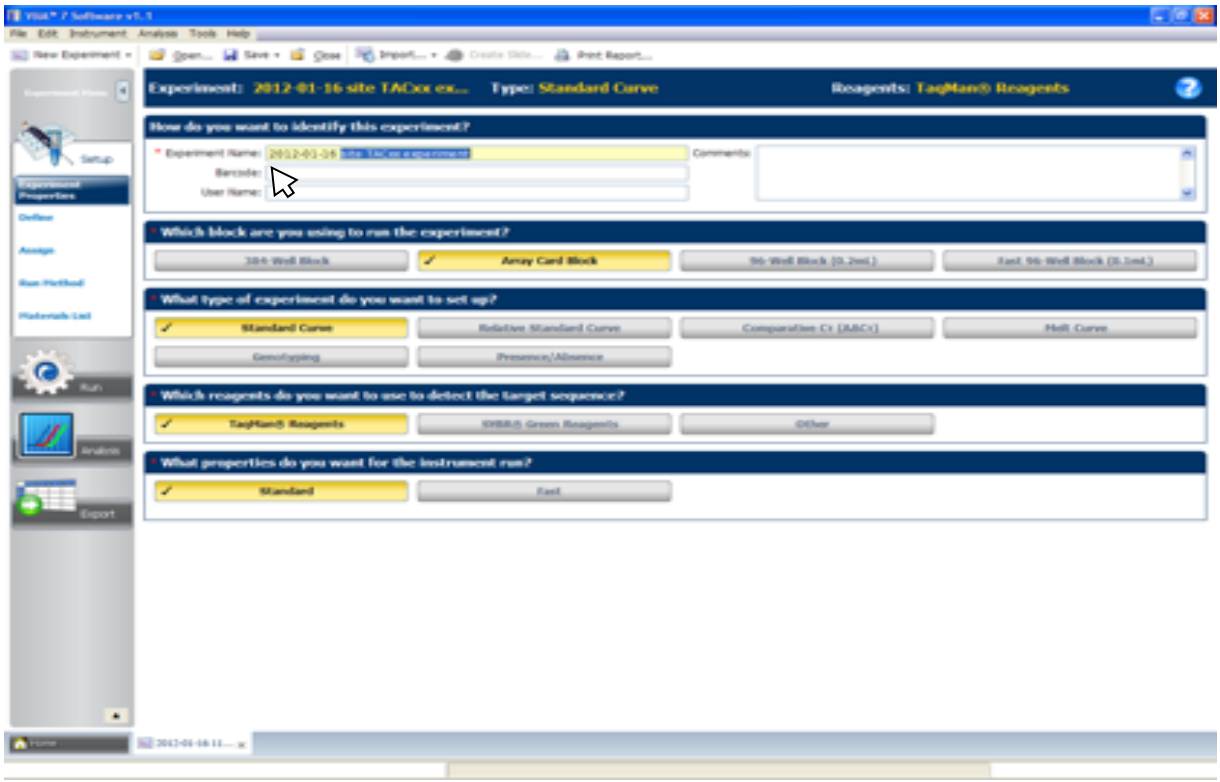
4.1.3. The Home screen will appear:



4.1.4. Ensure that the computer is communicating properly with the instrument. Click on the Instrument Console. When the computer properly connects with the instrument, the instrument icon will have a green check.

4.1.5. Set up a new experiment using UVA provided template. Open New Experiment from Template provided by UVA. Confirm experiment Properties: Array Card Block, Standard Curve, TaqMan Reagents, Standard.





4.1.6. Name the experiment by date (***keep the DATE automatically generated by the software***), site name, TAC, experiment description, such as **2025-03-05 GHRG_ETH_Card_1**.

4.1.7. Click “Define” on the left panel, and name the samples using SID (barcode scan). Name each blank with its extraction date and batch number, such as “**2025-03-05** Blank_1”. If there are multiple batches from one day, add 1, 2, 3… after the date as recorded. Note that the targets, fluorophores, passive reference dye have already been entered and assigned.

4.1.8. Confirm Cycling Conditions in the Run Method Screen:

Reaction volume: 1µL

Step	Temperature	Time
RT (1cycle)	45°C	20min
Denaturation (1 cycle)	95°C	10min
PCR (40 cycles)	95°C	15sec
	60°C	1min (data collection)

4.1.9. Save the experiment. The file name is automatically generated based on the experiment name.

5. PCR Reaction preparation:

- Keep all reagents on wet ice or on a cold block during assay set up.
- The enzyme mix is viscous because of glycerol; pipette slowly.
- Prepare Master Mix before loading the TAC cards.

5.1. **Prepare master mix for 8 samples:**

Ag-Path-ID 2X RT-PCR buffer	425µL
Ag-Path-ID Enzyme mix	34µL

5.2. Aliquot 54µL of master mix into each of eight (8) 1.5mL microcentrifuge tubes.

5.3. To each tube, add 20µL of total nucleic acid extract from **stool** specimens then supplemented with 26µL nuclease-free water, or 46µL of total nucleic acid extract from extraction blanks, or nuclease-free water (for NTC).

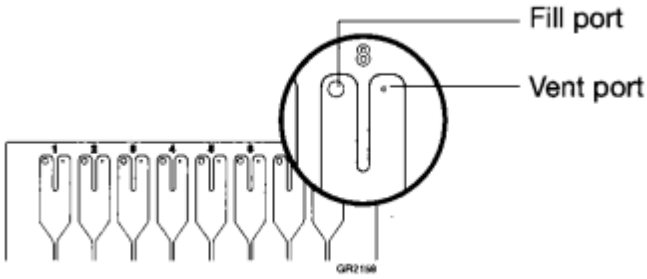
5.4. Mix gently, then centrifuge the tubes to eliminate air bubbles from the mixtures.

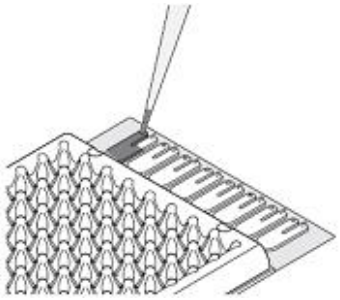
5.5. **Loading the TaqMan Array card**

5.5.1. Allow the TAC cards to reach room temperature in the original packaging.

5.5.2. Place the card on a lab bench, with the foil side down.

5.5.3. Transfer 100µL of each PCR reaction mix into the fill port. Hold the pipette in an angled position into the fill port (the larger hole on the left arm) and slowly dispensing the mix so that it sweeps in and around the fill reservoir toward the vent port without introducing air bubbles. Be careful when pushing the pipette plunger to its end as to avoid pushing the reaction mix out of the fill reservoir via the vent port or introducing bubbles.





5.6. **Centrifuging the TaqMan Array card**

5.6.1. Insert TaqMan Array Card into the array holder, ensuring that:

a. The filled reservoirs should project upwards out of the array holder.

b. The reaction wells face the same direction as the "This Side Out" label on the array holder.

c. Use blank arrays (*i.e.* empty cards with no spotted primers/probes) or used arrays to fill any remaining positions in the array holder. Failing to do so will impair the sample loading.

5.6.2. Place a filled array holder in the bucket so that the "This Side Out" label faces the front of the bucket.

5.6.3. Confirm the proper centrifuge settings. Set the bucket type:

Previous-Generation centrifuges: 15679

Current-Generation Centrifuges: 3618

5.6.4. Use following operation parameters:

Parameter	EASYSet (touchpad)	QUIKSet (knob-operated)
Up Ramp rate	9	3
Down Ramp rate	9	N/A
Rotational speed	1,200 rpm (331 xg)	1,200 rpm
Centrifuge time	2 X 1 min	2 X 1 min

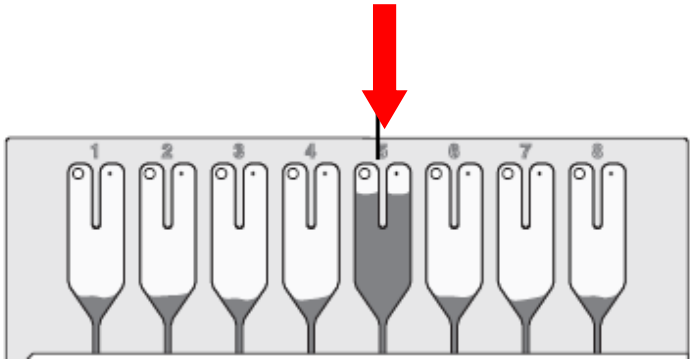
5.6.5. Place a loaded bucket onto an open rotor arm of the centrifuge. Make sure the bucket can swing easily within its slotted position on the rotor arm. The manufacturer recommends running the centrifuge with all four buckets, even if only two buckets contain cards. Make sure the buckets and their contents are balanced.

5.6.6. Centrifuge 1 minute. Wait for the centrifuge to stop, then repeat so that the cards are centrifuged for a total of two consecutive, 1-min spins.

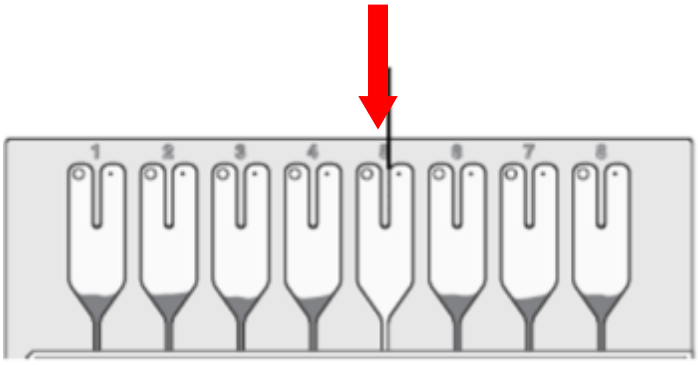
5.6.7. Remove the buckets from the centrifuge, then remove the array holders from the buckets. Remove the TaqMan array card(s) from the holders.

5.6.8. Examine the TaqMan Array card(s) to determine whether filling is complete. A small amount of sample will remain in the fill port, but it should be a uniform volume from reservoir to reservoir.

5.6.9. If there is excess reaction mix in a fill reservoir, centrifuge the card again for 1 additional minute. If the reservoir filling is still not complete, do not centrifuge again. Proceed with the card run but void the results for the affected sample. Excessive centrifugation speeds and times may deform the card, so do NOT exceed 1200rpm or accumulated centrifugation times of more than 3 minutes.



5.6.10. If a fill reservoir is completely drained, it is possible that some wells are not filled properly. Proceed with the card run but void the results for the affected sample.



5.7. **Sealing the TaqMan Array Card**

The sealer ("staker") isolates the wells of a card after loading with reaction mix. The sealer uses a precision stylus assembly ("carriage") to seal the main fluid distribution channels of the array. Proper operation of the sealer is critical to the successful use of the TaqMan Array Card.

5.7.1. Place the sealer on a sturdy lab bench, approximately waist high so that it can be easily used.

5.7.2. Make sure the carriage sits in the "starting position" which is closest to you. Never insert a card into the sealer if the carriage is NOT in its starting

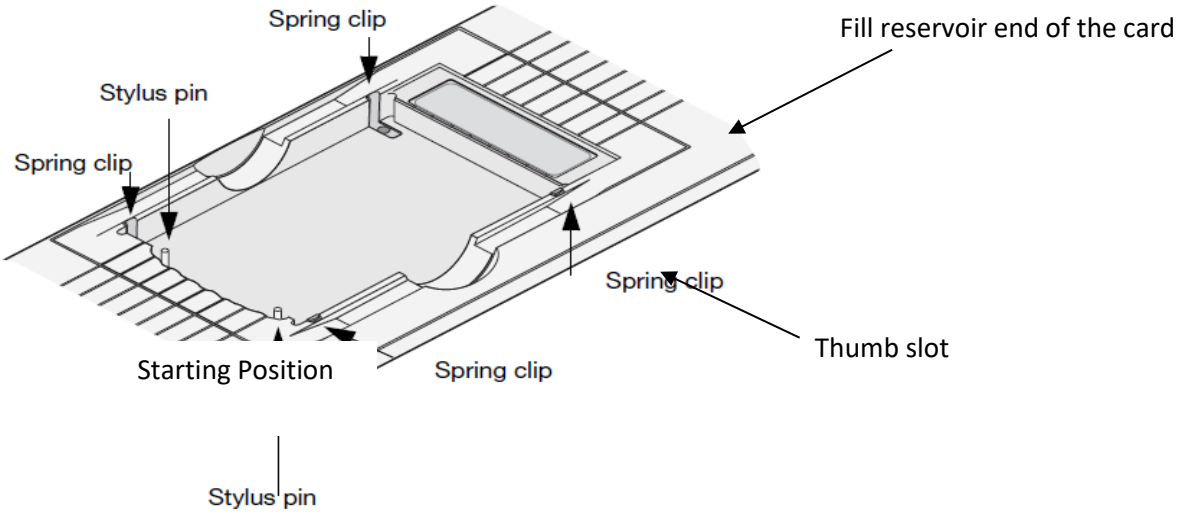
position. The card will be irreparably damaged if the carriage is moved across it in the wrong direction.

Carriage in starting position

5.7.3. Insert a TaqMan Array Card into the sealer.

a. Orient the card in the proper direction over the sealer's insert plate, **foil side up.** The fill ports should be at the farthest end of the sealer base (away from you).

b. Line up the card's rear pin groves to the stylus pins on the sealer.



c. Gently place the card on top of the insert plate and ensure that the front end of the card is held securely in place by the spring clips. Gently push the card until it is seated securely in the insert plate. When properly seated, the card's foil surface should be level with the base of the sealer. The four spring clips ensure that the card is held in the proper position.

5.7.4. Push the carriage across the base of the sealer in the direction of the arrow at the far end labeled with "Push to stake". Use a **slow, steady** and deliberate motion to push the carriage across the entire length of the card until the carriage reaches the mechanical stops. It is important to avoid moving the carriage rapidly across the card.

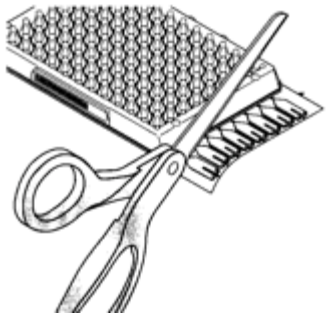
5.7.5. Remove the sealed card by grasping its sides and lifting it off the sealer's insert plate. In the middle of the sealer's insert plate, there is thumb slot to help you easily access one side of the card.

5.7.6. Inspect the card for proper sealing. The indentations from the stylus assembly should match up with the card's main channels. If the indentations do not match up or if the foil is in any way damaged, do not use the card.

5.7.7. Return the carriage to its starting position on the base of the sealer.

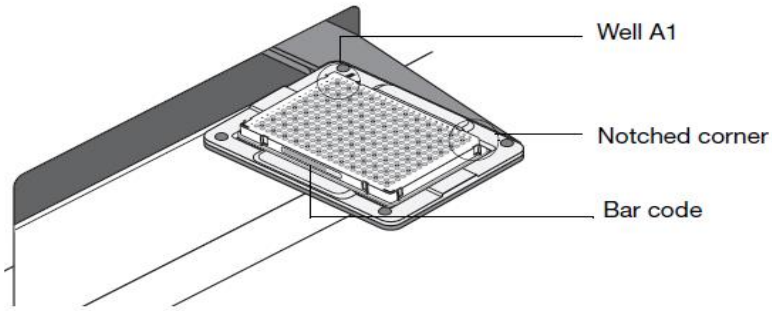
5.8. **Trim off the fill reservoirs**

Using scissors, trim the fill reservoirs from the card. Use the edge of the card's carrier as a guide.



5.9. **Run the experiment**

5.9.1. Place the sealed card in the instrument with well A1 at the top left corner of the tray and the notched corner at the top right, with the bar code facing toward the front of the instrument.



5.9.2. Within the software, click "Run" on the left panel, then the arrow next to "START RUN", select the instrument. You should be prompted to select a location to save the run file if the experiment hasn't been saved.

6.0. Analysis

Predefined thresholds are embedded in the run template and are used for all the assays. However, the baseline for each target may require adjustment. Refer to "TAC Raw Data Analysis" for the guidance on baseline adjustments as well as data validity and acceptance criteria.

III. Automated TAC Analysis (ATA)

1. PURPOSE

This procedure describes the steps for automated TAC analysis by updating raw TAC data

files into the UVA enteric shiny app.

2. INTRODUCTION/PRINCIPLE

The same software that was used to run your qPCR experiment is also capable of automatically analyzing the data and returning Ct (cycle threshold)/Cq (quantification cycle) values. The Enteric shiny app will be able to predict the correct Ct value without the need of manual adjustment of a baseline. This SOP describes the procedures for naming the samples and blanks when running TAC as well as the desired format for exporting raw data files.

Note: This protocol is written for ViiA 7 software v1.2.x, QuantStudio 12K Flex Software v1.2.x, and QuantStudio software v1.2 (for QuantStudio 7 Flex users).

3. NAMING OF BLANKS AND SAMPLE IDs

2.1. Blanks for each extraction batch should be labelled in ascending order.

- For the samples that have already been done the sites must go back and manually change the sample ID to include a blank in the raw files before exporting and uploading the files into Shiny app.
- **NOTE: numbers should be assigned as 1, 2, 3, etc. and not 01, 02, 03, etc.**

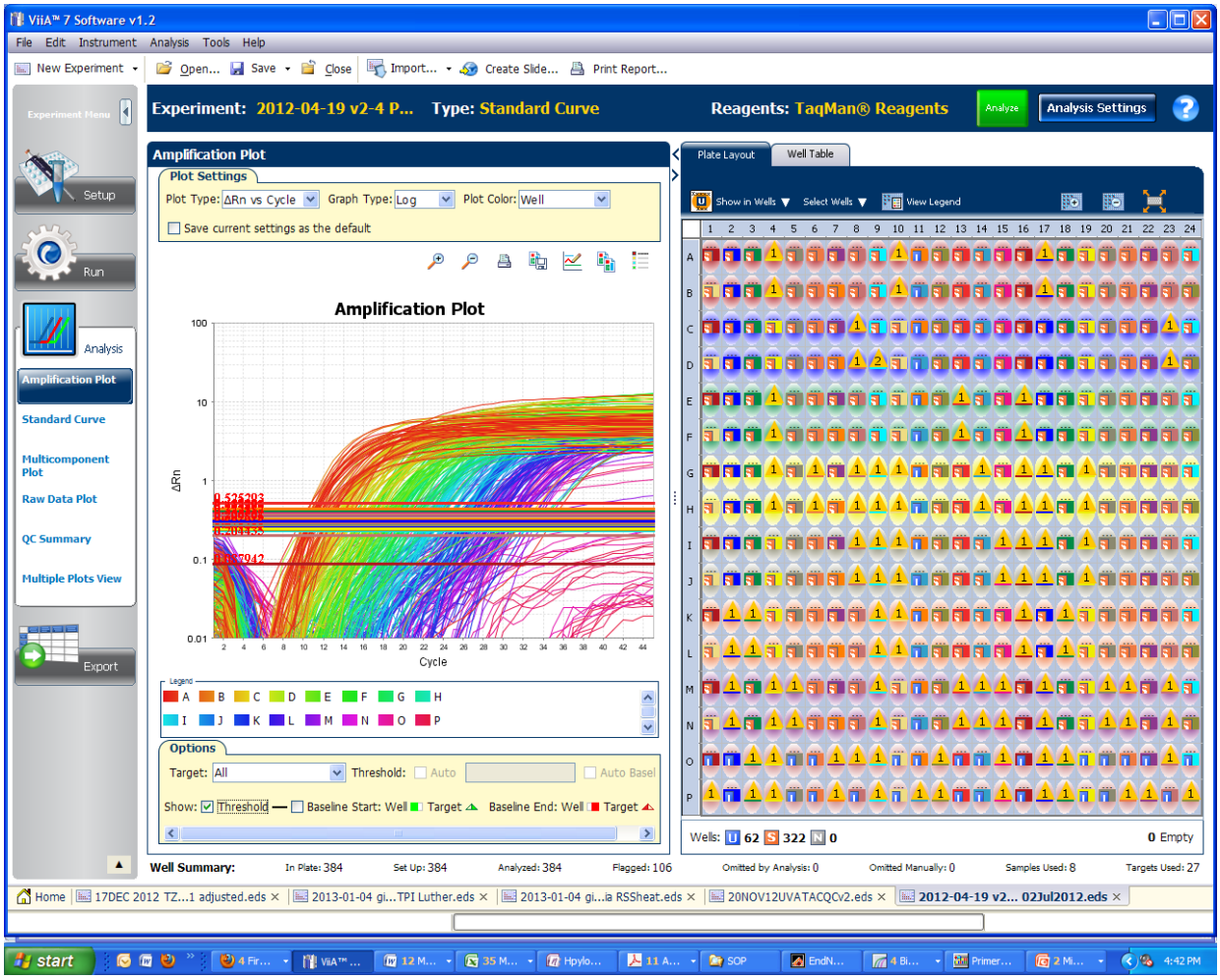
2.2. For each sample in an extraction batch must be explicitly linked to its respective blank by appending the blank number to the "sample name". The procedure below should be followed.

- Label the blank with date then extraction batch making it is clear which number it is. Please use the following format: Date Blank_Number, so for example "**2025-02-28_Blank_1**"
- All the sample IDs must be labelled to include the extraction blank for that batch in the assigning tab by adding underscore followed by the number of blanks for that batch. The example is given below. This has to be done on every sample

SAMPLE ID	**NEW SAMPLE ID**
BPH_ 001	BPH_ 001_1
BPH _001	BPH _001_1
BPH _024	BPH _024_2
BPH _025	BPH _025_2

- When all the sample names have been linked to the extraction blank EXPORT the card.

4. Export data from the ViiA7/QS7 Software for the RAW data files. **It is not necessary to perform any adjustments**. First, select the "Export" button at the bottom of the left column.



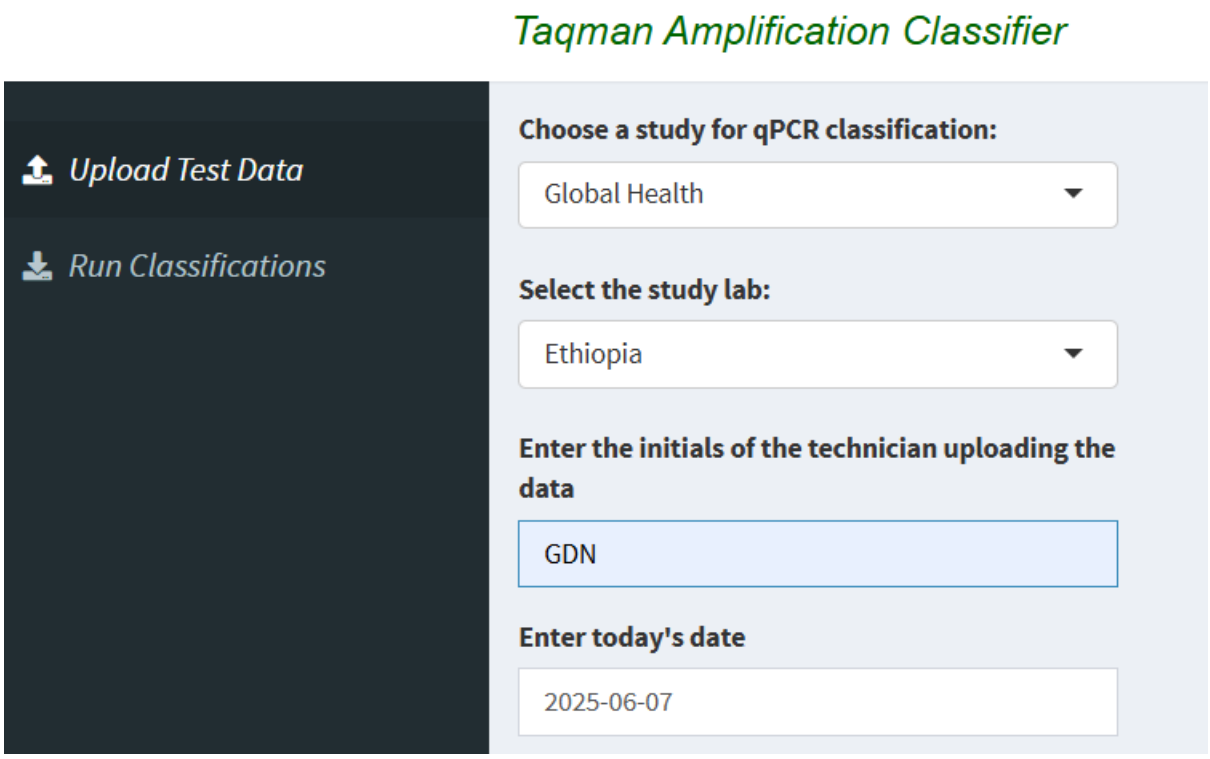
- On the export screen, select "Browse" to designate the folder where the export file will be saved.
- Name the export file: it is likely no name change will be needed as the Export File Name defaults to the "Experiment Name".

- Thus, if the experiment was named as directed in the TAC SOP i.e. “date site experiment”, such as “2025-02-28_GHRG Card_1”, the Export File Name is then automatically set as “2025-03-28_GHRG Card_1-ViiA7-export.
- 6Select the File Type *.xls (With the run template provided by UVA this is set by default. There should be no need to change)
- Select “Results” tab with Well Position, Sample Name, Target Name, and **REPORTER**. For some runs, there may be extra items after “Custom6”, including BADROX, NOISE, SPIKE etc. Please keep them if selected (if the QC Summary window noted a frequency other than 0 for these flags, they must be selected).
- Select **Multicomponent tab**. Ensure all tabs are selected (Well, well position, cycle, FAM, ROX and VIC). All other tabs should not be selected.

- Export the File by clicking “Start Export”.

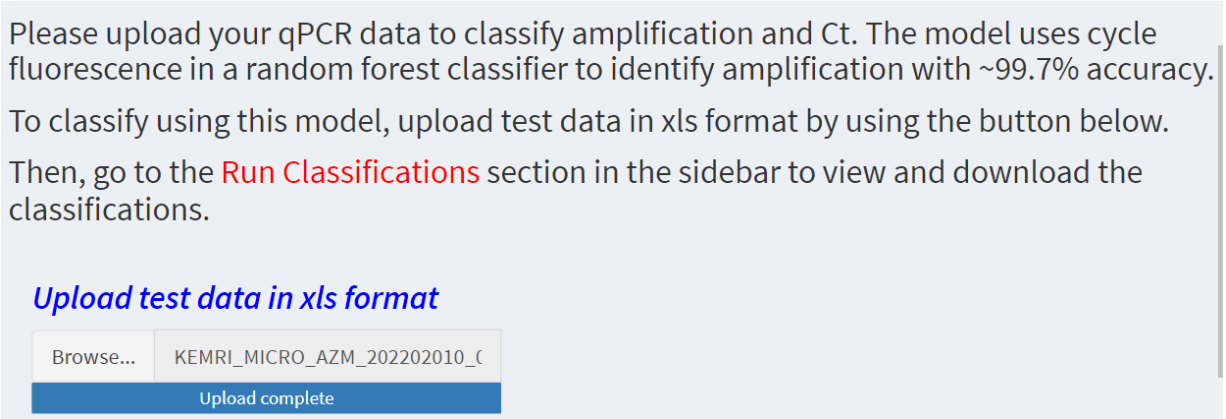
5. UPLOAD OF EXPORT FILES INTO SHINY APP

7.1 Open a web browser and navigate to http://shiny.uvaenterics.org:3838/TAC/ you should see the interface shown below.



7.2 Select respective study (GLOBAL HEALTH), year, study laboratory (country) and enter the 3 initials of the person uploading the data. Date is automatically entered.

7.3 Click browse and find and select the file to be uploaded, and when complete it will show upload complete as below.



7.4 On the left column, now choose “Run Classifications”. You will see curves for the first six targets that were classify as true amplifications. You can now download the classified data by clicking download classification. The data will be automatically saved on the UVA Enterics server for cleaning and sharing.

7.5 Click upload test data and repeat from step 7.3 to upload the next file.

AI Report

*% detected as AI

AI detection includes the possibility of false positives. Although some text in this submission is likely AI generated, scores below the 20% threshold are not surfaced because they have a higher likelihood of false positives.

Caution: Review required.

It is essential to understand the limitations of AI detection before making decisions about a student's work. We encourage you to learn more about Turnitin's AI detection capabilities before using the tool.

Disclaimer

Our AI writing assessment is designed to help educators identify text that might be prepared by a generative AI tool. Our AI writing assessment may not always be accurate (it may misidentify writing that is likely AI generated as AI generated and AI paraphrased or likely AI generated and AI paraphrased writing as only AI generated) so it should not be used as the sole basis for adverse actions against a student. It takes further scrutiny and human judgment in conjunction with an organization's application of its specific academic policies to determine whether any academic misconduct has occurred.

Frequently Asked Questions

How should I interpret Turnitin's AI writing percentage and false positives?

The percentage shown in the AI writing report is the amount of qualifying text within the submission that Turnitin's AI writing detection model determines was either likely AI-generated text from a large-language model or likely AI-generated text that was likely revised using an AI-paraphrase tool or word spinner.

False positives (incorrectly flagging human-written text as AI-generated) are a possibility in AI models.

AI detection scores under 20%, which we do not surface in new reports, have a higher likelihood of false positives. To reduce the likelihood of misinterpretation, no score or highlights are attributed and are indicated with an asterisk in the report (*%).

The AI writing percentage should not be the sole basis to determine whether misconduct has occurred. The reviewer/instructor should use the percentage as a means to start a formative conversation with their student and/or use it to examine the submitted assignment in accordance with their school's policies.

What does 'qualifying text' mean?

Our model only processes qualifying text in the form of long-form writing. Long-form writing means individual sentences contained in paragraphs that make up a longer piece of written work, such as an essay, a dissertation, or an article, etc. Qualifying text that has been determined to be likely AI-generated will be highlighted in cyan in the submission, and likely AI-generated and then likely AI-paraphrased will be highlighted purple.

Non-qualifying text, such as bullet points, annotated bibliographies, etc., will not be processed and can create disparity between the submission highlights and the percentage shown.

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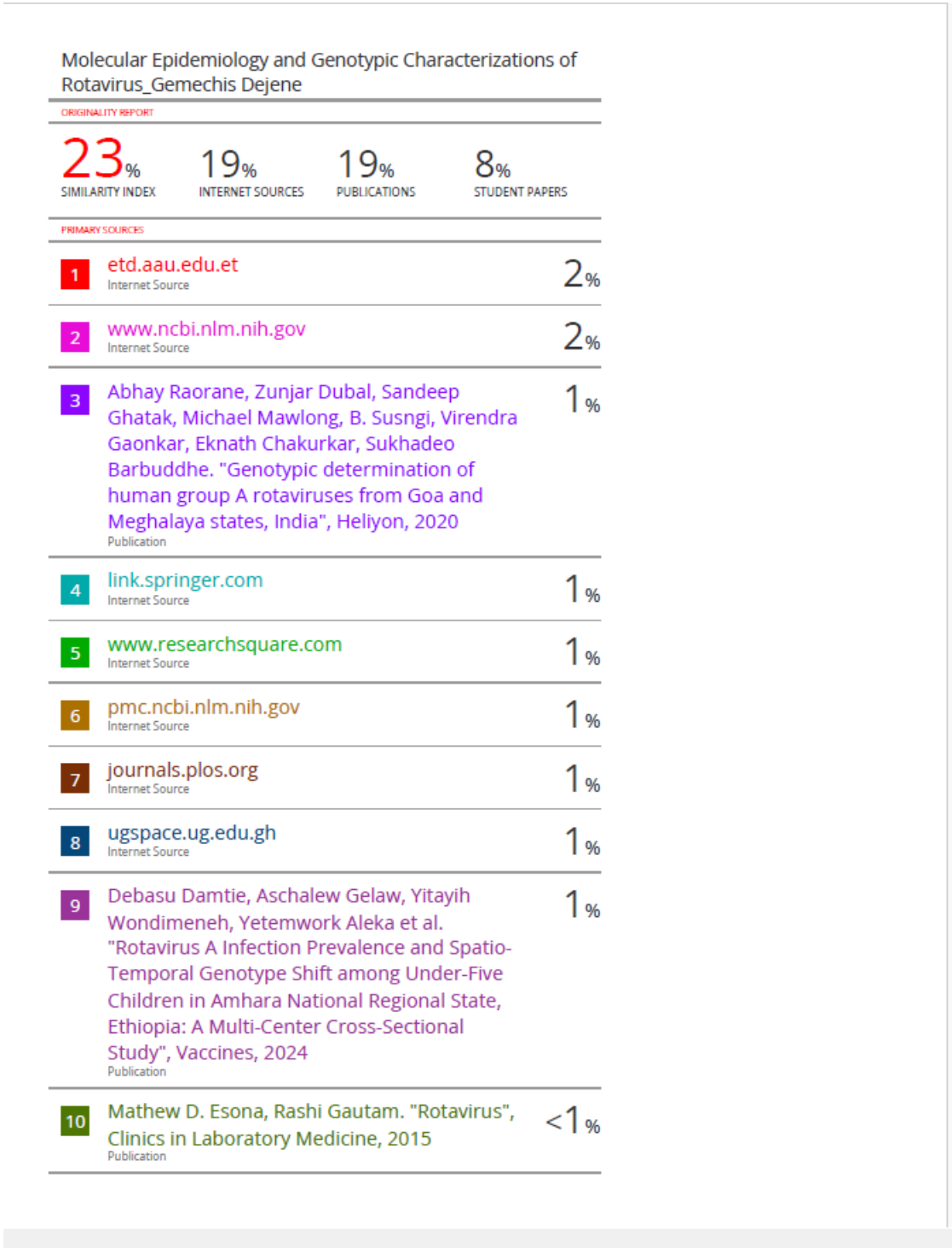

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DECLARATION SHEET

The undersigned MSc student of Medical Microbiology at Addis Ababa University declares that this research work is my original work in partial fulfillment of the requirement for the Master's Science degree in Medical Microbiology. Where the work of others has been used, it has been fully acknowledged and referenced according to requirements.

Name of the principal investigator:

1. Gemechis Dejene, B.Sc

Signature _____ Date August 15, 2025

Approval of the Advisors

This Research thesis has been submitted with the approval of advisors;

Advisors	**Signature**	**Date**
Prof. Daniel Asrat (MD, MSc, PhD)	_____	_____
Dr. Alem Abrha (B.Sc, MSc, PhD)	_____	_____