Enteric infections in Rwandan children under 5 years

Infection dynamics, association with diarrhea, and the role of host genetic factors (IFNL4 and FUT2)

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To my wife, my children, and my country

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ABSTRACT

Infectious diarrhea is a main cause of illness and death among children in poor countries, and enteric infections may also cause stunting as a result of impaired uptake of nutrients. This thesis investigated a broad range of enteric pathogens among children under five in Rwanda, focusing on their role in diarrhea, the rates of pathogen acquisition and clearance, and the potential impact of *IFNL4* and *FUT2* genetic variants on infection susceptibility. The findings in Paper I show high infection rates among 794 Rwandan children with *Shigella* and ETEC as the primary causes of diarrhea, while rotavirus was no longer the predominant etiology. However, as shown in Paper II, rotavirus remains an important cause of diarrhea despite vaccination since 2012. The longitudinal cohort study of 120 children described in Paper II also shows that new enteric infections occur very frequently, indicating heavy exposure, particularly in rural areas, but also that most infections are cleared within one month. Genetic variation in the *IFNL4* gene has recently been associated with the rate of diarrhea in a study from Mali, but Paper III did not identify any significant association between the *rs12979860* genotypes and any of a broad range of enteric pathogens. Paper IV showed that 'non-secretors' with a *FUT2* stop codon variant had a lower frequency of rotavirus but identified no association with other pathogens.
The results show that Rwandan children were heavily exposed to enteric pathogens, that this exposure was greater among children in rural areas, that *Shigella*, ETEC, and rotavirus were the main causes of diarrhea, and that children with inactivated *FUT2* (non-secretors) were less susceptible for rotavirus. The findings suggest that reducing exposure by improving living conditions should be a main priority to improve childhood health and growth in Rwanda.

Keywords: enteric infections, diarrhea, children under 5 years of age
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SAMMANFATTNING PÅ SVENSKA

Infektiös diarré är en viktig orsak till sjukdomar och död bland barn i fattiga länder, och tarminfektioner kan också orsaka tillväxthämning till följd av försämrat upptag av näringsämnen. Denna avhandling undersökte ett brett spektrum av tarmpatogener bland barn under fem år i Rwanda, med fokus på deras roll för diarré, nysmitta och eradikering av tarmpatogener, och om genetisk variation i generna för IFNL4 och FUT2 påverkar känsligheten för tarminfektioner. Fynden från analyser av 794 barn i delarbete I visar att Shigella och ETEC blivit de främsta orsakerna till diarré och att rotavirus inte längre dominerar. Som delarbete II visar är rotavirus dock alltjämt, trots att rotavirusvaccination infördes 2012, en viktig diarréorsak. Delarbete II visar också, genom upprepade prover från 120 barn i en longitudinell kohortstudie, att nysmitta med tarmpatogener sker mycket ofta, särskilt på landsbygden, men att de flesta av dessa infektioner läks ut inom en månad. Genetisk variation i IFNL4-genen har nyligen associerats med graden av diarré i en studie från Mali, men delarbete III påvisade inget signifikant samband mellan genotyper av rs12979860 (en position med genetisk variation i IFNL4-genen) och tarminfektioner. Delarbete IV visade att en stoppkodonvariant i FUT2-genen är associerad med en lägre frekvens av rotavirus men inte med andra patogener.

Resultaten visar att rwandiska barn är kraftigt exponerade för tarmpatogener, att barn på landsbygden är mer utsatta för denna exponering, att Shigella, ETEC och rotavirus är de främsta orsakerna till diarré hos barn i Rwanda, och att barn med inaktiverad FUT2-gen (’non-secretors’) har färre rotavirusinfektioner. Fynden talar för att minskad exponering genom förbättrade levnadsförhållanden bör vara en huvudprioritet för att förbättra barns hälsa och tillväxt i Rwanda.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Munyemana JB**, Kabayiza JC, Nilsson S, Andersson ME, Lindh M. Shigella and Enterotoxigenic Escherichia coli Have Replaced Rotavirus as Main Causes of Childhood Diarrhea in Rwanda After 10 Years of Rotavirus Vaccination. J Infect Dis. 2024 Nov 15;230(5):e1176-e1180. doi: 10.1093/infdis/jiae446. PMID: 39248312; PMCID: PMC11566240.

II. **Jean Bosco Munyemana**, Jean Claude Kabayiza, Eric Seruyange, Staffan Nilsson, Maria E Andersson, Magnus Lindh. A longitudinal study of acquisition and clearance of enteric pathogens in children under 5 years of age in Rwanda. (Manuscript submitted)

III. **Jean Bosco Munyemana**, Jean Claude Kabayiza, Eric Seruyange, Staffan Nilsson, Maria E Andersson, Anna Martner, Magnus Lindh. No association between *IFNL4*-related polymorphisms and enteric infections in Rwandan children under 5 years of age. (Manuscript submitted)

IV. **Jean Bosco Munyemana**, Jean Claude Kabayiza, Eric Seruyange, Staffan Nilsson, Gustaf Rydell, Maria E Andersson, Anna Martner, Magnus Lindh. Non-secretor status due to *FUT2* stop mutation is associated with reduced rotavirus infections but not other enteric pathogens in Rwandan children. (Manuscript submitted)

OTHER PAPERS PUBLISHED DURING THE THESIS WORK

I. **Munyemana JB**, Mukanoheli E, Nsabimana T, Niringiyumukiza JD. HCV Seroprevalence among HIV Patients and Associated Comorbidities at One Primary Health Facility in Rwanda. Am J Trop Med Hyg. 2021 Mar 15;104(5):1747-1750. doi: 10.4269/ajtmh.20-0500. PMID: 33720846; PMCID: PMC8103456.

II. **Munyemana JB**, Gatare B, Kabanyana P, Ivang A, Mbarushimana D, Itangishaka I, Niringiyumukiza JD, Musoni E. Antimicrobial Resistance Profile of Bacteria Causing Pediatric Infections at the University Teaching Hospital in Rwanda. Am J Trop Med Hyg. 2022 Oct 10;107(6):1308-1314. doi: 10.4269/ajtmh.22-0047. PMID: 36216320; PMCID: PMC9768258.

III. **Jean Bosco Munyemana**, Jonas, B., Evelyne, K., & Pauline, K. Urinary tract infection and antimicrobial resistance profile in patients attending Nemba District Hospital in Rwanda. Asian Journal of Medical Sciences, 11(6), 101–105. Retrieved from https://www.nepjol.info/index.php/AJMS/article/view/29921

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X. Nyirahabihirwe Françoise, **Munyemana Jean Bosco**, Nikuze Bellancille, Nsabimana Théoneste. HIV/AIDS and Syphilis Sero-prevalence Among Pregnant Women Attending Antenatal Care Center in Rwanda. American Journal of Laboratory Medicine, 5(4), 83-87. https://doi.org/10.11648/j.ajlm.20200504.11

CONTENTS

ABBREVIATIONS

ABI	Applied Biosystem
Ad OR	Adjusted Odd Ratio
AIEC	Adherent-Invasive *Escherichia coli*
CHUK	University Teaching Hospital of Kigali
CMHS	College of Medicine and Health Sciences
COVID 19	Corona Virus Disease 2019
DAEC	Diffusely Adherent *Escherichia coli*
DALYs	Disability-Adjusted Life Years
DEC	Diarrheagenic *Escherichia coli*
DNA	Desoxyribonucleic Acid
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EAEC	Entero-Aggregative *Escherichia coli*
EC	Ethics Committee
EED	Enteric Environmental Dysfunction
EHEC	Entero-Hemorrhagic *Escherichia coli*
EIEC	Entero- Invasive *Escherichia coli*
EPEC	Entero-Pathogenic *Escherichia coli*
ETEC	Entero-Toxigenic *Escherichia coli*
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
FUT2	Fucosyltransferase 2
FUT3	Fucosyltransferase 3
HBGA	Histo-Blood Group Antigens
HCV	Hepatitis C Virus
HUS	Hemolytic Uremic Syndrome

IFNL	Interferon Lambda
IGF-1	Insulin-like Growth Factor 1
IRB	Institutional Review Board
LMICs	Low- and Middle-Income Countries
MGB	Minor Groove Binding
mRNA	Messenger Ribonucleic Acid
NSP	Non-Structural Protein
OR	Odd Ratio
ORF	Open Reading Frame
PAF	Population Attributable Fraction
RDHS	Rwanda demographic health survey
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package for Social Sciences
ssRNA	Single Stranded Ribonucleic Acid
STEC	Shiga Toxin producing *Escherichia coli*
SP	Structural protein
UR	University of Rwanda
VP	Viral Protein
WASH	Water Sanitation and Hygiene

DEFINITIONS IN SHORT

ABO	The ABO is a blood group classification system that classifies blood groups into group A, group B, group AB, and blood group O. This classification is determined by the presence of A and B antigens on red blood cells. (Dean L. ABO Blood Group. 2012)
Allele	An allele is one of two or more versions of a DNA sequence at a specific genomic location that can differ by a single nucleotide or larger variations, and they determine the expression of genetic traits. Individuals inherit one allele from each parent for a given gene. (Gonzalez-Galarza FF 2011)
bfpA	bfpA is the structural subunit of bundle-forming pili (BFP), a key virulence factor in enteropathogenic Escherichia coli. It is encoded by the bfpA gene on the EPEC adherence factor plasmid and is essential for bacterial auto aggregation and localized adherence to epithelial cells. (Melo AR,2005)
DALYs	Disability-Adjusted Life Years is a measure of overall disease burden that combines the years of life lost due to premature death (YLL) and the years lived with disability or ill-health (YLD). One DALY represents one lost year of "healthy" life. DALYs help quantify the gap between the current health status and an ideal situation where everyone lives into old age free from disease and disability. This metric is widely used in public health to compare the impact of different diseases and health conditions across populations (WHO)

Dysentery	Dysentery is an inflammatory disorder of the intestine, especially the colon, causing severe diarrhea with blood and mucus, commonly caused by *Shigella dysenteriae* types 1 and 2 (*ET1 and ET2*) that are known for causing epidemic bacillary dysentery, characterized by severe bloody diarrhea and high virulence. (WHO)
eae	The eae gene in enteropathogenic Escherichia coli encodes intimin, the outer layer surface protein important for adherence to epithelial cells and the formation of attaching and a hallmark of pathogenesis. (Jerse AE 1991)
eltB	The eltB is a gene encoding the B subunit of heat labile toxin (LT) in E. coli, crucial for toxin binding to host cells. (Read LT 2014)
estA	A gene encoding heat-stable enterotoxin (ST) in enterotoxigenic E. coli, causing diarrhea by increasing intestinal cGMP levels. (Sjöling A 2006)
LT	Heat-labile enterotoxin produced by ETEC, consisting of A and B subunits, causing diarrhea by increasing intracellular cAMP. (Read LT 2014)
O157:H7	O157:H7 is a serotype of E. coli that produces Shiga-like toxins, causing severe foodborne illness. It can lead to bloody diarrheal diseases which sometime get complicated and results into hemolytic uremic syndrome (HUS), particularly in children and the elderly. (Rahal EA 2012)
PAF	Population Attributable Fraction (PAF): PAF is the proportion of disease cases in a population that can be attributed to a specific risk factor, representing the reduction in disease burden if that exposure were eliminated or reduced. (WHO)

SE/Se/se	Secretors (SE/Se) have at least one functional FUT2 gene allele, allowing them to secrete ABO blood group antigens in bodily fluids. Non-secretors (se/se) have non-functional FUT2 alleles and do not secrete these antigens. (Wacklin P 2011)
ST	Heat-stable enterotoxin produced by ETEC, a small peptide causing diarrhea by increasing intracellular cGMP in intestinal cells. (Aasland R2010)
T3SS	Type III Secretion System is a sophisticated, needle-like protein apparatus found in many Gram-negative bacteria, such as *Shigella*, *Salmonella*, and *Escherichia coli*, that functions as a molecular syringe, allowing bacteria to inject effector proteins directly from their cytoplasm into the host cell bypassing the extracellular environment. This system is crucial for bacterial virulence, and helps bacteria to manipulate host cell processes, evade immune responses, and establish infection. (Clin Microbiol Rev. 2007)
WASH	Water, sanitation, and hygiene is a program for providing communities with access to potable water, improved sanitation, toilets facilities and good hygiene practices, aiming to improve public health, and contribute to socioeconomic development. (Hutton G 2017)

1 INTRODUCTION

Enteric infections in childhood continue to pose a significant global health concern, leading to severe acute gastroenteritis, which is mainly characterized by watery diarrhea.[1] The diarrhea disease in children under 5 years of age is the second cause of death, resulting in 1.7 billion new cases and approximately 500,000 fatalities each year worldwide, with a higher distribution in developing nations, where hygiene, clean water, and sanitation are limited.[2,3]

Enteric pathogens include viruses: viruses: rotavirus, norovirus, adenovirus 40/41, and sapovirus; bacteria: *Shigella species*, *Escherichia coli* (*E. coli*), *Campylobacter*, *Salmonella*, *Vibrio*, and *Aeromonas*; and protozoa: Giardia, Entamoeba, Cryptosporidium, and Trichomonas.[4,5] These enteric pathogens are the main diarrheal agents and are mainly acquired through the fecal-oral route[6-8]

The main pathogens causing childhood diarrhea have been identified in previous studies, and the impact of rotavirus has been reduced by vaccination. Diarrhea remains a big concern, and a subclinical infection has been identified as an additional threat to health and growth, impacting physical and cognitive development in developing countries.[1,9-15]

Genetic variation plays a role in an individual's susceptibility to infection.[16] The fucosyltransferase 2 (*FUT2*) gene, which encodes an enzyme involved in producing histo-blood group antigens (HBGA), has been linked to norovirus and rotavirus infection susceptibility.[17,18] Furthermore, variations in interferon lambda (IFNL), a cytokine known to play the role of stimulating and activating the immune system, predict viral clearance and hepatitis C viral (HCV) treatment response.[19-21]

1.1 ROTAVIRUS

The rotavirus is a double-stranded ribonucleic acid (dsRNA) virus belonging to the Reoviridae family. The genome is segmented into 11 fragments, each encoding either the structural (SP)or nonstructural proteins (NSP). Rotavirus is categorized into seven serogroups from A to G, with serogroup A being responsible for 90% of human rotavirus infections. The viral proteins 7 (VP7) and viral protein 4 (VP4) are the basis for rotavirus genotyping into G and P types, respectively.[22,23]

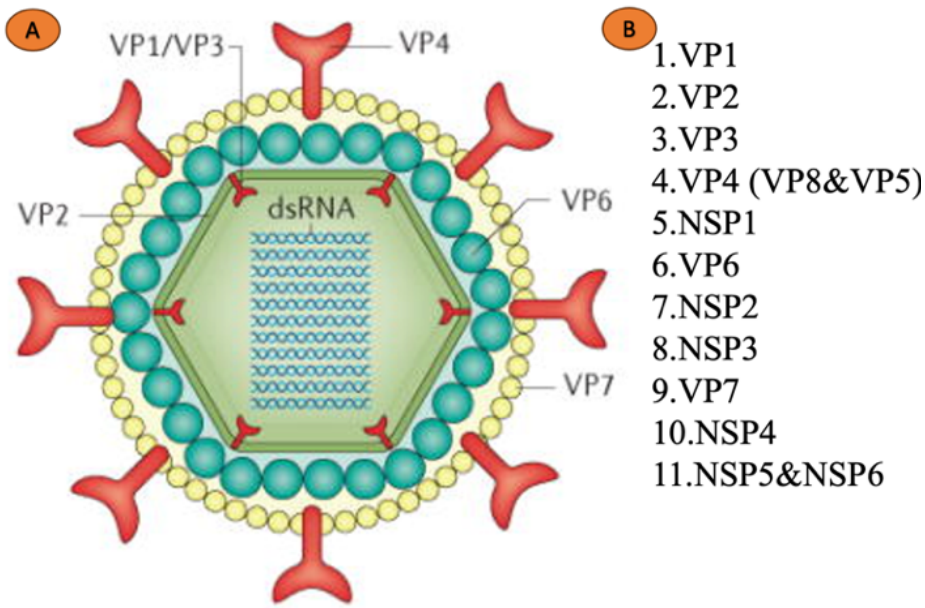

***Figure 1**: Rotavirus structure and genome. **A**: a viral diagram showing the inner core (VP2, VP1, VP3, genome), middle layer (VP6 for species specificity), and outer layer (VP7 and VP4, triggering immune response). The VP4 cleaves into VP8 and VP5. **B**: Genome segments that encode either viral protein (VP1 to VP4, VP6 and VP7) and nonstructural proteins (NSP1 to NSP 5).*

1.1.1 EPIDEMIOLOGICAL STATUS

Rotavirus prevalence and its association with severe gastroenteritis have dramatically decreased. Over the past three decades, the number of deaths has dropped from over 600,000 in 1990 to around 200,000 in 2019.[24] However, rotavirus infections are still responsible for approximately one-fifth of all diarrhea-related fatalities globally and remain a significant threat in developing countries, with around 130.000 deaths annually.[24,25]

A systematic review covering different studies done between 1990 and 2017 which was done in Northern part of Africa and the Middle East region reported varying rates of rotavirus prevalences in children with diarrhea, with an estimated mean prevalence of 35.9% in Egypt (range: 11%–76.9%), 33.8% in Morocco (range: 17.2%–44%), 23.0% in Tunisia (range: 6.1%–33.9%), 28.3% in Saudi Arabia (range: 3.9%–65.5%), 34.9% in Iran (with some studies reporting up to 70.2%), and 31.4% in Pakistan (range: 8.2%–65.9%).[26]

Similarly, a hospital-based study in India found that 24% of children under two years old with diarrhea tested positive for rotavirus. Among those infected, 61.5% experienced severe gastroenteritis, with a higher prevalence in hospitalized children (45.2%) compared to those who were ambulatory (15.25%). Another study conducted in Shanghai, China (2012–2018), found that 31.7% of outpatient children under five with acute diarrhea tested positive for group A rotavirus.[27,28]

In Kenya, an East African country, between 2008 and 2012, the global enteric multicenter study (GEMS) identified rotavirus in 15% of children under 5 years of age with diarrhea, with peak prevalence observed in infants aged 0–11 months.[29,30]

In addition, the vaccine impact on diarrhea in Africa (VIDA) study (2019–2022), which was a follow-up study of GEMES conducted across Mali, Kenya, and Gambia, sought to reassess the etiology of diarrheal disease following the introduction of rotavirus vaccines and underscored the significant burden of rotavirus-associated diarrhea, with infection risks influenced by age, socioeconomic status, and geographic factors. [31,32]

Different studies have reported varying prevalences. For example, a study done in Malawi reported varying detection rates between vaccinated and unvaccinated populations and higher detection rates of rotavirus in diarrheal cases compared to controls. The post-vaccination data showed rotavirus detection rates of 21.9% in diarrhea cases versus 3.4% in controls, with another analysis reporting 19.4% versus 5.4% in diarrhea and control groups, respectively, highlighting its significant contribution to acute gastroenteritis (AGE), [33,34] despite higher vaccination coverage (>90%).[34] In Mozambique, in pre-vaccination periods, rotavirus was detected at 29% in diarrhea cases and 15% in controls, which slightly decreased post-vaccination to 25% and 11%, respectively.[35]

Moreover, a study conducted in 2017 in Moshi, Tanzania, after the introduction of rotavirus vaccination with Rotarix in 2013, found that mortality among children under five due to diarrhea significantly declined, from 72% of diarrhea-related deaths in 2008 to 36% in 2013. Before vaccination, rotavirus infection rates among children under five ranged from 18.1% to 43.4%, and post-vaccine implementation, it dropped and ranged between 17.4%–29%. Another study conducted in Tanzania found a rotavirus detection rate of 26.4% in symptomatic children (73/277). [36,37]

In Rwanda, rotavirus epidemiology has undergone significant changes since the introduction of the pentavalent RotaTeq vaccine in 2012. In a report from the WHO 4 regions, rotavirus detection rates for Rwanda were 59.5% overall but showed a reduction to 19.2% in children eligible for vaccination compared to 32.2% in those not eligible (above the vaccination age).

Despite the vaccine's impact, rotavirus has continued to be one of the primary pathogens associated with diarrhea, with detection rates ranging from 16.1% to 34.7% in diarrheal cases versus significantly lower rates (1.4%-9.8%) in controls across various studies. [25] Another study in Rwanda reported that pre-vaccination (2009-2012), rotavirus caused 47% severe diarrheal cases, with genotypes shifting from G2P4 and G12P8 to G9P8 and G1P8 dominance. After vaccination, rotavirus prevalence declined to 34% two years later.[33,38-43]

1.1.2 INFECTION AND PATHOGENESIS

Rotavirus in the gut targets primarily the mature enterocytes, specifically in the jejunum and ileum of the small intestine. The virus attaches to host cells using specific glycans like sialic acid, HBGA, and cell surface integrins, entering via receptor-mediated endocytosis. Once internalized, the virus undergoes uncoating in the endosome, releasing double-layered particles into the cytoplasm. Viral replication occurs in specialized cytoplasmic regions called viroplasms, where viral RNA is synthesized, and new viral particles are assembled. [23]

Rotaviruses evade host immune responses, targeting interferon regulatory factors and hijacking cellular translation machinery. The viral enterotoxin NSP4 disrupts cellular mechanisms by inducing chloride secretion and inhibiting sodium-glucose transporters, contributing to secretory diarrhea.

Then NSP4 also stimulates serotonin released from enterochromaffin cells, to activate the enteric nervous system and increase intestinal motility, which further exacerbates diarrhea. Moreover, vomiting during early infection may result from cytokine release or vagal nerve activation triggered by serotonin signaling.[44-47]

An individual's susceptibility to rotavirus infections has been reported to depend on the variations in *FUT2* and *FUT3* genes. These genes encode the enzymes that build HBGA, resulting in the synthesis and expression of H, AB, and Lewis antigens on the mucosal surface, thus determining the so-called secretor status of an individual. The expression of these antigens has been associated with differences in rotavirus infection susceptibility, including differences in rotavirus genotype affinity. For instance, rotavirus genotypes P*[4]* and P*[8]* have a binding affinity to Lewis b (Le b) and H type-1 antigens, while the genotypes of P*[9]*, P*[14]* and P*[25]* prefer Lewis A (Le a) antigen. Additionally, studies have shown that the secretors of HBGA s are more susceptible to rotavirus infections compared to non-secretors.[17,46,48] This HBGA individual variation has also been shown to correlate with symptomatic infections, where individuals with non-secretor phenotypes had fewer symptomatic rotavirus infections.[49]

1.1.3 PREVENTION AND VACCINE UPDATES

Since mid-2006, rotavirus vaccines have become established as crucial tools in preventing pediatric diarrheal diseases. Two primary vaccines, RotaTeq (manufactured by Merck & Co, Inc, Pennsylvania, USA) and Rotarix (developed by Glaxo Smith Kline, Biological, Rixensart, Belgium), have been globally licensed and endorsed by the World Health Organization

(WHO) for integration into national immunization strategies which have demonstrated comparable protective capabilities.[23,50] Moreover, additional vaccines have recently gained international recognition, expanding prevention options. ROTAVAC produced by Bharat Biotech, RORASIIL produced by Serum Institute of India, and Rotavin-M1, by PolyVac, have demonstrated protective efficacy against childhood rotavirus-associated gastroenteritis.[51,52]

Rwanda introduced rotavirus vaccination on 25th May 2012, with the live oral pentavalent RotaTeq given at weeks 6, 10, and 14 after birth, achieving 99% coverage in 2013, with a substantial impact in reducing diarrhea requiring hospitalization. In April 2017, Rwanda shifted to a two-dose Rotarix given at weeks 6 and 10 after birth.[53,54]

1.2 NOROVIRUS

Norovirus belongs to the family of *Caliciviridae*. It is a single-stranded RNA (ssRNA) virus, positive-sense, non-enveloped, with a genome that is organized into three open reading frames (ORFs) that encode SP and NSP. Two clinically significant genogroups (G), including GI and GII, account for approximately 95% of human infections.[55]

The molecular flexibility of noroviruses enables them to adapt rapidly to host immune responses and genetic diversity, primarily occurring at the junction between ORF1 and ORF2, contributing to the virus's evolutionary potential and challenges in developing comprehensive prevention strategies.[56]

1.2.1 EPIDEMIOLOGICAL STATUS

Globally, norovirus I and II cause approximately 685 million cases annually, predominantly affecting children under five in developing countries.[57] Norovirus global prevalence is estimated at 19% in cases of acute gastroenteritis.[58]

Globally, norovirus genogroup II.4 is the most prevalent, found in approximately 42% of norovirus cases. A systematic review on the burden of acute gastroenteritis due to norovirus in China reported a norovirus detection rate of 19- 21%, and the most frequent were norovirus genogroup II.4 (70%) and genogroup II.3 (13%).[59] In Sub-Saharan Africa, norovirus epidemiological surveillance is limited, with available data suggesting a prevalence of around 10-20% in diarrhea cases.[60] A systematic review reported a pooled prevalence of 13.5% of norovirus infection in symptomatic children from 14 African countries, with norovirus GII.4 being reported as predominant.[61] Other studies in Africa reported different prevalences, with 37% in South Africa and 16% in Kenya.[40,62,63] In Rwanda, norovirus II was more often detected in sick children than asymptomatic ones (8.1 vs 4.3%) while norovirus GI was less frequent.[64]

1.2.2 INFECTION AND PATHOGENESIS

Norovirus is a highly infectious enteric pathogen that primarily spreads through person-to-person contact in addition to contaminated food, water, surfaces, and fomites, which makes it exceptionally contagious and challenging to control.[65] Upon infection, the virus binds to HBGAs on host enterocytes, allowing entry and replication. Noroviruses infect different types of cells, such as B cells, Dendritic cells, macrophages, and enterocytes, utilizing microfold (M) cells to overcome epithelial barriers.[66,67]

Several studies have shown that susceptibility to human norovirus infection is associated with genotypic and phenotypic diversity of HBGA expression, which confer resistance or susceptibility to norovirus infection. Both homozygote Secretors (SE) and heterozygote secretors (Se) express functional *FUT2* and produce α1,2-fucosylated HBGAs, which serve as receptors for many norovirus strains, particularly the globally dominant GII.4 genotype. Thus, secretor-positive individuals are highly susceptible to GII.4 infections, which are associated with more severe symptoms. A systematic review of studies that investigated host genetic susceptibility to enteric infections showed that the SE/Se were 9.9 times infected by norovirus GII.4 times and 2.2 times by just norovirus GII when compared with their counterparts, non-secretors.[18,68]

Conversely, individuals with non-functional FUT 2 genes (non-secretors) are less susceptible to norovirus genogroup II.4. but remain vulnerable to other genogroups, such as GI.3 and GII.2, which exhibit secretor-independent binding patterns. The interaction between noroviruses and HBGAs is strain-specific; for example, GI.1 Norwalk virus binds selectively to type O secretors but not to non-secretors or individuals with type B blood. Additionally, Lewis antigens (*FUT3*-dependent) may act as alternative receptors for certain strains, broadening the complexity of norovirus-host interactions. These genetic factors underscore the influence of HBGAs in norovirus infection susceptibility across diverse populations and viral genotypes.[18] The typical infection develops 12-48 hours after exposure and is characterized by non-bloody diarrhea, vomiting, and stomach pain. Symptoms usually resolve within 1-3 days, though vulnerable populations like children and the elderly may experience more severe complications of acute gastroenteritis and dehydration.

A critical aspect of norovirus transmission is its prolonged shedding period. Infected individuals can continue to spread the virus for up to 60 days and more especially in immunocompromised individuals, even when asymptomatic.[69,70] This characteristic, combined with its high infectivity, with at least 10 virions, is enough to cause infection, making norovirus a significant public health challenge.[66]

1.2.3 PREVENTION AND VACCINE UPDATES

Norovirus infection prevention strategies primarily focus on rigorous hygiene practices, including thorough handwashing, careful food preparation, surface disinfection, and isolation of infected individuals.[71,72] Vaccine development has made significant progress, with several promising candidates in clinical trials. There are two multivalent vaccines developed by Moderna Inc., including the mRNA 1405 pentavalent and mRNA 1403 trivalent. HilleVax is advancing HIL-216, a hexavalent vaccine targeting six common norovirus genotypes, while Vaxart is developing an orally administered bivalent vaccine targeting GI.1 and GII.4 strains.[73,74] Despite these advances, to date, there is no WHO-licensed norovirus vaccine, and the primary challenge remains the virus's extensive genetic diversity, which complicates the creation of a broadly effective vaccine. Additionally, the virus’s low infectious doses and easy transmission, coupled with its environmental resistance, continue to pose a global health challenge.

1.3 ESCHERICHIA COLI

The Escherichia coli (*E. coli*) represents a complex group of bacterial pathogens causing a significant global diarrheal disease burden, particularly in developing countries. It is a Gram-negative rod belonging to the *Enterobacteriaceae* family with different epidemiological characteristics and a substantial impact on child health and nutrition.

E. coli comprises multiple pathotypes that are categorized into two groups, including extraintestinal *E. coli* and diarrheagenic *E. coli* (DEC). The DEC includes seven key pathotypes: enterotoxigenic (*ETEC*), enteroinvasive (*EIEC*), enteroaggregative (*EAE*C), enteropathogenic (*EPEC*), Shiga toxin-producing/ enterohemorrhagic (*STEC/EHEC*), diffusely adherent (*DAEC*), and adherent-invasive (*AIEC*) *E. coli*.[75,76] These *E. coli* strains have acquired resistance genes that help them evade the immune system and are responsible for childhood acute gastroenteritis.[77]

1.3.1 EPIDEMIOLOGICAL STATUS

ETEC has emerged as the most prevalent *DEC*, causing approximately 196 million episodes annually. This pathotype predominantly affects children under five years in the African and Eastern Mediterranean regions, contributing significantly to childhood morbidity and mortality. In 2016, ETEC was the 8th cause of diarrheal deaths with 51.186 death cases, accounting for about 3.2% of diarrhea-related deaths globally.[78] In children under five years old, ETEC contributed to approximately 4.2% (2.2–6.8%) of diarrhea deaths.

While mortality rates for those pathogens have declined over time due to improved sanitation and access to healthcare, their impact on morbidity remains significant in low- and middle-income countries.[79] Other DECs demonstrate variable prevalence rates, ranging from 1.6% to 9.1% across different geographical settings. EAEC consistently shows the highest prevalence, accounting for approximately 5.7% of diarrheal cases in multiple epidemiological studies, such as the study conducted in Nigeria, where 41.3% of DEC was reported.[1,79,80] In a study conducted in Mozambique, ETEC was identified among the most common causes of moderate to severe diarrhea (MSD), while in GEMS, ETEC was associated with MSD than less severe diarrhea (LSD).[81,82]

A longitudinal study in Peru has reported that ETEC was responsible for more than 70% of childhood diarrheal episodes, with infections occurring early after birth, contributing to 10% of the population-attributable fraction (PAF) of diarrheal burden in neonates while infection rates increased with age, peaking at 17% PAF at around 2 years of birth, with the ST-ETEC being the most prevalent type with 32.1%.[83] In a previous study conducted in Rwanda, ETEC-*estA* was detected in 21 vs 9% is sick children compared to the healthy control respectively with a significant association with symptoms while ETEC-*eltB* was equally detected in both symptomatic and asymptomatic children (29% vs 30%)[64] Another study done in Rwanda in 2014 on diarrheagenic microbes in children under 5 years, found the overall prevalences of 31.3% ETEC-*eltB*, 19% ETEC-*estA*, 25% EPEC-*eae*, and 14% EPEC-*bfpA*. In that study, ETEC was more frequently detected in symptomatic children and was associated with severe gastroenteritis and dehydration.[41]

1.3.2 INFECTION AND PATHOGENESIS

*ETE*C primarily causes watery diarrhea through the secretion of enterotoxins that interfere with intestinal ion transport and cause fluid loss and dehydration. Some strains produce the heat-stable toxin, ST, coded by the *estA* gene; others produce the heat-labile toxin, LT, coded by the *eltB* gene, and some produce both. Those pathogenic ETEC strains also need colonization factors (CF) that help them attach to the intestinal mucosa. Both CF and LT, but not ST, induce immune responses that provide at least partial protection and limit symptoms in case of re-infection. This makes ETEC eltB more symptomatic in younger children for their first exposure. *EPEC* may cause diarrhea by adhering to intestinal epithelial cells and injecting effector proteins by using the type III secretion system (T3SS) that manipulates the cell signaling pathway of the host, resulting in the effacement of microvilli and increased permeability of the intestinal barrier.[40,84-86]

EHEC, notably the O157:H7 strain, produces Shiga toxins and prevents synthesis of protein synthesis, which causes bloody diarrhea and severe complications such as hemolytic uremic syndrome (HUS).[87] The EIEC invades intestinal epithelial cells similarly to *Shigella*, causing inflammation and ulceration, while *EAEC* forms biofilms on the intestinal mucosa, leading to persistent diarrhea through a combination of adherence factors and enterotoxins.[88,89]

1.3.3 PREVENTION AND VACCINE UPDATES

E. coli prevention strategies focus on multiple approaches, with hygiene and food safety being critical components. Regular handwashing, particularly before food handling and after animal contact, remains a fundamental preventive measure.

In addition, the water, sanitation, and hygiene (WASH) program has shown a significant impact on DEC and other enteric pathogen prevention.[90] Researchers have explored various vaccine strategies, including a promising live attenuated vaccine like ACE527. The vaccine targets multiple surface colonization factors and could potentially provide coverage against 80% of clinical ETEC strains. Moreover, the ongoing research focuses on innovative vaccine platforms, such as recombinant bacterial vectors.[91,92] These approaches aim to develop safe, effective vaccines that can protect against different *DECs*, with particular emphasis on *ETEC* and *EHEC* strains that pose significant public health challenges. However, to date, there is no WHO-approved *E. coli* vaccine.[92]

1.4 SHIGELLA

Shigella belongs to the Enterobacteriaceae family. It is a non-motile Gram-negative bacterium that is highly infectious, causing bacillary dysentery. The *Shigella* genus comprises four serogroups, including *Shigella dysenteriae* (Serogroup A), *Shigella flexneri* (Serogroup B), *Shigella boydii* (Serogroup C), and *Shigella sonnei* (Serogroup D). *Shigella flexneri* and *Shigella sonnei* are the most isolated species, while *S. dysenteriae* is associated with severe acute gastroenteritis and dysentery outbreaks.[93]

1.4.1 **EPIDEMIOLOGICAL STATUS**

Globally, *Shigella* remains a critical public health challenge, annually affecting 270 million people with an estimated 212,000 deaths,[94] primarily affecting children under five years old. According to the Global Burden of

Disease report, in 2019, *Shigella* was the leading cause of diarrheal deaths after rotavirus, with 8.4 million disability-adjusted life years (DALYs).[95] Two years before (in 2016), *Shigella* caused around 60,000 deaths among children under five years of age and was reported to be associated with diarrheal diseases in all age groups.[79] Another GEMS study reported *Shigella* as a leading cause of moderate-to-severe diarrhea, accounting for 26.6% of diarrhea cases in under 5 years of age children, with prevalences varying by age group and location, with older children (24–59 months) experiencing the highest proportion of *Shigella*-attributable diarrhea (37%), compared with 9% in infants (0–11 months). The pathogen was detected in 16% to 83% of diarrhea cases across different sites but had a significantly lower prevalence in controls, emphasizing its association with symptomatic disease.[1]

A recent study has reported a prevalence of 10% of *Shigella* in Asia, with a higher detection rate from rural areas, and *Shigella flexneri* accounting for 58% of the *Shigella* cases.[96] The GEMS has reported that *Shigella* bacterial load was associated with diarrhea, which increased by age with a maximum peak at 24 to 35 months of age, and the greatest relationship was found in Bangladesh (OR=13.2).[97] In Africa, *Shigella* prevalence varies significantly across countries and different detection methods, with around 10% in children under five years. [98] In a previous study done in Rwanda, *Shigella* was detected in 13 vs. 10 % symptomatic and asymptomatic children, and Ct (pathogen load) values were lower for sick in multiple logistic regression analyses with age, *Shigella* was significantly associated with diarrhea [64] Another study conducted in Rwanda revealed a *Shigella* prevalence of 17.5%, which correlated with bloody diarrhea, elevated C-reactive proteins (CRP), and an increase in *Shigella* infection with age.[41]

1.4.2 INFECTION AND PATHOGENESIS

Shigella is transmitted through the fecal-oral route and is very infectious, requiring as few as 10-100 bacterial cells to initiate an infection. The bacteria primarily target the colonic epithelium, entering through M cells located in lymphoid follicles and utilizing T3SS to enter the host cell. [99] Upon entering the host, *Shigella* rapidly lyses phagosome compartments in macrophages and escapes into the cytosol. The bacteria release the pro-inflammatory cytokines (IL-18 and IL-1β) to induce macrophage apoptosis and then force their way into epithelial cells through micropinocytosis, using T3SS effector proteins to manipulate the host cell cytoskeleton. Inside the cell, *Shigella* replicates in the cytosol, uses actin-based motility to move between cells, and spreads to neighboring epithelial cells, causing extensive tissue destruction.[99]

Clinical manifestations include bloody diarrhea, fever, nausea, stomach cramps, and potentially dysentery. Symptoms typically emerge 2-4 days after ingestion and can last several days to weeks. In rare cases, particularly in young children, the infection can lead to severe complications like seizures and reactive arthritis. Different *Shigella* species produce varying toxins, with *S. dysenteriae* generating Shiga toxin, potentially causing hemolytic-uremic syndrome, while *S.* flexneri produces ShET1 and ShET2 toxins contributing to diarrheal symptoms. The PCR assays for *Shigella* often target the ipaH gene because it is present in four copies per bacterium. However, since this target is also present in EIEC, PCR results are sometimes reported as Shigella/EIEC.[99,100]

1.4.3 PREVENTION AND VACCINE UPDATES

Shigellosis prevention primarily requires comprehensive public health measures and personal hygiene practices, and WASH program interventions, especially in resource-limited settings, are important in preventing infection transmission. Currently, there are promising S*higella* vaccine candidates which include *Shigella* 4V2 (S4V2), a tetravalent bioconjugate vaccine (Valneva and Limma Tech), which is in Phase 2b human challenge studies; LimmaTech S4V, another tetravalent bioconjugate vaccine targeting multiple *Shigella* serotypes, with phase 2 results reported in June 2024; GVGH altSonflex1-2-3, a quadrivalent vaccine using OMV/GMMA technology, which reported Phase 1 results in June 2024; and Sf2aWC, an inactivated whole-cell *Shigella flexneri* 2a vaccine showing promise in Phase 1 trials; Invaplex *Shigella* vaccine administered with the dmLT adjuvant which in phase I clinical trial in Netherland and Zambia. However, none of these have been licensed for use by the WHO.[94,101]

1.5 OTHER ENTERIC PATHOGENS

Some other clinically significant enteric pathogens that cause childhood diarrhea and associated consequences are discussed below. In this section, the focus is on sapovirus, astrovirus, adenovirus, *Salmonella*, *Campylobacter,* and *Cryptosporidium*. Other enteric pathogens, such as Giardia, Entamoeba histolytica, *Vibrio cholerae*, *Aeromonas*, hepatitis A virus, and helminth infections, will not be discussed primarily due to their lower detection rates in previous studies conducted in Rwanda or no detection in the current study.

1.5.1 SAPOVIRUS

Sapovirus belongs to the Caliciviridae family like norovirus. It has a single-stranded positive-sense RNA genome organized into two to three ORFs, depending on genogroup (G1, G4, and G5 have 3 ORFs while G2 and G3 have 2 ORFs). The ORF1 encodes NSP and VP1 capsid protein, while ORF2 encodes a minor structural polyprotein. The ORF 3 encodes a 161 amino acid protein with an unknown function so far.[102] Sapovirus has 19 genogroups, with G1, G2, G3, and G4 being capable of infecting humans. These four groups correspond to four antigenically distinct strains: Sapporo, Houston, London, and Stockholm. At least 17 genotypes have been identified, with new variants continuously emerging in America, Asia, and Europe.[103]

Epidemiologically, sapovirus causes significant childhood infections. Studies show nearly one infection episode per child in children under 2 years, with the highest incidence between 6-23 months. About 60% of children experience sapovirus diarrhea in their first two years of life. Currently, no specific vaccine for sapovirus exists.[103,104]

1.5.2 HUMAN ASTROVIRUSES

Human astroviruses belong to the Astroviridae family and are small, non-enveloped, with a positive-sense sense single-stranded RNA genome. Human astroviruses were first discovered in 1975 following a diarrhea outbreak. Astrovirus infection has a seasonal pattern, with a maximum peak in winter and the beginning of spring. Previous studies have revealed a prevalence of 2-9% in childhood acute gastroenteritis. Other studies reported that astrovirus was detected in 4.2% and 2.3% of children with diarrhea.[40,105-107]

Currently, no specific vaccine for astroviruses exists. Previously, a two-subunit vaccine candidate combining the hepatitis E virus (HEV), rotavirus, and astrovirus has been tried in mice, showing a promising trivalent vaccine. Research continues to focus on understanding viral diversity, genetic characteristics, and potential vaccine development strategies.[106,108]

1.5.3 ADENOVIRUSES

Adenoviruses are the only enteric viral pathogens with a DNA genome. They are big viruses (90-100nm), non-enveloped, and classified into seven species (A-G), comprising 88 human adenovirus types. Clinically significant species include respiratory pathogenic adenovirus (B and C), conjunctive pathogenic adenovirus (D), and gastroenteritis pathogenic adenovirus (F), with types 40/41 of subgenus F causing diarrhea,[109] accounting for 7.2% of diarrheal cases in children. [109-111] Currently, no specific vaccine exists for adenovirus 40/41. Research continues to understand their molecular epidemiology, with studies highlighting their significant role in pediatric gastrointestinal infections across different geographical regions.

1.5.4 SALMONELLA

Salmonella belongs to the *Enterobacteriaceae* family and is a genus of rod-shaped, gram-negative bacteria. There are two species, including *Salmonella enterica* and *Salmonella bongori. Salmonella enterica* is the primary human pathogen, which is further divided into six subspecies containing over 2,650 serotypes. These bacteria are classified into two main categories: *Salmonella typhi* and non-typhi *Salmonella*.[112]

It is an invasive bacterium, acquired through the fecal-oral route by eating or drinking contaminated food or drinks. In the gut, it invades the mucosal tissue and produces toxins. This invasion stimulates the release of proinflammatory cytokines, triggering inflammatory reactions that damage the intestinal mucosa. Symptoms typically manifest in 6-48 hours and can persist for 4-7 days. These symptoms primarily include abdominal pain, diarrhea, fever, and vomiting. In some cases, *Salmonella* enters the bloodstream and leads to septicemia.[112,113]

Salmonella enterica serovar Typhi causes typhoid fever and has a WHO-licensed vaccine; however, non-typhoidal *Salmonella,* responsible for gastroenteritis, does not yet have an approved vaccine, and prevention relies on hygiene.[114,115]

1.5.5 CAMPYLOBACTER

Campylobacter is a microaerophilic, gram-negative, spiral-shaped bacterium that causes foodborne illnesses worldwide. Enteric infection with *Campylobacter* is common in several animal species, including poultry, and most human infections are zoonoses, while transmission between humans is rare. Transmission may occur by consumption of undercooked poultry, contaminated water, or contamination of surfaces used for the preparation of food. Small children can also become infected by oral exposure to animal feces in the environment.[116,117] *Campylobacter jejuni* is the most common species causing human infections, responsible for approximately 90% of cases. Campylobacteriosis typically presents with gastrointestinal symptoms such as diarrhea, abdominal pain, and fever.[118]

Studies on campylobacter and diarrhea in South Asia reported a prevalence ranging from 3 to 17.4% in children with diarrhea and 0-13% in asymptomatic children [119] A systematic review has reported 9.9% of pooled prevalence of *Campylobacter* in sab-Sahara Africa with no major variations in region and some studies *Campylobacter* was associated with diarrhea mainly in children.[120] A study conducted in Malawi showed a significantly higher detection rate in symptomatic (21%) than in asymptomatic (14%) children, with *Campylobacter jejuni* in 85% of cases.[121]

In Rwanda, the prevalence among children under five years old with or without symptoms was 15.5%, and living with animals was identified as a major risk factor.[7,117,122] Another study in Rwanda found a detection rate of 14% and 17% in sick and controls, respectively.[64] Prevention strategies should focus on improving biosecurity in poultry farms, enhancing hygiene during food processing, and promoting proper food handling practices by consumers, while living with animals should be prevented. Vaccine development against *C. jejuni* has been challenging due to its antigenic diversity and complex pathogenesis.[40,123]

1.5.6 CRYPTOSPORIDIUM

Cryptosporidium is an intracellular apicomplexan protozoan responsible for both respiratory and gastrointestinal cryptosporidiosis. The parasite measures about 4.2 to 5.4 µm in diameter and has a complex life cycle within a single host. Transmission occurs mainly through contact with infected people or animals and eating or drinking contaminated water or food. Oocysts are highly infectious, with as few as 10 capable of causing illness in humans.

The infection begins with the release of sporozoites from the ingested oocysts, which then attach to and invade intestinal epithelial cells. It resides in a parasitophorous vacuole derived from the host cell membrane, which makes it intracellular but extracytoplasmic, a feature that limits exposure to immune responses like antibodies and phagocytes. The parasite causes villous atrophy, crypt hyperplasia, and inflammation in the intestines, increasing intestinal permeability and chloride secretion, which in turn result in watery diarrhea. *Cryptosporidium* is an important cause of childhood diarrhea in LMICs.[124]

In 2016, *Cryptosporidium* was the fifth cause of childhood diarrhea, with around 48.000 attributed deaths and impaired growth.[79] In a study done in Israel, Cryptosporidium was identified as the second cause of hospitalization due to gastroenteritis in children.[125] A study on the etiology of acute watery diarrhea reported an attributable fraction (AF) of 5.8, indicating the pathogen-causal role of watery diarrhea in children under 5 years.[25] In the study conducted in Sudan, *Cryptosporidium* was reported in 27.1% of children with diarrhea compared to 8.8% of children without diarrhea, with a statistically significant association with symptoms.[126] In Malawi, *Cryptosporidium* was detected in 9.1% of symptomatic children,[127] while the study on diarrheagenic microbes done in Rwanda showed a detection rate of 7.8% in children with or without diarrhea.[41] The prevention focuses on hygiene practices, avoiding contaminated water and food sources, and animal contact prevention, as it is a zoonotic parasite. There is no vaccine for human cryptosporidiosis, although research is ongoing.[124,128,129]

1.6 SINGLE NUCLEOTIDE POLYMORPHISM

Host genetic factors, including single-nucleotide polymorphisms (SNPs) in the *IFL4* gene and variations in histo-blood group antigens (HBGAs), may influence susceptibility to infections and clinical outcomes[130,131] by influencing pathogen recognition, immune responses, disease progression, and treatment responses. For example, hepatitis C clearance and treatment responses are strongly influenced by human genetic variation related to lambda interferon responses (*IFNL4*), and variants influencing the synthesis of HBGAs may alter the binding efficiency of certain rotavirus and norovirus subtypes. Their role underscores the importance of genetic profiling in understanding infection risks and advancing personalized prevention or treatment strategies.[20,132]

1.6.1 IFL4 SNP AND ENTERIC INFECTIONS

The SNP of *rs12979860*, located in intron 1 of the *IFNL4* gene, is in strong linkage disequilibrium with *rs368234815*. These genetic variations have been shown to significantly influence hepatitis C virus (HCV) treatment response and viral clearance. Surprisingly, the 'defective' TT genotype at *rs368234815* is associated with better outcomes in HCV clearance and treatment response, possibly by indirectly activating stronger *IFNL-3* responses.[20,21]

The genetic variations in the *IFNL4* gene show distinct population-level patterns, with the less favorable dG genotype at rs368234815 (corresponding to the TT at *rs12979860*) being twice as prevalent in African populations compared to European populations. This disparity may explain ethnic differences in infection outcomes.

The potential impact of *IFNL*-related SNPs has been reported to influence other infections, including hepatitis B and COVID-19, showing uncertain or smaller impacts than on hepatitis C. A recent study found that Rwandan children with the TT genotype at *rs12979860* exhibited reduced viral respiratory tract infection clearance than those with the CT or CC *IFNL4* genotypes [20,21,132]. A study from Mali, done on 914 children, found that the IFNL4 gene variant rs368234815-dG allele (producing IFN-λ4) significantly increases the risk of gastrointestinal infections (OR=1.53) and malaria (OR=1.30), and is linked to earlier onset of gastrointestinal infections. The IFNL3 variant rs4803217 showed weaker associations, implicating IFN-λ4 (not IFN-λ3) as the primary genetic driver of these infection risks.[133] The role of IFNL4 variants for enteric infections has, to our knowledge, not been investigated elsewhere.

1.6.2 FUT2 SNP AND ENTERIC INFECTION

The FUT2 and FUT3 genes, located on chromosome 19q13, regulate the expression of histo-blood group antigens (HBGAs) and play a crucial role in determining susceptibility to enteric infections. The fucosyltransferase enzyme encoded by the *FUT2* gene is responsible for the expression of ABO HBGAs on mucosal surfaces, which serve as attachment sites for various pathogens. Mutations in the *FUT2* gene can introduce premature stop codons or cause frameshifts, resulting in a non-functional enzyme that cannot add fucose in the position necessary for the synthesis of the H antigen, a precursor of the A and B blood group antigens in epithelial cells. Most *FUT*2 mutations are single-nucleotide polymorphisms (SNPs) that can be either missense or nonsense, affecting enzyme function.

The most well-known is the nonsense mutation *428G>A* (*rs601338*), which leads to a nonfunctional enzyme and results in the non-secretor phenotype. Other notable mutations include the missense mutation *385A>*T (*rs1047781*), common in East Asian populations, as well as variants such as *739G>A* and *839T>C*, which also reduce or eliminate enzyme activity and contribute to non-secretor status. There are also population-specific polymorphisms, with some variants conferring secretor status and others leading to loss of function. These mutations not only determine secretor status but also influence susceptibility to various infections and diseases.[134,135]

Accordingly, individuals with nonfunctional *FUT2* alleles cannot secrete H, A, or B antigens in the saliva or on the intestinal surface and are called non-secretors (se). Since these HBGAs are needed for attachment of some viruses, non-secretors may have reduced susceptibility to these infections, and several studies have shown that non-secretors are resistant to norovirus GII.4 and rotavirus P[8] infections.[16-18] Around 20% of Caucasians and Africans are homozygous for the A allele at SNP rs601338 in *FUT2*, which introduces a stop codon and confers lower susceptibility to rotavirus and norovirus. However, data from different regions show inconsistencies, possibly due to variations in host genetics or circulating viral strains. Similarly, *FUT3,* which regulates Lewis antigen expression, impacts susceptibility to specific enteric pathogens.[17,18] In addition to this genetic impact on infection susceptibility [132,135], the phenotypes of *FUT2* and *FUT3* in breastfeeding mothers may influence the composition and concentration of human milk oligosaccharides in breast milk, which can shape or impact the infant's microbiome composition and potentially enhance or alter the resistance to gastrointestinal infections or time to first gastroenteritis. These oligosaccharides can act as decoy receptors by binding to pathogens like rotavirus, preventing them from causing symptomatic infections.[136-138]

In a previous longitudinal cohort study that done at three sites, including Bangladesh, Peru, and Tanzania, on 827 children and their mothers, the prevalence of non-secretor children was between 19 and 22.5%, and in this study, they found a strong association between HBGAs and pathogen-specific infections, particularly for noroviruses, rotaviruses, ETEC, and *Campylobacter* and maternal secretor-positive had a 38% risk of symptomatic infection in their children.[136]

Other previous studies consistently demonstrated that secretor-positive individuals are more susceptible to rotavirus and norovirus infections.[17,18,139] However, norovirus GII.4 showed an exclusive infection in the secretors,[16] while rotavirus P[8] more often causes a symptomatic infection in *FUT2*-positive individuals than in *FUT2*-negative individuals. [140] The difference in findings and larger genetic variability, coupled with a broader range of enteric pathogens, need further investigation. To the best of my knowledge, there is no longitudinal study that evaluated the association of HBGAs and enteric infection in Rwandan children.

1.7 SYMPTOMATIC INFECTION

Symptomatic infections are typically characterized by acute gastroenteritis, with diarrhea as its primary symptom. Diarrhea consists of three to more passages of watery, loose, or liquid stools within 24 hours, possibly accompanied by blood or mucus. Globally, it is estimated that diarrhea accounts for 1.7 billion illnesses and approximately 500,000 deaths annually among children under 5 years of age, with 90% of cases being recorded in developing countries.

Despite progress in medical care, sanitation, and good vaccination coverage, diarrhea continues to be among the most common causes of childhood mortality and hospital visits, and it is ranked as the second (globally) and third (in Rwanda) cause of death in children under 5 years of age.[3,15,82]

Dehydration, coupled with electrolyte imbalances, constitutes a severe complication of diarrhea and is associated with poor outcomes if not treated rapidly.[141] Specific pathogens have demonstrated distinctive virulence characteristics that enable their pathogenic potential. Rotavirus, for instance, represents a globally predominant diarrheal agent characterized by exceptional transmission capabilities and widespread impact. *Shigella dysenteriae* type 1 showed remarkable invasive properties, producing potent Shiga toxin that triggers extensive mucosal inflammation and severe intestinal damage. ETEC generates, stimulating excessive intestinal fluid secretion and precipitating profuse watery evacuations.[15,142]

Efforts such as rotavirus vaccination and other interventions, such as improved life conditions, clean water availability, and access to health, have significantly reduced infection prevalence and severe gastroenteritis; however, diarrhea continues to pose a significant threat to global public health.[33,143,144]

1.8 SUBCLINICAL INFECTION

Subclinical enteric infections are infections without apparent symptoms and are very common in developing countries, affecting both rural and urban populations. These infections often pass unnoticed but leave severe consequences for the host, including gut inflammation, growth faltering, stunting, malnutrition, and immunosuppression, which further lead to long-term consequences such as cognitive impairment, reduced school performance, and decreased adult productivity.[145]

1.8.1 INFECTION AND IMMUNOSUPPRESSION

Continuous enteric infections cause chronic gut inflammation, leading to villous atrophy, crypt hyperplasia, and increased intestinal permeability, which impair gut absorption and result in nutrient deficiency, including crucial micronutrients like zinc, copper, selenium, and magnesium, which in turn compromise immune system function through reduced IgA secretion, impaired T-cell activity, altered cytokine responses.[146-148] Nutritional deficiencies also weaken physical barriers such as skin and mucous membranes, which are the primary barriers against infecting agents.
This interaction between infections, malnutrition, and immunosuppression impacts the overall child health, including stunting, cognitive delays, and intellectual performance, highlighting the need for consideration and intervention.[149-151]

1.8.2 INFECTION AND GROWTH IMPAIRMENT

The children's innate immunity can clear enteric infections within a short period, as previously reported in a study conducted in Zanzibar, where enteric pathogens were cleared in 14 days.[152]

However, children from developing countries experience continuous exposure to pathogens due to poor sanitation and limited hygiene. Despite mounting immune responses that protect against symptomatic infection in some cases, persistent exposure leads to chronic intestinal inflammation, resulting in the condition known as environmental enteric dysfunction (EED).[153]

The EED consists of intestinal mucosal inflammation, high gut permeability, and altered microbiota composition, causing significant gut dysfunction and reduced nutrient absorption capacity. The body's constant immune system response diverts energy and nutrients away from growth processes, directly contributing to linear growth faltering. Pathogens cause mucosal wall damage, reducing the available surface area for nutrient absorption. This combination of malabsorption, chronic inflammation, and microbiota alterations affects insulin-like growth factor 1 (IGF-1) production, which is critical for linear growth.[142,153-155]

1.8.3 STUNTING IN RWANDA

Childhood stunting is a more pronounced issue in Rwanda, a landlocked country in East Africa, divided into five provinces and 30 districts, which is home to approximately 14.6 million people as of March 2025, with a young population of a median age of 25.3 years.

The stunting prevalence is 33% among children under 5 years old, with notable geographic and socioeconomic disparities throughout the country.[156] The rural districts in Western and Northern provinces bear the highest burden of childhood growth impairment, with some districts reporting rates exceeding 50%, while in the Kigali capital city, the stunting rate is considerably lower, with some sectors reporting around 20%.[156,157]

A cross-sectional study conducted in five Rwandan districts has underscored several risk factors associated with childhood stunting, and inadequate illness, coupled with inadequate sanitation facilities, were highlighted as individual and community-level variables that were associated with childhood growth impairment.[158]

These observed disparities and identified risk factors highlight the need for targeted interventions, especially in rural and economically disadvantaged areas. Addressing socio-demographic and environmental factors in reducing enteric infections could be vital in fighting poor childhood linear growth in Rwanda.[159]

2 AIM

The main aim of this project was to investigate enteric infections and their association with *IFNL4* and *FUT2* in children under 5 years of age in Rwanda.

2.1 SPECIFIC AIMS

- In paper I, we aimed to investigate the causes of childhood diarrhea in Rwanda almost 10 years after the introduction of rotavirus vaccination.
- In paper II, we aimed to analyze enteric infection acquisition and clearance in children from rural and urban settings in Rwanda.
- In paper III, we aimed to investigate the possible association of the SNPs at *rs12979860* of *IFNL4* with enteric infections.
- In paper IV, we aimed to evaluate the association between G428A at *rs601338* (stop codon variant) in the *FUT 2* gene and a range of enteric pathogens in children under 5 years of age.

3 PATIENTS AND METHODS

This thesis is based on two studies conducted at different health facilities across Rwanda in 2021 and 2022, recruiting children under five years of age and collecting rectal swab samples for a broad range of enteric pathogen analysis and human DNA genotyping. In Paper I, we report the findings from Study I, while in Paper II, we report the findings from Study II. However, Studies III and IV report the findings from samples collected in Studies I and II, where enough human DNA material was available for genotyping the *FUT2* and *IFNL4* genes, to determine their SNPs, to analyze their association with enteric infections and diarrhea.

3.1 STUDY DESIGN AND SITES

Initially, a cross-sectional study was conducted from September 14 to November 5, 2021, and recruited children under five years old from nine health facilities. In Kigali City, participants were recruited from the University Teaching Hospital of Kigali, known as CHUK (Center Hospitalier Universitaire de Kigali), Kacyiru Hospital, Legacy Clinic, Nyacyonga Health Center, and Kagugu Health Center. In the Northern Province, participants were recruited from Ruhengeri Level II Teaching Hospital. In the Southern Province, participants were recruited from the Kabutare District Hospital. In the Eastern Province, participants were recruited from Rwamagana Level II Teaching Hospital, and in the Western Province, participants were recruited from Gisenyi District Hospital.

Secondary, a longitudinal study of five months follow-up from June 12, 2022, to December 15, 2022, was conducted at two health centers: a rural Gataraga Health Centre, located in Musanze District, Northern Province, and at a peri-urban Nyacyonga Health Centre, located in Gasabo District, Kigali City.

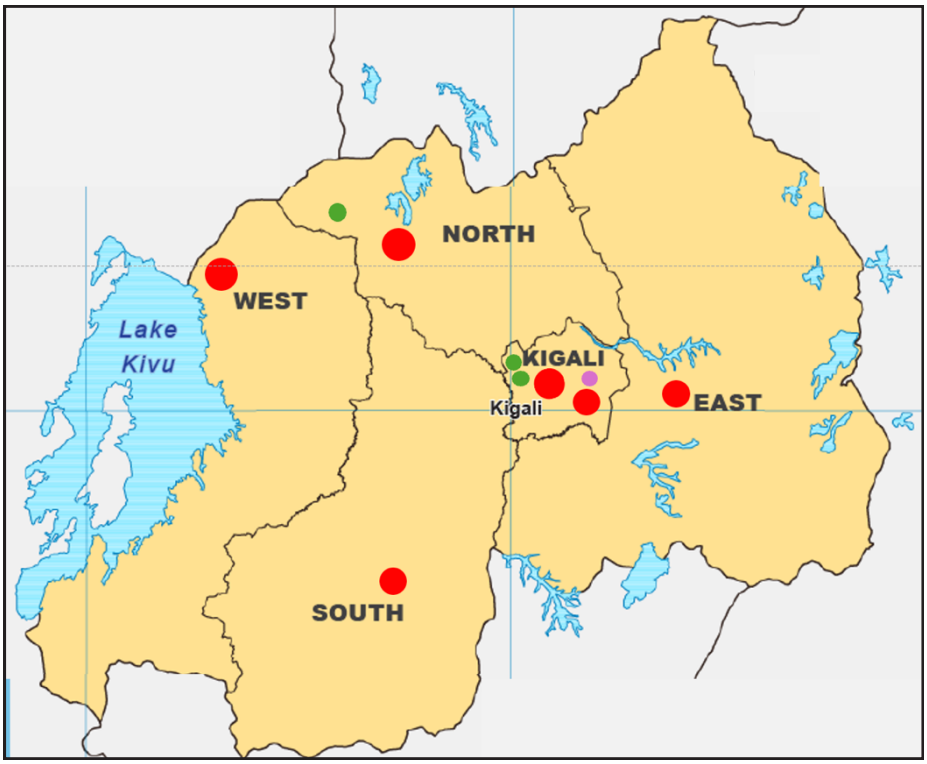

Figure 2: Map of Rwanda showing all the ten study site locations across Rwanda for both study I and study II. Study 1 was conducted at nine health facilities while Study I was conducted at two health facilities including one from Study I

Hospitals *Health Centers* *Private clinic*

3.2 PARTICIPANTS AND SAMPLES

3.2.1 CROSS-SECTIONAL STUDY

In study I, we enrolled 794 children, including 496 with diarrhea and 298 without diarrhea from nine health facilities, as presented in Table 1.

Table 1: Characteristics of participants in the cross-sectional study

Variables		Patients	Controls	Total
Study Sites	CHUK	60	51	111
	Gisenyi DH	56	0	56
	Kabutare DH	104	0	104
	Kacyiru Hospital	26	37	63
	Kagugu HC	5	93	98
	Legacy Clinic	30	0	30
	Nyacyonga HC	37	63	100
	Ruhengeri RH	93	7	100
	Rwamagana PH	85	47	132
Sex	Female	239	155	394
	Male	257	143	400
Age	Mean age (months)	18.8	14.8	17.3
	Median age (months)	14.6	9.2	13.0
Age groups	Under 6 months	119	122	241
	6–11 months	108	49	157
	12–23 months	146	62	208
	24–35 months	62	32	94
	36–47 months	32	21	53
	48–59 months	29	12	41
HIV Status	Negative	335	219	554
	Positive	9	1	10
	Not known	150	76	226
Total		496	298	794

CHUK, University Teaching Hospital of Kigali; DH, District Hospital; HC; Health Centre; RH, Referral Hospital; PH, Provincial Hospital; HIV, Human Immunodeficiency Virus.

3.2.2 LONGITUDINAL STUDY

In study II, we recruited and enrolled 120 children, including 60 from a rural health center (Musanze District/Gataraga Health Center) and 60 from Peri-urban Kigali City (Nyacyonga Health Center), all with diarrhea at their first visit. The children were followed up 4 times more, and rectal swab samples were collected monthly and at any time when diarrhea was present.

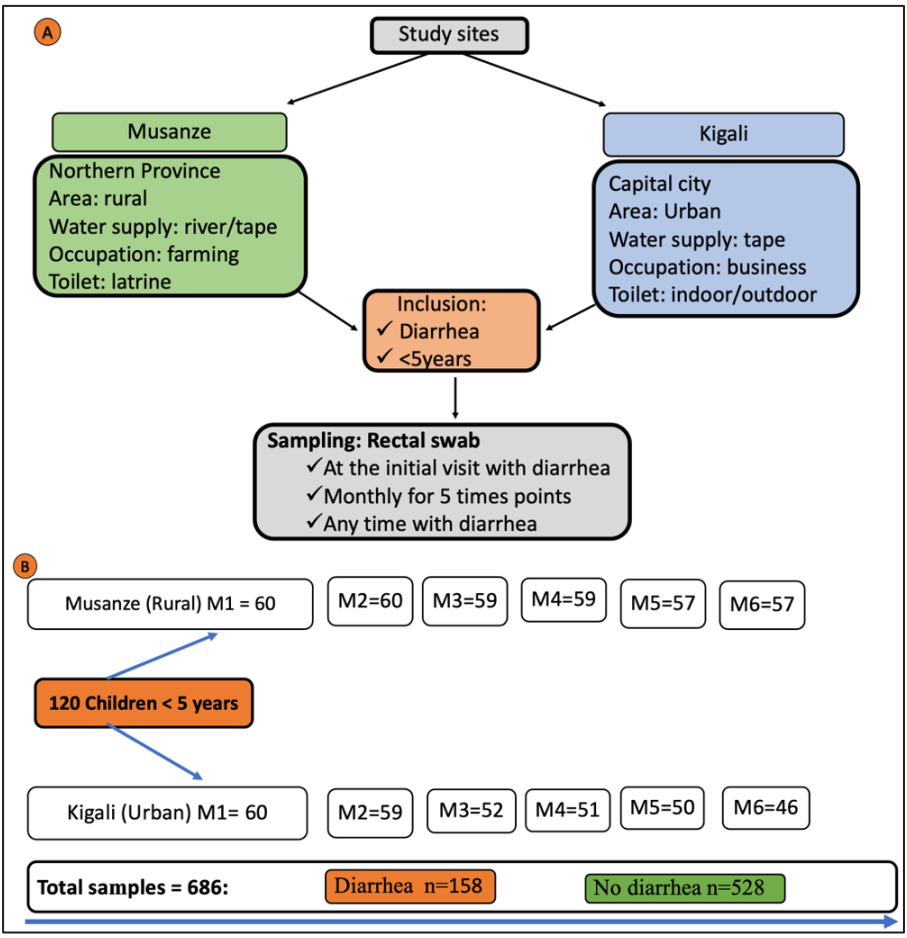

Figure 3: Longitudinal-observational-cohort study, June to December 2022, at rural (Musanze) and peri-urban (Kigali) sites. A: Study site differences, inclusion, and method. B: collected samples at each site at each time point. M: month, D: diarrhea

In Musanze 57/60 (95%) children have completed the follow-up, with 3/60 (5%) dropping out. In Kigali, 46/60 (76.7%) %) of children completed follow-up, with 14/60 (23.3%) dropping out. In Figure 2, we show all participants who attended the sample collection time and the number of participants who had diarrhea during the study period. The 17 diarrheal samples were collected at any time outside regular visits when diarrhea occurred, while 21 additional diarrheal cases were collected during the scheduled visit concurrently with diarrheal symptoms. In total, 686 samples were collected at 6 time points, including 158 samples collected when diarrhea was present, and 528 samples collected when diarrhea was absent, as presented in Figure 3.

3.3 SAMPLE COLLECTION

Children with diarrhea in studies I and II were recruited during their clinical visits at a health center or hospital when diarrhea was the chief complaint. In contrast, the asymptomatic children in Study I were recruited during vaccination visits, while in Study II, the children were sampled monthly over the follow-up time regardless of diarrhea presence. A rectal swab sample was collected from each participant in a tube containing 1 mL of sterile saline, stored at temperatures below +8 $^{\circ}$C for several hours, and then sent to the University Teaching Hospital of Kigali, where it was stored at -80 $^{\circ}$C until transported to the Department of Infectious Diseases at the University of Gothenburg, Sweden, for PCR analysis.

Table 2. Primers and probes for PCR pathogen detection

Pathogen	Forward primer	Reverse primer	Probe
Norovirus GII	TGGAYTTTTAYGTGCCCAG	CGACGCCATCTTCATTCAC	AGCCAGATTGCGATCGCCC
Rotavirus	AACCATCTACACATGACCCTCTATGA AACCATCTTCACGTAACCCTCTATGA	GGTCACATAACGCCCCTATAGC	CAATAGTTAAAAGCTAACACTGTCAAA
Astrovirus	GACTGCWAAGCAGCTTCGTGA	GCTAGCCATCACACTTCTTTGGTCCT	TCACAGAAGAGCAACTCCATCGCATTTG
Sapovirus	TTGGCCCTCGCCACCTAC GAYCASGCTCTCGCYACCTAC	CCCTCCATYTCAAACACTA	CCRCCTATRAACCA
Norovirus GI	TGGCAGGCCATGTTCCGCT	TTTGKTGGGGCGTCCTTAGAC CGCTTGATGTAGCGTCCTTAGAC	ATTGCGATCTCCTGTCCA
Campylobacter	ATGCAAACCATAATTGGGTTTCAAC	CGAGTATCAGCAACTTCTTCTACAGCT	TTGCCACCAAAACCAAAACT
Salmonella	CGGGTTGCGTTATAGGTCTGA	TGAAATACGATGCGAACAACATC	AATACTGCGCTGCCAGAT
ETEC-*estA*	AAGCATGAATAGTAGCAATTACTGCT	TTAATAGCACCCGGTACAAGCA	AACAACACAATTCAC
ETEC-*eltB*	TCCGGCAGAGGATGGTTACA	CCAGGGTTCTTCTCTCCAAGC	AGCAGGTTTCCCACCGGATCACC
Shigella/EIEC	ACCGGCGCTCTGCTCTC	GCAATGTCCTCCAGAATTTCG	CTGGGCAGGGAAATGTTCCGCC
Cryptosporidium parvum/hominis	CAAATTGATACCGTTTGTCCTTCTG	TGGTGCCATACATTGTTGTCCT	TGTCCTCCTGGATTCA
EPEC-*eae*	ACATGACCGATGACAAGGCA	CGCGACTGAAGCTGGCTAC	TCGCCGCCTGTTGTGCCG
EPEC-*bfpA*	GGTCTGTCTTTGATTGAATCTGCA	GCAGACTGGTAGTAAAACATCACACC	GCGCTTGCTGCCACCGTTACCG
Adenovirus 40/41	TGCCCGCGCCACCGAT	GAGCCACAGTGGGGTTTCTG	CCAGGCTGAAGTACG

MGB, minor groove binding.

3.4 REAL-TIME PCR AND TARGETS

We used PCR in all studies. The targets included ETEC-*eltB* or ETEC-*estA*, *Shigella/EIEC*, *Salmonella*, *Campylobacter*, Cryptosporidium, rotavirus, adenovirus 40/41, astrovirus, norovirus GI and II, sapovirus, EPEC-*bfpA,* and EPEC-*eae*. We used 250 µl of rectal swab mixed with 2 mL of lysis buffer for nucleic acid extraction in the EasyMag instrument (Biomerieux, Marcy l'Étoile, France). The nucleic acids were eluted in a 110 µl volume, and 5 µl was used for PCR. Amplification was done in the QuantStudio 6 Flex (Applied Biosystems, Foster City, CA) in multiple parallel 20-µL reactions of oligonucleotides and TaqMan Fast Virus 1-step master mix (ABI, for RNA targets) or universal master mix (ABI, for DNA targets). A 2-step amplification (15 seconds at 95°C, 60 seconds at 56°C) was run for 45 cycles after an initial 10-minute denaturation at 95°C and a 30-minute reverse transcription step at 46°C.[38]

3.5 ROTAVIRUS GENOTYPING

As per papers I and IV, the 74 samples that were positive for rotavirus were subjected to genotyping by real-time PCR, identifying the main G and P subtypes with type-specific primers and probes as previously described.[38]

3.6 NOROVIRUS GENOTYPING

A PCR specific for norovirus GII.4 was applied to samples that were positive for norovirus GII in multiplex PCR, using forward primer GATGGGTCCA-CAGCCAACCT; reverse primer CCGCTACAGGTGCCGCAA; and probe CGGGCTCCAAAGCCATAACCTCATT.

3.7 GENOTYPING IFNL4 AT RS12979860

The SNP at *rs12979860* of the *IFNL4* gene was genotyped using PCR. First, human DNA was extracted from the rectal swab samples as described above. The target region containing the rs12979860 was then amplified using specific primers in a PCR reaction, including forward primer: GTGCCTGTCGTGTACTGAACCA; reverse primer: AGCGCGGAGTGCAATTCA, in the TaqMan SNP genotyping master mix. Two differentially labeled allele-specific probes, including the C allele, FAM-CCTGGTTCGCGCCTT-MGB, and the T allele, VIC-CCTGGTTCACGCCT-MGB, were used to identify these specific alleles. The results were then analyzed to determine the genotype at the rs12979860 locus, which included GG, GC, or CC as previously described.[160]

3.8 GENOTYPING FUT2

The G428A at rs601338 (stop codon variant), a segment of the FUT2 gene, was genotyped after a human DNA extraction as described above. The coding region of the *FUT2* gene was amplified using PCR 45 cycles (15 seconds at 95 °C, and 60 seconds at 60 ºC with specific primers, including forward primer: GCAGAACTACCACCTGAACGACT; reverse primer: GTGGTCGTGCAGGGTGAAC and probe: FAM-MGB-G allele: CTGCTCCTGGACCTT and VIC-MGB-A allele: CTGCTCCTAGACCTT in a Taqman genotyping master mix (Applied Biosystems). The amplified products were analyzed to determine the *FUT2* genotype, which includes alleles such as *AA*, *AT*, or *TT*.

3.9 DATA ANALYSIS

The collected information was entered into a spreadsheet using Microsoft Excel to create a study database and subsequently transferred to the IBM SPSS Statistics 28.0 software (IBM Corporation, New York, USA) for statistical evaluation. Demographic and clinical profiles of the participants, along with pathogen identification frequencies, were analyzed using frequency distributions and descriptive statistical methods. To compare the proportions of PCR-positive and PCR-negative samples between groups, Fisher's exact test was employed. The population attributable fraction for each pathogen was calculated as PAF = prevalence x (1–1/OR) in paper I. The relationship and association analysis were analyzed by the chi-square test and multiple logistic regression analysis. Throughout the analysis, a p-value below 0.05 was deemed statistically significant.

3.10 ETHICAL CONSIDERATION

This study has received ethical clearance from multiple institutions in Rwanda, including the National Health Research Committee (NHRC/2021/PROT/033) and several institutional ethics panels. These comprised the Ministry of Health's review board (20/5722/DPMEHF/2021), the University of Rwanda's ethics committee (226/CMHS IRB/2021), and the University Teaching Hospital of Kigali's ethics council (EC/CHUK/110/2021). Before sample collection, researchers obtained voluntary agreement from the children's parents or legal guardians. Participation was entirely optional, and subjects were free to discontinue their involvement at any point without facing any repercussions.

4 RESULTS

4.1 PAPER I

4.1.1 PATHOGEN DETECTION RATES

In Paper I, we investigated the pathogen detection rates in children with (n=496) and without (n=298) diarrhea and found at least one pathogen in 68% of children in the diarrhea group, compared to 54% in the non-diarrhea group (p<0.001). Overall ETEC-*eltB* and *Shigella* were the most detected pathogens (21.3% and 12.8% respectively), with *Shigella* appearing in 17.3% of symptomatic children versus 5.4% of controls (p<0.001), followed by ETEC-*eltB* (26.6% vs 12.4%, p=0.003) and rotavirus (10.9% vs 4.7%, p=0.002). *Campylobacter* (8.7% vs 3%, p=0.011), ETEC-*estA* (10.9% vs 3.7%, p=0.037), and Cryptosporidium (3% vs 0.7%, p=0.04) also showed significant differences in detection rates between symptomatic and asymptomatic children.

Table 3. Pathogen detection rates

Pathogens	All	No Diarrhea n=298 (%)	With diarrhea n=496 (%)	P
All pathogens	498 (62.7)	162 (54)	336 (68)	<0.001
Shigella	102 (12.8)	16 (5.4)	86 (17.3)	<0.001
Rotavirus	68 (8.6)	14 (4.7)	54 (10.9)	0.002
ETEC-*eltB*	169 (21.3)	37 (12.4)	132 (26.6)	0.003
Campylobacter	52 (6.5)	9 (3)	43 (8.7)	0.011
ETEC-*estA*	65 (8.2)	11 (3.7)	54 (10.9)	0.037
Cryptosporidium	17 (2.1)	2 (0.7)	15 (3)	0.04
Astrovirus	28 (3.5)	7 (2.3)	21 (4.2)	0.2
Norovirus GI	30 (3.8)	9 (3)	21 (4.2)	0.44
Sapovirus	89 (11.2)	28 (9.4)	61 (12.3)	0.25
Norovirus GII	75 (9.4)	25 (8.4)	50 (10.1)	0.46
Salmonella	52 (6.5)	19 (6.4)	33 (6.7)	1
Adenovirus 40/41	56 (7.1)	21 (7)	35 (7.1)	1

The data are presented as frequency and (%). Odds: Odd Ratios, P: p values

4.1.2 ASSOCIATION WITH DIARRHEA

We further compared the odds ratios for pathogen detection and diarrhea. Our findings showed that *Shigella* (OR=3.9; P<0.001), rotavirus (OR=2.6; P=0.002), and ETEC-*eltB* (OR=2.4; P=0.003) had higher odd ratios, indicating that they were main causes of diarrhea in the studied population, followed by *Campylobacter* (OR=1.9; P=0.011), ETEC-*estA* (OR=1.4; P=0.037), and *Cryptosporidium* (OR=1.3; P=0.04), as shown in Figure 4.

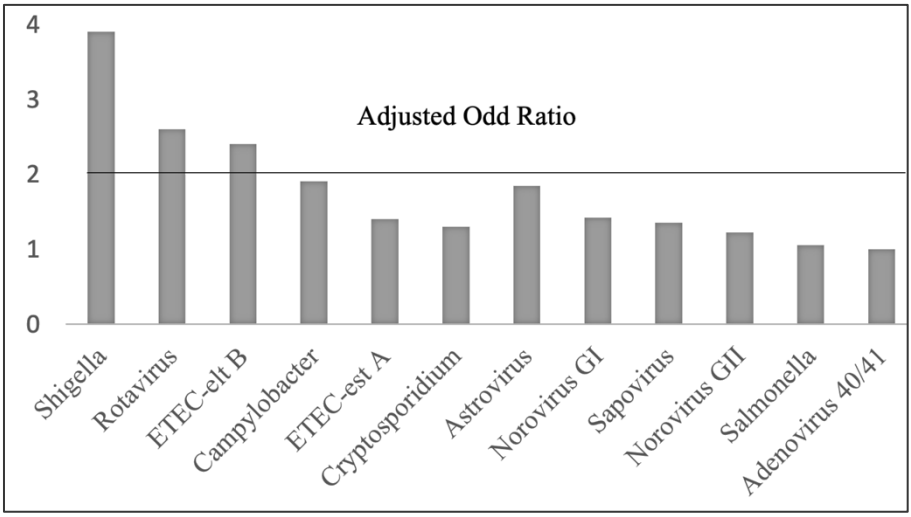

Figure 4: Comparison of pathogen detection in the diarrhea group and control group to determine the pathogen associated with diarrhea. Data are presented as adjusted odds ratios.

4.1.3 ROTAVIRUS VACCINATION AND DIARRHEA

Our analysis of the effect of rotavirus vaccination on symptomatic infection shows a reduced rotavirus infection in children with diarrhea. As shown in Table 4, rotavirus vaccination was significantly associated with a lack of diarrhea (OR 0.41, 95% CI: 0.22–0.76), both in those who were rotavirus positive or negative by PCR.

Table 4. Association of rotavirus vaccination status with diarrhea

Variables		Rotavirus vaccinated		P.value	OR
		No	Yes		
Rotavirus Negative	Diarrhea (n=441)	45	396	0.01	0.46
	No diarrhea (n=282)	14	268		
Rotavirus positive	Diarrhea (n=52)	8	44	0.19	0.47
	No diarrhea (n=14)	0	14		
All	Diarrhea (n=493)	53	440	0.002	0.41
	No diarrhea (n=296)	14	282		

The association between vaccination status and clinical presentation was investigated. Data are presented as frequencies. Vaccination status not known (n=5), OR: odds ratio

4.2 PAPER II

4.2.1 INFECTION AT M1 VS M2-M6

In Paper II, we conducted a longitudinal investigation of enteric infections at enrollment and at subsequent follow-up time points. As presented in Figure 5, the most common pathogens identified at the first visit (M1) when diarrhea was present included ETEC-*eltB* (34%), EPEC-*eae* (31%), rotavirus (24%), ETEC-*estA* (22%), and norovirus GII (19%). In the following months (M2-M6) during follow-up when diarrhea was usually absent, the rate of pathogen detection was significantly lower for rotavirus (OR=8.53; P<0.0001), ETEC-*estA* (OR=2.71; P<0.0001), norovirus GII (OR=2.45; P<0.0001), astrovirus (OR=2.62; P=0.00037), and *Cryptosporidium* (OR=2.5; P=0.00045).

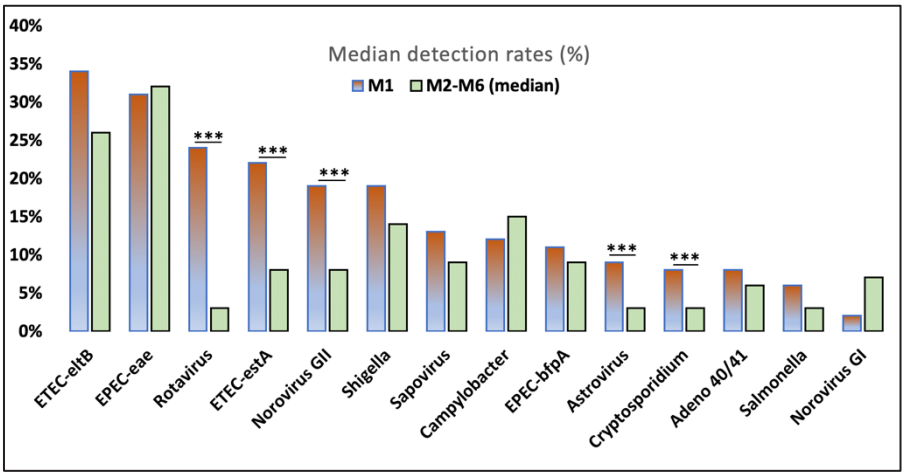

*Figure 5. Median detection rates at baseline compared with follow-up time points. Data are presented as percentages %. M: Month, ***: P values less than 0.001*

4.2.2 PATHOGEN ASSOCIATION WITH DIARRHEA

In total, 158 diarrhea episodes were recorded, including 120 at enrollment and 38 additional cases recorded during the follow-up period. The pathogen presence at these time points, when diarrhea was present, was compared with time points without diarrhea after excluding pathogen representing persistence (those detected at a preceding time point, assuming that diarrhea would be due to a newly acquired pathogen). Rotavirus (OR=7.24, $p<0.0001$), ETEC-*estA* (OR=3.84, $p<0.0001$), norovirus GII (OR=2.74, $p=0.0003$), ETEC-*eltB* (OR=1.99, $p=0.001$) and sapovirus (OR=2.05, $p=0.02$) demonstrated a significant link to diarrhea. These findings underscore the remaining importance of rotavirus for childhood diarrhea, despite 10 years of vaccination, and show that ETEC and norovirus GII were major causes of diarrhea in this cohort.

Table 5. Pathogen detection and association with Diarrhea

Pathogens	Diarrhea n=158	No diarrhea n= 528	OR	P
Rotavirus (n=45)	18.4	3	7.24	<0.0001
ETEC-*estA* (n=64)	20.3	6.1	3.84	<0.0001
Norovirus GII (n=65)	17.7	7.2	2.74	0.0003
ETEC-*eltB* (n=139)	31.6	16.9	1.99	0.001
Sapovirus (n=60)	13.9	7.2	2.05	0.02
Cryptosporidium (n=28)	7	3.2	2.24	0.06
Astrovirus (n=32)	7.6	3.8	2.09	0.05
Adenovirus 40/41 (n=39)	8.2	4.9	1.73	0.12
Shigella/EIEC (n=87)	17.7	11.2	1.58	0.08
EPEC-*bfpA* (n=54)	11.4	7	1.65	0.10
Salmonella (n=26)	5.1	3.4	1.53	0.34
EPEC-*eae* (n=151)	27.2	20.5	1.25	0.28
Campylobacter (n=60)	10.8	8.3	1.23	0.53
Norovirus GI (n=34)	1.9	5.9	0.31	0.06

Table 5. Frequency of pathogens (not detected in a preceding sample) at time points with or without diarrhea.

4.2.3 CT VALUE AND DIARRHEA

We further performed the analysis of PCR cycle threshold (Ct) values and found that lower Ct values (indicative of higher pathogen loads) were associated with diarrhea for ETEC-*estA*, rotavirus, norovirus GII, ETEC-*eltB*, astrovirus, and *Shigella*/EIEC. These differences were statistically significant, even after adjustment for potential confounders, showing that Ct values may improve the distinction between symptomatic and asymptomatic infections. Lower Ct values in diarrheal cases suggest a higher pathogen burden, which may contribute to symptom manifestation.

Table 6. Detected pathogen Ct values and association with diarrhea

Pathogens	With Diarrhea[b]	No diarrhea	P value	Adjusted P value[c]
ETEC-*estA* (n=64)	24.46	32.50	<0.0001	<0.0001
Rotavirus (n=45)	29.34	35.69	<0.0001	0.001
Norovirus *GII* (n=65)	25.10	30.93	0.0003	0.0004
ETEC-*eltB* (n=139)	28.55	32.68	0.002	0.0003
Astrovirus (n=32)	19.22	29.09	0.02	0.008
Shigella/EIEC (n=87)	29.78	33.66	0.03	0.02

Median Ct value. Only pathogens not detected in a preceding sample were considered in this analysis because diarrhea would be caused by a newly acquired pathogen. b Including the initial visit (M1) and time points with diarrhea during follow-up (at planned visits or at extra visits because of diarrhea). c Adjusted for age and study site by multiple logistic regression.

4.2.4 LONGITUDINAL INFECTION ACQUISITION

Over time, several pathogens were acquired in most of the children. The highest rates of new infections (10-19%) were observed for bacteria such as EPEC-*eae*, ETEC-*eltB*, and *Shigella*, whereas viruses were less often acquired (≈5%) in the subsequent follow-up time. Figure 6.

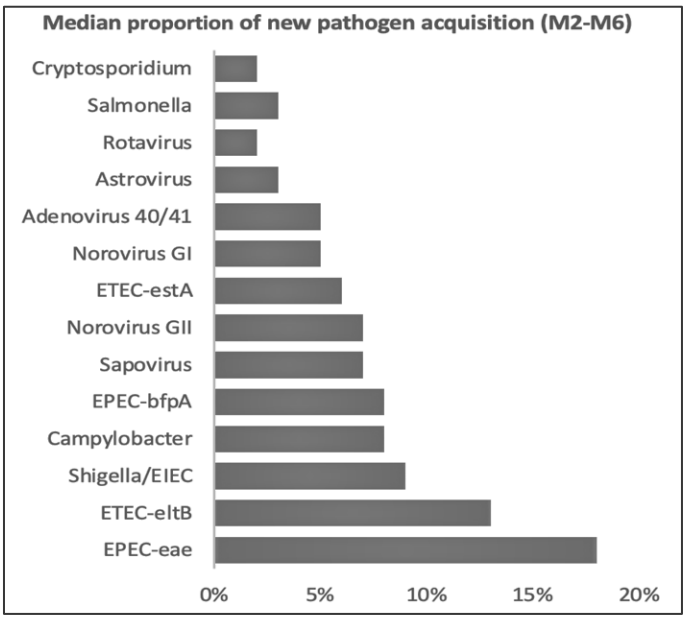

Figure 6: New infection acquired during follow-up, defined as when a pathogen was detected and had not been detected at the preceding time point.

4.2.5 LONGITUDINAL INFECTION CLEARANCE

At each subsequent sampling time point (M2-M6), the proportion of infections that were present at the previous time point and had been cleared was assessed, showing that almost all viral infections (78-100%) and 47-86% of bacterial infections were cleared between consecutive sampling time points, except a lower clearance or persistence rate of *Campylobacter* (47%). These findings highlight a higher pathogen clearance rate for viral agents. The clearance would be underestimated if reinfection happened between scheduled sampling time points. This might explain the low clearance of *Campylobacter,* which was observed only in rural areas, possibly due to a maintained exposure risk by the presence of household animals. Figure 7.

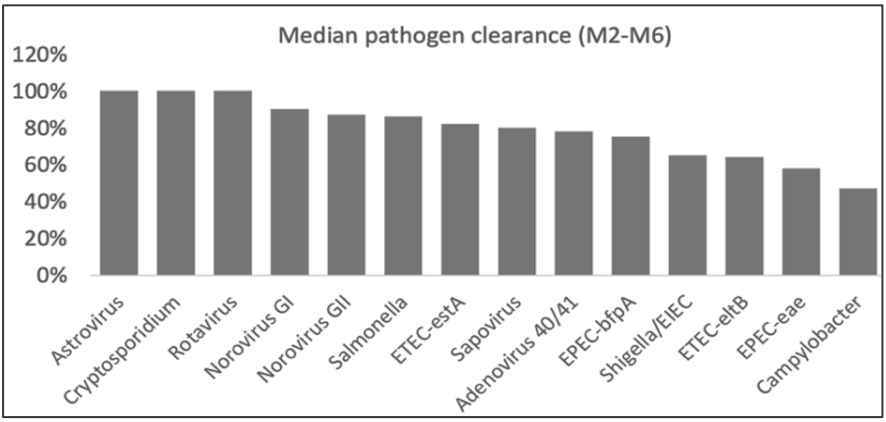

Figure 7. Median proportion of infections that had been cleared during follow-up time points. M: Month

4.2.6 PATHOGEN ACQUISITION SITE DISPARITIES

We further investigated the impact of living in rural areas vs urban areas, social category differences, water supply, and toilet type on infection acquisition. Our analysis shows that Cryptosporidium (OR=8; P=0.0003) and several bacteria such as *Campylobacter (OR=4; P=0.0001)*, *Shigella (OR=2.88; P<0.0001),* ETEC-*estA (OR=2.2; P=0.011)*, and ETEC-*eltB*

(OR=1.94; P<0.0001) were more often detected at follow-up time points M2-M5 among the children from rural areas as compared with urban (Figure 8). The significant association and odds ratios remained when multiple logistic regression analysis was used to adjust for age, socioeconomic class, water supply, or toilet type, highlighting that other factors possibly play a role in infection acquisition at these study sites.

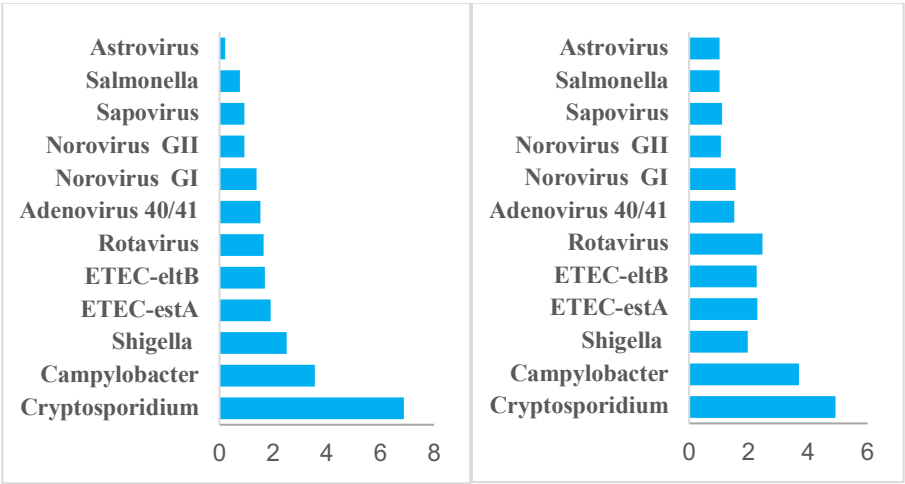

Figure 8. The left panel shows a comparison of frequency ratios (rural vs urban districts) for pathogens detected during follow-up (M2-M6) in Study II. The right panel shows the odds ratios when comparing samples from Kigali vs. rural areas in study I.

4.2.7 SITE DIFFERENCES IN DIARRHEAL CAUSES

The analysis of diarrheal causes across rural and urban sites has some differences. Diarrhea among the children in Kigali was strongly linked to norovirus GII (OR=4.12, p=0.0001) and *Cryptosporidium* (OR=11.8, p=0.0001). Diarrhea in Musanze was associated with bacteria such as EPEC-*bfpA* (OR=2.49, p=0.02), ETEC eltB (OR=2.30, p=0.004), and ETEC-*estA* (OR=5.56, p<0.0001), but also with viruses such as astrovirus (OR=5.75, p=0.009) and sapovirus (OR=3.63, p=0.001). Rotavirus (p<0.0001) remained significantly associated with diarrhea across both settings.

Table 7. Rural-urban differences in diarrhea causes

Pathogens	Urban		Rural	
	OR	P	OR	P
Adenovirus 40/41	1.30	0.58	2.5	0.11
Astrovirus	1.18	0.80	5.75	0.009
Norovirus *GI*	0.55	0.74	0.17	0.06
Norovirus *GII*	4.12	0.0001	1.42	0.25
Rotavirus	10.3	<0.0001	5.82	<0.0001
Sapovirus	1.02	1.00	3.63	0.001
Campylobacter	1.19	0.78	1.27	0.57
EPEC-*bfpA*	0.84	1.00	2.49	0.02
EPEC-*eae*	1.18	0.65	1.30	0.28
ETEC-*eltB*	1.69	0.10	2.30	0.004
ETEC-*estA*	2.17	0.11	5.56	<0.0001
Salmonella	1.99	0.32	1.09	1.00
Shigella/EIEC	2.20	0.07	1.32	0.40
Cryptosporidium	11.8	0.001	0.95	1.00

Only pathogens not detected in a preceding sample are considered. Data are presented as odds ratios and P-values.

4.3 PAPER III

4.3.1 FREQUENCY OF RS12979860 GENOTYPES

In paper III, we investigated whether the SNP at *rs12979860* of IFNL4 is associated with enteric infection in 795 children from Rwanda. In this study group, any virus was detected in 323 (41%), any bacterium was detected in 338(43%), and any parasite was detected in 23 (3%) of the participants. The frequency of the C allele was 41.7%, while the frequency of the T allele was 58.3%. As presented in Figure 6, the *rs12979860* genotype distribution was 17.4% CC, 48.9% CT, and 33.7% TT. Figure 9

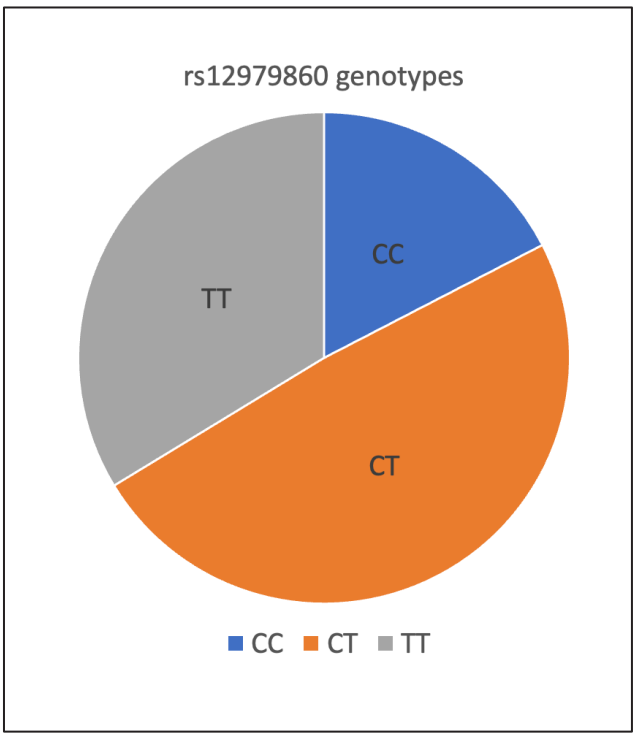

Figure 9: Frequency of rs12979860 genotypes of IFNL4 in 795 children from Rwanda

4.3.2 INFECTION AND RS12979860 GENOTYPES

Figure 7 shows that for most pathogens, the odds ratios for CC vs. CT/TT, as well as for TT vs. CC/CT, were close to 1 with P values above 0.1, indicating no difference in risk of infection between individuals with CC, CT, or TT genotypes. The exceptions (with borderline statistical significance) were norovirus GII with OR 1.75 (P=0.05) for the CC genotype and Cryptosporidium with OR=0.29 for the TT genotype (P=0.04). Figure 10

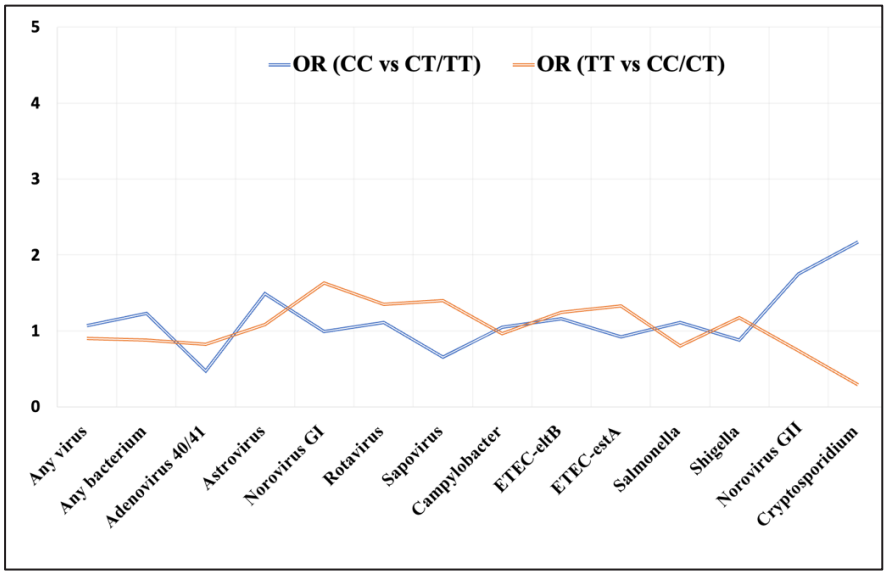

Figure 10. Association of pathogen detection and rs12979860 genotypes. OR: Odds Ratio

4.3.3 RS12979860 GENOTYPES AND DIARRHEA

The analysis of the association between the *rs12979860* genotype and diarrhea shows that there is no significant association between the *rs12979860* genotype of *IFNL4* and diarrhea for the overall pathogen (p = 0.25, OR = 0.83) or any specific pathogen (all p-values > 0.05). These findings suggest that the *rs12979860* genotype does not influence susceptibility to diarrhea or specific pathogen-related diarrhea in this cohort. Table 8

Table 8: Diarrhea and *rs12979860* genotypes of IFNL4

Pathogens	TT with diarrhea	CT&CC with diarrhea	P.value	Odd Ratio
All (n=795)	179	373	0.25	0.83
Adenovirus 40/41 (n=54)	12 (75%)	26 (68%)	0.75	1.38
Astrovirus (n=34)	9 (75%)	19 (86%)	0.64	0.47
Norovirus GI (n=29)	9 (69%)	13 (81%)	0.67	0.52
Norovirus GII (n=82)	16 (70%)	49 (83%)	0.23	0.47
Rotavirus (n=80)	28 (88%)	43 (90%)	1.00	0.81
Sapovirus (n=96)	30 (77%)	43 (75%)	1.00	1.09
Campylobacter (n=61)	17 (85%)	37 (90%)	0.67	0.61
ETEC-*eltB* (n=180)	60 (85%)	100 (84%)	1.00	1.04
ETEC-*estA* (n=86)	31 (91%)	46 (88%)	1.00	1.35
Salmonella (n=48)	10 (71%)	24 (71%)	1.00	1.04
Shigella (n=114)	35 (83%)	67 (93%)	0.12	0.37
Cryptosporidium (n=23)	2 (67%)	20 (100%)	0.13	0.00

Odds ratios for diarrhea among children with TT vs. CC/CT rs12979860 genotypes of IFNL4 in Rwanda

4.4 PAPER IV

4.4.1 PATHOGEN DETECTION AND FUT2 STATUS

In paper IV, study participants whose rectal swab samples contained enough human DNA to do SNP genotyping (n=668) were included. SNP analysis showed that 19% were homozygous for the *FUT2 rs601338* A allele (introducing a stop codon) and were thus defined as non-secretor or *FUT2⁻ (se)*, while 42% had a GA genotype and 39% a GG genotype and thus were defined as secretors or *FUT2⁺(SE/Se)*. As presented in Figure 9, rotavirus detection had the highest odds ratio (OR = 2.55) with a significant P-value of 0.019, reflecting that rotavirus was significantly more common among secretor-positive individuals. For the rest of the pathogens no significant association with *FUT2* status. Figure 11

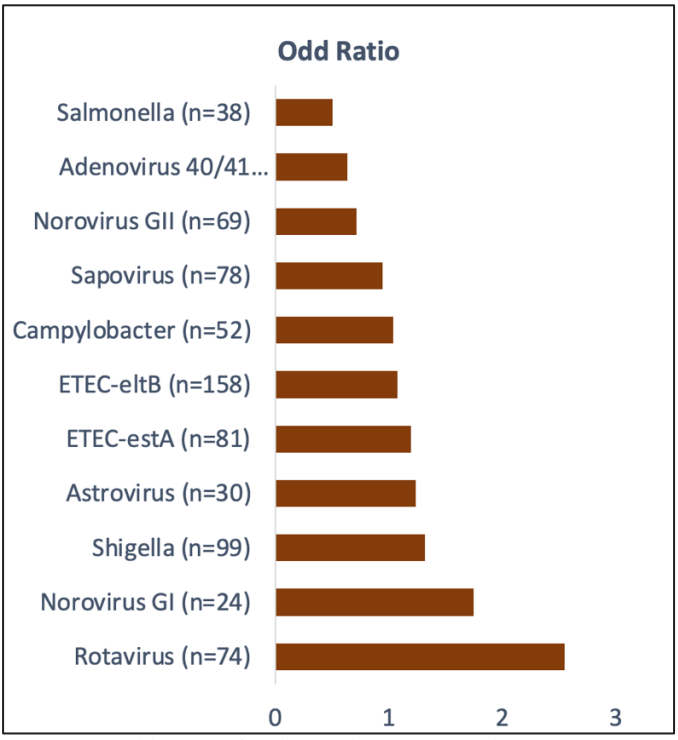

Figure 11: Odd ratios of pathogen detection rates by FUT2 genotype

4.4.2 DIARRHEA AND FUT2 STATUS

The proportion of non-secretors tended to be lower among children with diarrhea than among children without diarrhea (18% vs. 23%, age-adjusted OR=0.73, P=0.13), suggesting that non-secretors might have a slightly lower risk for diarrhea in general.

We then investigated the impact of secretor status on symptomatic infection. We assumed that if non-secretors had been protected from diarrhea, then the odds ratio would be lower than for *SE/Se*. This was seen only for rotavirus (OR 1.35 vs. 3.84, P=0.30). The other pathogens associated with diarrhea had a similar elevation of odds ratio in the *SE/Se* and *se groups* (but with P above 0.05 in the *se* group, likely reflecting the low number of cases, Table 9). In summary, we did not find any significant association between FUT2 status and pathogen-specific diarrhea.

Table 9. Association of symptomatic infection and secretor status

Pathogens	With vs without diarrhea (All)		With vs without diarrhea in SE/Se		With vs without diarrhea in se		P int.
	OR	P.value	OR	P.value	OR	P.value	
Rotavirus	3.37	0.0002	3.84	0.0003	1.35	1.0	0.30
Campylobacter	3.47	0.0023	3.13	0.012	5.25	0.16	0.65
ETEC-*eltB*	3.07	<0.0001	3.67	<0.0001	1.95	0.21	0.25
ETEC-*estA*	3.80	<0.0001	3.83	0.0003	3.57	0.14	0.94
Salmonella	1.38	0.47	2.25	0.18	0.73	0.75	0.17
Shigella	4.39	<0.0001	4.37	<0.0001	4.28	0.052	0.98
Cryptosporidium	9.21	0.007	7.86	0.02		0.54	0.62
Adeno 40/41	1.15	0.75	1.30	0.58	0.96	1.0	0.67
Astrovirus	1.72	0.31	1.62	0.50	2.20	0.66	0.80
Norovirus GI	1.27	0.82	1.28	0.81	1.07	1.0	0.90
Norovirus GII	1.58	0.16	1.74	0.15	1.33	0.79	0.69
Sapovirus	1.08	0.89	1.05	1.0	1.20	1.0	0.84

P int., P value for the potential impact of the interaction term (FUT2 status) on diarrhea.

4.4.3 FUT2 IN ROTAVIRUS/NOROVIRUS INFECTION

The samples for positive for rotavirus were further analyzed by genotyping (Table 10). Rotavirus P8 was the most prevalent P-type and was found in 50 of the 61 (82%) rotavirus-positive samples that could be genotyped. The number of cases was too low to analyze differences in FUT2 associations between the P genotypes. As shown in Table 5, the P8 genotype of rotavirus was detected in 2.3% (3/132) of *FUT2*$^{-}$ as compared with 8.8% (47/536) of *FUT2*$^{+}$ children (P=0.009), supporting that at least infection by this subtype is restricted by secretor status.

Norovirus GII was detected in 52 of 536 secretors (9.7%) and 17 of 132 non-secretors (13%). These 69 samples were further analyzed by a PCR that is specific for GII.4, identifying the GII.4 genotype in 2/17 non-secretors (12%), as compared with 12/52 secretors (23%, P=0.49). Thus, we did not find any significant association between FUT2 status and norovirus GII.4, a finding that might be due to the low number of cases.

Table 10. Secretor status in rotavirus and norovirus GII infections

Viruses	Genotype	SE/Se	se
Norovirus	Noro GII (All)	52/536 (9.7%)	17/132 (13%)
	Noro GII.4	12/52 (23%)	2/17 (12%)
Rotavirus	Rotavirus (All)	67/536 (13%)	7/132 (5.3%)
	Rotavirus *[P8]*	47/536 (8.8%)	3/132 (2.3%)
	Rotavirus *[P4]*	7/536 (1.3%)	0 (0)
	Rotavirus *[P6]*	2/536 (0.4%)	1/132 (0.8%)
	Rotavirus *[P9]*	1/536 (0.2%)	0 (0)

Data are presented as frequency and %. SE/Se: secretor or FUT2^{+}*. se : non-secretor or FUT2*

5 DISCUSSION

5.1 PAPER I

In paper I, we reported the presence of diarrheal pathogens in 68% of patients and 54% of controls, with *Shigella*, ETEC-*eltB*, and rotavirus being the most prevalent pathogens associated with diarrhea. These findings align with the previous findings in Malawi, where rotavirus, ETEC, and *Shigella* were among the pathogens associated with diarrhea.[33] Our findings are also consistent with the findings in Kenya,[161] Tanzania[162] , and elsewhere in a GEMS study done in 4 sites in Africa and three in Asia.[1]

This study confirms the importance of *Shigell*a and ETEC as the main pathogens associated with diarrhea reported by others.[163] In most previous studies, ETEC producing the heat-stable toxin (ETEC-*estA*) has been strongly associated, while a weaker or absent association has often been observed for ETEC-*eltB.* [1,64,164,165] However, an association between ETEC-*eltB* and diarrhea, especially during the first year of life (first exposure), has been reported.[164] We think that the restrictions introduced during COVID-19 might have delayed the first encounter with ETEC-*eltB.* Accordingly, a higher proportion would not have acquired adaptive immunity, resulting in a higher rate of symptomatic infections than in previous studies.

In our study, rotavirus was still detected in 10.9% of diarrheal cases and was significantly associated with symptoms despite 10 years of vaccination. Compared with a 2011-2012 study in Rwanda, rotavirus impact on diarrhea has substantially dropped with a reduction in the odd ratio from 23.5 to 2.6, indicating vaccination effectiveness.[64] This aligns with global findings showing a decline in rotavirus infection and a reduction of severe diarrheal

disease [166,167], probably mainly an effect of vaccination.[24] In a study conducted in Rwanda in 2014, just two years after rotavirus vaccination, the proportion of rotavirus detection in symptomatic children was still high (34%),[38] however, an additional seven years could have reduced the virus circulation in the community coupled with improvement in living condition, and likely with SARS-Cov2/COVID-19 restriction measure which was in place during the study time.

Even though findings confirm the importance and effectiveness of rotavirus vaccination in preventing childhood acute gastroenteritis and its severity, continued investigations are warranted in LMICs where rotavirus vaccine efficacy has been reportedly poor, with equal pathogen detection before and after vaccine introduction, and in sick and control groups. [33,35,168,169] The poor vaccine performance in developing countries could be due to vaccination dropout with incomplete doses, poor vaccine immunogenicity in undernourished children, or even a mismatch between vaccine and rotavirus genotype circulating in the area, as well as virus reassortment.[38,170]

5.2 PAPER II

In study II, we investigated the pathogen acquisition over time, pathogen clearance rate, and differences between rural and urban study sites. Our findings provide insights into the dynamics of continuous exposure to enteric infection and higher pathogen acquisition rates, but with the potential to clear the infection before the next sampling month, with urban-rural disparities in risk of infection exposure and pathogen responsible for diarrhea. These findings align with the previous longitudinal study in Egypt.[171] Additionally, our findings align with other studies that longitudinally investigated

infections due to enteric pathogens and found the diversity and complexity of enteric pathogen infections in children from LMICs.[172-174] In the current study, the samples collected at the baseline time point when diarrhea was present revealed a high prevalence of ETEC-*eltB* (34%), EPEC-*eae* (31%), rotavirus (24%), ETEC-*estA* (22%), and norovirus GII (19%), of which all but EPEC-*eae* were significantly associated with diarrheal symptoms. These findings are consistent with our results in paper I. The importance of *Shigella* as a cause of diarrhea is well established[64,163,175-178]. In Paper II, lower *Shigella* Ct values but not detection itself were associated with diarrhea (P=0.02). An association between lower Ct values (higher viral load) has been found in previous studies for several pathogens, suggesting that it could potentially help to discriminate and improve the identification of clinically relevant infections.[164,175,179,180]

The comparisons with other longitudinal studies revealed comparable detection rates of ETEC and EPEC across Rwanda, Guatemala, and Ghana, but with notably higher prevalence in rural Guatemala than in other sites. *Shigella* detection was also highest in rural Guatemala (52.3%), followed by Rwanda (19%) and Ghana (8%). *Campylobacter* was highly prevalent in rural Guatemala (60%) but less so in the other sites. These patterns likely reflect geographic and environmental differences in exposure to pathogens but may also be influenced by seasonal variation.[172,173]

New pathogen acquisition was relatively common for bacteria (13%-18%) and uncommon for viruses (5%) during follow-up time points, with EPEC-*eae*, ETEC-*eltB*, and *Shigella*/EIEC being the most common acquired pathogens. In general, the new infections were not accompanied by diarrhea. These results align with a previous longitudinal study in Egypt[181] and other longitudinal studies reporting that new pathogen acquisition was common

without causing symptoms,[172,173] and mirror findings from other LMIC studies[174] with continuous exposure and frequent asymptomatic infections, pointing to the importance of acquired immunity.

We observed higher pathogen clearance rates ranging between 78% to 100% for viruses and 47% to 85% for bacteria before the next sampling time points, consistent with prior findings that viral infections are typically short-lived while bacterial pathogens, like *Shigella* and *Campylobacter*, may persist.[174] These patterns observed in our study align with the findings in Zanzibar/Tanzania, where often cleared within 14 days.[152] A recent study by McMurray et al. reported the duration of post-diarrheal enteric pathogen carriage in young children from LMICs and found that shedding is variable between pathogens, with rotavirus being cleared in a week compared to the prolonged shedding of *Shigella* and *Cryptosporidium* in some children.[174] Prolonged shedding was also noted in some children in a study done in Ghana, with many children still harboring pathogens after 28 days. These pathogen clearance differences indicate that immune protection is partial and variable and highlight persistent transmission risks in high-burden settings, especially in LMICs.

In our study, most pathogens were more common in rural than urban areas, with *Campylobacter* (OR=4; ad.p =0.001), Cryptosporidium (OR=8; ad.p<0.025), and ETEC-*eltB* (1.94 adj. P =0.0056) being mostly detected in the children from the rural area together with ETEC-*estA* (OR=2.2;p=0.016), and *Shigella* (OR=2.88; p=0.0025). The associations remained after adjustment for age, socioeconomic status, location of the house (rural vs urban), water supply, and toilet types, suggesting that factors not evaluated in the current study are important. These possible factors may include living with animals, which is more possible in rural areas than urban areas,

sanitation standards, and an individual's hygiene and behaviors. Our results align with other studies that reported rural-urban disparities in infection distribution, with rural children being at higher risk of infection than their urban counterparts.[182,183] For example, *Campylobacter,* ETEC-*eltB*/*estA,* and *Shigella*/EIEC were consistently more prevalent in rural areas, as seen in a study from Guatemala.[172] In contrast, urban settings showed higher detection of norovirus and *Salmonella*, likely due to person-to-person transmission. Comparing our findings and the findings from the Guatemala study,[172] Cryptosporidium patterns were country-specific, further supporting the influence of local environmental conditions and possibly food or water contamination. *Campylobacter* was almost detected all the time from baseline to the last month of follow-up in our study, reflecting possible reinfection possibly due to animal exposure, especially in rural areas (OR=4; p<0.0001), and this is supported by a previous study which shows that animal exposure or household animal presence such as chickens, pigs, goats is a risk factor for *Campylobacter* infection.[184]

Additionally, the causes of symptomatic infection differed between the settings, with rotavirus being associated with diarrhea in both settings, while in urban diarrhea was strongly linked to norovirus GII (OR=4.12, p=0.0001) and *Cryptosporidium* (OR=11.8, p=0.0001), likely reflecting fecal-oral transmission amplified by overcrowding, contaminated water, and poor sanitation. Rural living conditions were associated with astrovirus (OR=5.75, p=0.009), sapovirus (OR=3.63, p=0.001), EPEC-*bfpA* (OR=2.49, p=0.02), ETEC-*eltB* (OR=2.30, p=0.004), and ETEC-*estA* (OR=5.56, p<0.0001), possibly driven by zoonotic exposure, open defecation, and limited clean water access. Our findings align with previous studies on the causes of childhood diarrhea.[1,5,15,25,32,41,64,81,173]

Despite high rates of enteric infection acquisition, symptomatic infection was not common during follow-up, likely due to acquired immunity reducing clinical presentation, and this is in line with Rogowski et.al, who have described the protection of natural immunity against enteric infection and pathogen-specific diarrheas [185]. However, the subclinical infections that were more common in our study participants have been recently reported to be associated with EED with intestinal inflammation, compromised nutrient absorption, and impaired child linear growth. [154,155,186]

This growth problem is an alarming situation in Rwanda, where 33% of children are stunted countrywide, according to the 2019 national demographic health survey.[156] The rural districts, including the Northern province where our study was conducted, bear the highest burden of childhood growth impairment, with some districts reporting rates exceeding 50%. In contrast, in the capital city (Kigali), the stunting rate is considerably lower, with some sectors reporting around 20%.[156,157] A cross-sectional study done in Rwanda has found several risk factors associated with childhood stunting and gastrointestinal illness, coupled with inadequate sanitation facilities, were highlighted as individual and community-level variables that were associated with childhood growth impairment.[158] Our findings also support a previous report on the association of prolonged episodes of diarrhea of 7 to 13 days with reduced child growth and increased risk of persistent diarrhea and malnutrition.[187] Moreover, Schnee et.al. have previously reported that *Campylobacter* infection is associated with child growth faltering and EED,[188]highlighting its implication in child malnutrition, which was higher in rural than in urban areas in Rwanda, and *Campylobacter* detection at baseline and follow-up correlated in the current study, indicating maintained risk.

These observed disparities between rural and urban areas in childhood enteric infection acquisition, and pathogens associated with symptomatic infections, together with previous findings on childhood stunting rate in Rwanda and associated risk factors, highlight the need for targeted interventions, especially in rural and economically disadvantaged areas. Addressing socio-demographic and environmental factors in reducing enteric infections could be vital in fighting poor childhood linear growth and improving overall child health in Rwanda and other similar settings, as previously reported that community-level sanitation access is associated with child health outcomes.[159,189]

5.3 PAPER III

The IFNL4, a member of the type III interferon family, plays a complex role in immune defense against infections, exhibiting well-documented antiviral properties alongside paradoxical clinical outcomes. Extensively studied in HBV, HCV, and respiratory infections, the *IFNL4* has demonstrated tissue-specific activity through epithelial receptors (*IFNLR1/IL10R2*), influencing mucosal immunity.[190] The *IFNL4* genotypes at rs12979860 paradoxically correlated with higher spontaneous HCV clearance,[21] predicted respiratory RNA viral infection clearance,[20] HCV treatment outcomes,[191,192] association with HBV chronic infection risk,[193] and COVID-19 susceptibility.[194] While the role of SNPs of *IFNL4* at *rs12979860* in hepatitis viral and respiratory viral infections is established, however, its role in enteric pathogen infections remains unexplored. In this paper III, we investigated the potential link between *IFNL4 rs12979860* genotypes and enteric infections in Rwandan children, a population exposed to high levels of enteropathogens.

In our study, we find the frequency of genotype distribution of 17.4% CC, 48.9% CT, and 33.7% TT, which is similar to the previously reported 17% CC, 49% CT, and 34% TT in similar settings,[20] and well-established frequency in African descendants.[195,196] Our findings show that, for most pathogens, the odds ratios comparing rs 12979860 genotypes (CC vs CT/TT and TT vs CC/CT and enteric infection were closer to 1, with p-value exceeding 0.1, suggesting no significant difference in infection risk across genotypes in our study population. In the previous study done in Rwanda, the TT variant was linked to impaired respiratory virus clearance in young children,[20] but our analysis found no connection between this genotype and enteric infections.

Further analysis revealed no association between *rs12979860* genotype and diarrhea, whether overall or pathogen-specific, as reflected by a p-value of 0.25 and an odds ratio of 0.83 for overall diarrhea risk. This suggests that genetic variability in *IFNL4* does not substantially influence susceptibility to diarrhea or pathogen-specific diarrhea in this studied population. Our findings are in contrast with the findings from Mali, which showed that the *rs368234815 dG* genotype (corresponding to *rs12979860 TT*) correlated with reduced early-life gastroenteritis risk.[133]

In that study conducted in Mali, they analyzed genetic variants in *IFNL4 (rs368234815)* and *IFNL3* (rs4803217) in 914 children, revealing allele frequencies of 72.2% for *IFNL4-dG* and 66.7% for *IFNL3*-T. These variants showed strong linkage disequilibrium, with the *IFNL4-rs117648444*-A variant (P70S) occurring exclusively on haplotypes carrying the *IFNL4-dG* allele. The *IFNL4-dG* allele was significantly associated with the risk of gastrointestinal infections and malaria, with earlier onset of gastrointestinal episodes. Associations for *IFNL3-rs4803217* were weaker and lost

significance when adjusted for *IFNL4*. Respiratory infections showed no significant links, and recurrence rates were unaffected, implicating *IFN-λ4,* not *IFN-λ3,* as a key genetic factor in early-life infection susceptibility[133]

Discrepancies between the Mali findings and other studies might, to some extent, arise from differences in allele frequencies (e.g., 51% dG in Mali vs. 34% TT in other cohorts), study designs (longitudinal vs. cross-sectional), or population-specific genetic architectures. For instance, the Mali study focused on *IFNL4-rs117648444* (exon 2) and *IFNL3-rs4803217* (3' UTR), while other studies examined *IFNL4-rs12979860* (intron 1), which exhibits strong linkage disequilibrium with related variants in European populations but weaker associations in African cohorts.[19] The high *IFNL4-dG* frequency in Mali (~72%) contrasts with lower frequencies in non-African populations. These differences may reflect that *IFN-λ*4's effects are dual and context-dependent – detrimental in early-life infections but potentially protective in chronic viral settings. Also in the Mali study, respiratory infections showed no significant links with *IFNL* SNPs, while it was previously reported that *IFNL4* genotypes predict RNA viral infection clearance.[20]

The *IFNL4-rs117648444-A* variant reduces IFN-λ4 activity through a P70S substitution, while the *IFNL4-dG* allele enables *IFN-λ4* production. This protein's rapid induction of immune regulators like SOCS1 and USP18 may explain its paradoxical effects: enhancing antiviral responses in chronic infections (e.g., HCV) but increasing susceptibility to acute pediatric infections. Population-specific linkage patterns and allele frequencies further modulate these outcomes, underscoring the need to consider genetic architecture when interpreting IFN-λ4's role in infection.[133]

Nonetheless, the use of rectal swabs offered a promising non-invasive method for collecting human DNA suitable for SNP analysis, leveraging an easy, affordable, and simple method that could enable efficient detection of SNPs associated with disease susceptibility, microbial interactions, or population genetics, particularly in studies requiring minimal invasiveness. The method's compatibility with commercial PCR reagents and tolerance to fecal matter suggests its adaptability for different applications, where it could complement existing SNP detection methods like allele-specific PCR or sequencing, providing an alternative to blood or saliva sampling in clinical or epidemiological settings.

5.4 PAPER IV

Secretor status, determined by the *FUT2* gene, dictates whether individuals secrete ABO blood group antigens into body fluids and mucus. Secretors express these antigens, which act as receptors for pathogens such as norovirus and rotavirus, making them significantly more susceptible to infections. Non-secretors, lacking these antigens, exhibit reduced pathogen binding in the gut, lowering their infection risk. This genetic dichotomy influences strain-specific susceptibility, underscoring the role of host genetics in shaping enteric infection outcomes.[16,197-199] However, this could be population-specific and depend on the pathogen or the pathogen genotype. Therefore, it is also of interest to investigate the possible relation between secretor status and other pathogens than those already investigated. In our paper IV, we investigated the potential impact of *FUT-2* rs 601338 SNP genotype on a broad range of enteric infections among children in Rwanda, where such data are lacking.

Our findings showed a frequency of 19%,42%, and 39% AA, AG, and GG genotypes in the studied population in Rwanda which is in line with a previous study in Burkina Faso and South Africa, where they found that 21% and 17% curries AA genotypes, respectively, and are non-secretors.[200,201] Detection of rotavirus was less likely to be found in non-secretors (OR=0.39, P=0.019), which is consistent with other studies.[202,203] Moreover, the previous report has shown that non-secretors confer complete protection against rotavirus P[8] strain infections.[17,202] However, subsequent reports, including our findings, suggest that P[8] can also infect non-secretors. Three cases (2.3%) of rotavirus P[8] were identified in non-secretors (3/132) compared to 47/536 secretors (8.8%; OR=0.24; P=0.009), consistent with recent findings that non-secretors are significantly but completely protected from rotavirus P[8] infection.[204,205]

The lower rates of rotavirus detection in non-secretors observed in our study reflect the situation among vaccinated children, who constitute 93% of the children in our study. Non-secretors might develop a poorer response to the vaccine,[205] and it would be interesting to compare the impact of *FUT2* status in vaccinated and unvaccinated children; however, the latter group was too small to allow this analysis. Our study revealed an expected prevalence of norovirus GII in non-secretors (13%), compared to secretors (9.7%) in Rwanda, challenging the notion that non-secretor status protects against all norovirus GII infection. This observation was surprising and suggests that secretor-independent strains of norovirus GII were more common than secretor-dependent strains in Rwanda at the time of our study. Norovirus binds to *FUT2*-dependent glycans in a strain-dependent manner,[206,207] and these non-secretors are protected from infection by strains from GII.4[208] as well as GII.3 strains.

In contrast, other less frequent strains, such as GII.2, may infect individuals regardless of their *FUT2* genotype status.[209] This possibility is supported by the detection of GII.4 in only 14 out of 71 (19.7%) norovirus GII-positive cases, where 2 of these GII.4 strains were found in non-secretors (2 out of 17; 12%) compared to 12 in secretors (12 out of 52; 23%), suggesting that non-secretors are less susceptible to norovirus GII.4 than to other norovirus GII strains in our studied population.

Previous studies have reported *FUT2* activity to be associated with inflammatory bowel disease (IBD),[210] and *Campylobacter* infection,[211] and secretors from Bangladesh have been reported to be less likely to have symptomatic enteric infections by certain ETEC strains.[197,212] We found that the odds ratios for diarrhea among secretors compared to non-secretors were elevated for rotavirus, ETEC-*eltB*, and *Salmonella* (with P values ranging from 0.10 to 0.20), suggesting that non-secretors might be protected from symptomatic infections by these pathogens. Further studies, including a larger number of non-secretors and a more detailed characterization of pathogens, such as rotavirus and norovirus typing, are needed to clarify if and how secretor status may be linked to enteric infection and diarrhea.

6 CONCLUSION

6.1 PAPER I

In a cross-sectional study of 794 Rwandan children, we found that *Shigella* and ETEC were the main causes of diarrhea, while rotavirus was no longer the predominant etiology, although it remains an important cause of diarrhea despite vaccination since 2012.

These findings underscore the success of the rotavirus vaccine program but also highlight the need for targeted interventions against *Shigella* and ETEC. Continued surveillance, including monitoring of rotavirus genotypes, remains essential to inform public health strategies and adapt preventive measures to the evolving landscape of childhood diarrheal diseases in Rwanda.

6.2 PAPER II

In a longitudinal cohort study of 120 children followed over five months, we found that new enteric infections were frequently acquired, indicating heavy exposure, particularly in rural areas, but also that most infections are cleared within one month. The results emphasize the need for additional studies that identify the factors that promote the spread of enteric infections and that explain the much higher rates in rural areas. This is necessary for strategic intervention to reduce childhood diarrhea and improve growth and development.

6.3 PAPER III

Study III, which included 795 children, showed that the *IFNL4*-related r*s12979860* genotype frequencies were 17% CC, 49% CT, and 34% TT. The findings indicate that genetic variation in the *IFNL4* related to *rs12979860* likely does not play a role in determining enteric infection susceptibility or infectious diarrhea among children

6.4 PAPER IV

The findings in study IV, which included 668 children, showed that 'non-secretors' with a *FUT2* stop codon variant constituted 19% of the population and had a lower frequency of rotavirus, specifically rotavirus [P8], in line with previous reports. The study identified no association with a broad range of other pathogens, results that have not been described before.

6.5 OVERALL CONCLUSION

Taken together, the findings in all these studies emphasize the clinical importance of *ETEC*, *Shigella*, and rotavirus as main causes of diarrhea in developing countries, the children's ability to effectively clear most infections, and the heavy exposure to enteric pathogens that children in Rwanda face, especially those in rural areas. The findings suggest that improved living conditions should probably be a main priority. Furthermore, genetic individual susceptibility to rotavirus *[P8]* was observed in sectors of individuals.

7 FUTURE PERSPECTIVES

- Expanded surveillance of rotavirus genotype dynamics post-vaccination, incorporating larger, geographically diverse cohorts and advanced genomic sequencing to clarify vaccine-driven selection pressures and long-term strain evolution trends, as well as vaccine effectiveness in terms of infection and symptom prevention.

- Long-term cohort studies assessing genetic determinants of susceptibility to symptomatic vs. asymptomatic enteric infections, including host-pathogen interactions influencing pathogen clearance and persistence or reinfection.

- Design and establish an advanced, rapid, and applicable diagnostic platform combining pathogen-specific biomarkers and host response signatures to (1) distinguish viral, bacterial, and parasitic infections requiring differential treatment, and (2) differentiate persistent infections from reinfections, enabling precision therapies that curb unnecessary antibiotic use and combat antimicrobial resistance.

- Pathway investigations into how enteric pathogen infections interfere with growth during a developmental stage for targeted intervention

- Combined interventional trials targeting environmental enteric dysfunction (EED) and mitigating infection-related developmental delays

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