Molecular Epidemiology of Noroviruses in Ghanaian Children

A Dissertation Submitted to the University of Ghana, Legon in Partial Fulfillment of the Requirement for the Award of PHD in Molecular Cell Biology of Infectious Disease Degree

By

Belinda Naa Larteley Lartey
(10551338)

JULY, 2020

Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Sciences, University of Ghana

DECLARATION

I hereby declare that this thesis is the result of my own research undertaken at the Regional Rotavirus Reference Laboratory in the Department of Electron Microscopy and Histopathology at the Noguchi Memorial Institute for Medical Research (NMIMR) under the supervision of Prof. G. E. Armah (NMIMR), Dr. Osbourne Quaye of the Department Of Biochemistry, Cell And Molecular Biology, University Of Ghana, Legon and Dr. Francis E. Dennis (NMIMR). I further declare that other people's work have been duly acknowledged by reference and that no material presented in this work has been previously submitted to any other institution for acquiring a degree.

BELINDA NAA LARTELEY LARTEY .....
(CANDIDATE)

DATE: 21-07-20

PROFESSOR GEORGE E. ARMAH .....
(PRINCIPAL SUPERVISOR)

DATE: 21- July 2020

DR. OSBOURNE QUAYE .....
(SUPERVISOR)

DATE: 21-07-2020

DR. FRANCIS E. DENNIS .....
(SUPERVISOR)

DATE: 21 July 2020

DEDICATION

To all children under five years of age who continually suffer and die from vaccine-preventable diseases.

ACKNOWLEDGEMENTS

I could not have imagined having a better advisor and mentor for my Ph.D. study. I am highly indebted to my life–coach, Professor George Enyimah Armah whose expertise, understanding, generous guidance, and support have ensured the successful completion of this work.

The opportunity I had with the virology lab was a great chance for my learning and professional development. I express my gratitude and appreciation to my supervisors Dr. Osbourne Quaye and Dr. Francis Dennis for always making time off their busy schedules to give me the necessary advice and guidance as well as resources to make my research work a success. Without your assistance, constructive criticisms and dedicated involvement in every step of my research processes, this work would never have been completed.

Besides my advisor and supervisors, I am immensely grateful to Dr. Susan Damanka, and Dr. Chantal Agbemabiese and for their insightful comments, technical support, encouragement, and also for their tough questions which incented me to widen my research from various perspectives.

I acknowledge the financial support received from the West African Center for Cell Biology of Infectious Pathogens (WACCBIP) that saw my research through and the AMED project for paying my salary.

I am forever thankful to Sabina Kwofie “my lab partner”- no matter the time or how long we needed to be there on the bench to get an assay working or done, you were always ready and willing to offer a helping hand.

To the staff of the Department of Electron Microscopy and Histopathology, NMIMR: Fred, Clement, Armachie, Yamoah, Jennifer, Elijah, Michael, Abigail, and Afua- I say thank you so much for your support, encouragement, and concern for my work.

I acknowledge the support of the staff of WACCBIP, the Department Of Biochemistry, Cell and Molecular Biology, members of the Virology Lab, and the companionship of my colleagues.

Most importantly, none of this could have happened without my family. To my best friend Nee Martey, my parents, and my siblings, this dissertation stands as an attestation for your unconditional love, support, and encouragement. Your confidence in me means so much.

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LIST OF ABBREVIATIONS

AGE	Acute Gastroenteritis
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BEAST	Bayesian Evolutionary Analysis by Sampling Tree
°C	Degree Celsius
Cap	Capsid gene
cDNA	Complimentary deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ESS	Effective sampling sizes
FUT2	α-1,2-fucosyltransferase
FUT3	α-1-3(4)-L-fucosyltransferase
G	Genogroup
GE	Gastroenteritis
GI	Genogroup one
GII	Genogroup two
HEL	Helicase
MCMC	Markov chain Monte Carlo
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
NS	Non-structural protein
nt	Nucleotide
NTP	Nucleoside triphosphate
NTPase	Nucleoside triphosphatase
ORF	Open Reading Frame
NoV(s)	Norovirus(es)
P	Protruding domain
PBS	Phosphate buffered saline
PABP	Poly (A)-binding protein
P/Pol	Polymerase
PRO	Proteinase
RdRP	RNA dependent RNA polymerase
RNA	Ribonucleic acid

µl	Microliter
(m)RNA	Messenger Ribonucleic acid
(sg)RNA	Sub-genomic Ribonucleic acid
(ss)RNA	Single stranded Ribonucleic acid
RDP4	Recombination Detection Program
RPM	Revolutions per minute
RT – PCR	Reverse transcription-polymerase chain reaction
RT	Reverse Transcription
S	Shell domain
UV	Ultraviolet
V	Volts
VP	Viral protein
VPg	Virion Protein Genome
PCR	Polymerase Chain Reaction
WHO	World Health Organisation

ABSTRACT

Background:

The human noroviruses are a highly diverse group of diarrheagenic RNA viruses which are globally distributed, and a cause of acute gastroenteritis in all age groups with the elderly and young children usually experiencing severe clinical outcomes. To date, at least 40 different genotypes of noroviruses belonging to two major genogroups have been observed in humans. These different genotypes have been suggested to be associated with different transmission patterns and disease severity in humans. Also, host genetic factors including the presence of ABO antigens and mutations in the fucosyltransferase (*FUT2* and *FUT3*) genes affect the susceptibility of individuals to infection with these diverse norovirus genotypes. This has raised questions on whether the prevalence, as well as the percentage of circulating Histo-Blood Group Antigen (HBGA) mutations within a population, would influence the prevalence of specific norovirus genotypes as a function of their ability to infect certain HBGA types. Moreover, the continuous changes observed in the genetic diversity of the noroviruses highlight the need for sustained surveillance to provide a full overview of norovirus epidemiology for future vaccine policy decisions. The overall aim of this thesis was to get a better understanding of norovirus infection dynamics, strain diversity, evolutionary dynamics, and the host genetic factors associated with the risk of norovirus infection in the Ghanaian pediatric population.

Methods:

A chronologically comprehensive 10-year study was conducted with diarrheic stool samples collected during active surveillance for diarrhoea in Ghanaian children between January 2008 and December 2017. A total of 1,337 stool specimens were obtained and subjected to RT-PCR and partial nucleotide sequencing for the typing of the polymerase and capsid genes of the norovirus

genome. Phylodynamic and evolutionary relationships were performed using MEGA 6.0 and BEAST software, respectively, to analyze sequences that overlapped at open reading frame (ORF) 1 and ORF2 regions. The entire coding region of the *FUT2* gene was also amplified from saliva samples collected from a cohort of the study population and genotyped using RLFP.

Results:

Overall positivity for norovirus infection among the Ghanaian pediatric population was 36.2%. Infection was most commonly (82.7%) observed in children aged between 6-24 months, suggesting that 0-6 months would be the most appropriate age range for effective norovirus vaccination, as early prevention is critical. Results from this thesis showed broad norovirus genotype diversity characterized by the circulation of both GII.4, and non-GII.4 strains.

The evolution of these norovirus strains was usually a result of both intra- and intergenic recombination that occurred within the capsid and polymerase genes. A total of 25 capsid/RdRP combinations were detected with GII.4[P4] (25.9%); GII.4[P16] (9.2%); GII.3[P21] (6.3%); GII.4[P31] (4.6%) and GII.6P[7] (4.0%) being the most common norovirus strains. Children infected with non-GII.4 norovirus strains recorded equally severe clinical illness comparable to that caused by GII.4 norovirus strains. Data from this thesis also suggest that most norovirus strains circulated at low prevalence within the population before their recognition as epidemic and pandemic strains associated with increased norovirus outbreaks.

The study results further indicate that neither secretor status nor genotype difference affects the susceptibility of an individual to norovirus infection in Ghana. More than half (62.0%; 8/13) of symptomatic patients were found to be carriers of the G428A mutation for inactivation of the FUT2 enzyme. Comparing norovirus genotype GII.4 with non-GII.4 genotypes, we observed that GII.4

norovirus strains infected more secretor-positive children who possessed heterozygous allele of the *FUT2* gene than non-GII.4 strains (60.0% vs 40.0%, p=0.035) whereas non-GII.4 norovirus strains had a preference for secretor-positive children with the homozygous allele of the gene (62.5% vs 37.5%, p<0.05) in the study population.

Conclusions:

In summary, the study confirmed the significant role that noroviruses play in the cause of acute gastroenteritis among Ghanaian children and further contributes to our understanding of the epidemiology and evolution of the virus which hopefully can lead to better preventive measures for norovirus disease as well as baseline data for vaccine policy decisions. Since the epidemiology of norovirus changes rapidly, the establishment of systematic surveillance within sentinel sites across the country would enhance the monitoring of circulating norovirus strains and allow us to have a continuous understanding of the current state of norovirus infection within our settings.

CHAPTER ONE

General Introduction

1.0. Background

1.0.1 Diarrhoeal disease: A Common Illness

Diarrhoea is usually a symptom of the secretory response of the gastrointestinal tract to a variety of external stimuli which can either be infectious (when caused by bacteria, viruses, and parasites) or non-infectious (when caused by chemical toxins or medications) (Mathan, 1998). It is a common cause of illness in individuals across all age groups with potential complications occurring in very young children and elderly people (Troeger et al., 2018a). As defined by the World Health Organisation (WHO) and Infectious Diseases Society of America (IDSA), diarrhoea is the passage of 3 or more loose or liquid stools within 24 hours (WHO, 2017).

Diarrhoea caused by micro-organisms is characterized first by the inflammation of the gastrointestinal tract (gastroenteritis) as a result of its colonization by the infecting pathogen. Subsequently, there is a disruption in the balance of intestinal fluid absorption and secretion mechanism, which then results in watery stool, discharged frequently in large volumes (Mathan, 1998). Poor sanitation, lack of access to safe and clean drinking water, improper food preparation, and inadequate hygiene practices are the main risk factors for diarrhoeal disease (WHO, 2011). The term “diarrhoeal disease” would be used synonymously with “acute gastroenteritis (AGE)” in this thesis.

1.0.2 Global Burden of Diarrhoeal Disease: Decreasing but Still a Leading Cause of Death

Despite improvement in its management and prevention, diarrhoeal disease continues to have a marked effect on global health. Since 1990, it has been listed as one of the top 10 cause of morbidity and mortality in persons of all age groups (Bern et al., 1992; Troeger et al., 2017a) and one of the five leading cause of deaths in children under five years of age (Kosek et al., 2003; Liu L et al., 2016). The incidence of diarrhoeal disease is global and has been estimated to be responsible for nearly 1.6 million deaths in the year 2016 (Troeger et al., 2018a). More than a quarter of these deaths (27.0%) occurred among children under five years of age of which approximately 90% occurred in 65 countries in sub-Saharan Africa and South-East Asia [Fig 1.1; (Troeger et al., 2018a)].

1.0.3 Etiological Agents of Diarrhoea

Several etiological agents including a variety of bacteria, viruses, parasites (protozoans), and fungi have been identified as causing diarrhoea disease (Mathan, 1998). However, viruses are associated with the majority of diarrhoeic infections globally as shown in Fig 1.2 (Bányai et al., 2018). At times, based on location and patient population, about 50% of diarrhoeal cases may remain undiagnosed using conventional laboratory diagnostic techniques (Mokomane et al., 2018; Simpson et al., 2003; Thompson et al., 2015).

1.0.3.1 Etiology of Bacterial Diarrhoea

Diarrhoea caused by bacteria accounts for approximately 20% - 40% of all diarrhoeal cases (Hosangadi et al., 2019; Nic Fhogartaigh & Dance, 2013; Prudden et al., 2020) and can range from mild to severe diarrhoea. The clinical symptoms of bacterial diarrhoea include but not limited to watery stool, vomiting, nausea, blood, or pus in

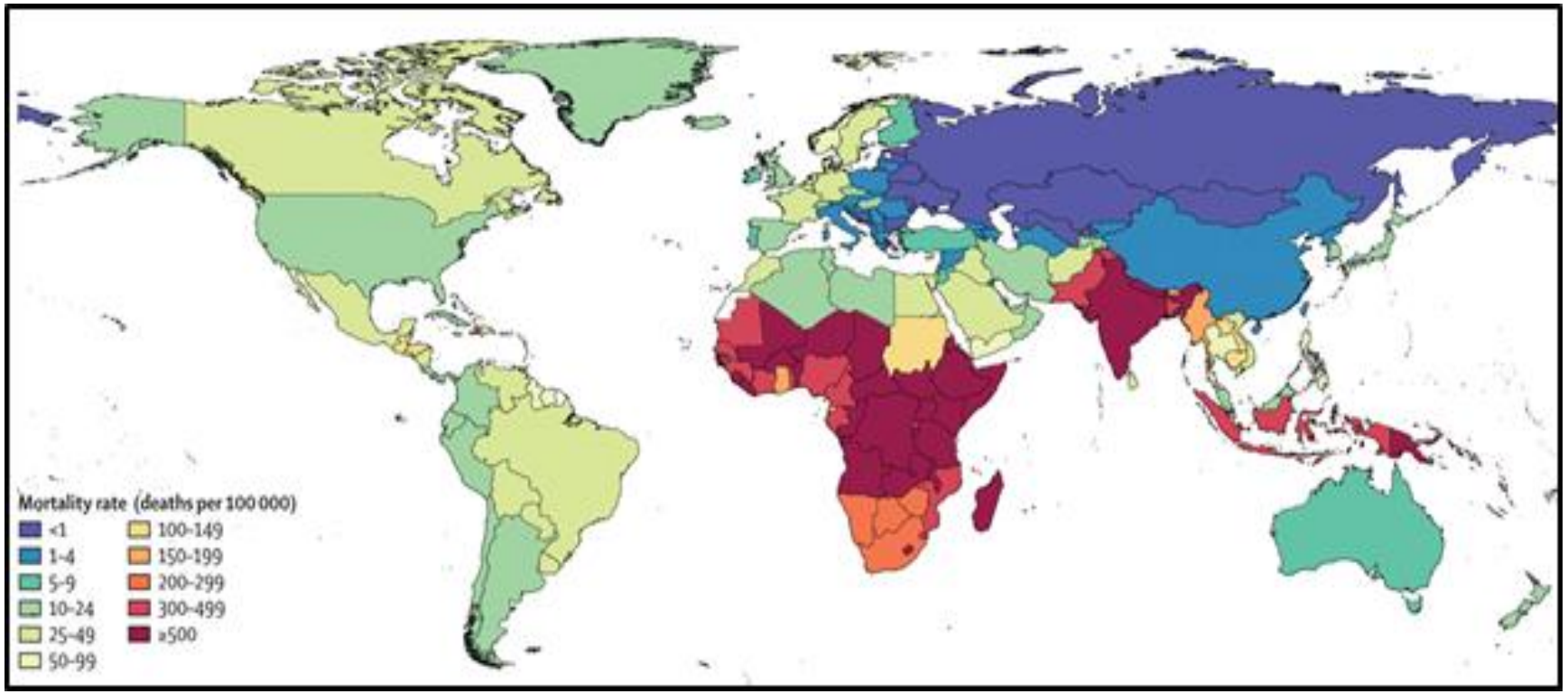


Fig 1.1: Global rates of diarrhoeal mortality in children less than 5 years in 2016

Children within sub-Saharan Africa and South East Asia accounted for nearly 90% of deaths

Adapted from (Troeger et al., 2018a)

stool, abdominal pain, abdominal bloating, and cramps. Common bacterial agents associated with diarrhoea include diarrheagenic *E.coli, Shigella spp, Campylobacter jejuni,* non-typhoidal *Salmonella spp,* and *Vibrio spp*. Diarrheagenic *E. coli* is further classified into 6 major pathovars based on some specific virulence properties. These are enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), diffusely adherent *E. coli* (DAEC), and the recently described adherent invasive *E. coli* (AIEC) pathotype (Croxen et al., 2013; Khalil et al., 2019). Recent findings from the Global Enteric Multi-Center Study (GEMS) which sought to estimate the burden of pediatric diarrhoeal disease in Africa and Asia showed that diarrheagenic *E.coli* and *Shigella spp* were amongst the four main causative agents of moderate to severe diarrhea in children (Kotloff et al., 2013).

1.0.3.2 Etiology of Parasitic Diarrhoea

Parasitic or Protozoan diarrhoea have also been determined as an important cause of diarrhoea (Fig 1.2). The common clinical features associated with parasitic diarrhoea include diarrhoea with abdominal cramping, vomiting, flatulence, and weight loss (Mulatu et al., 2015). Symptoms can be severe in younger children, undernourished, and immunocompromised patients. *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia lamblia* have been reported as the most common protozoan pathogens causing diarrhoea (Ahmed et al., 2016; Huh et al., 2009). *Cyclospora cayetanensis*, *Isospora belli*, *Blastocystis hominis*, *Strongyloides stercoralis,* and *Microsporidium* are other less commonly isolated diarrheagenic parasitic agents. According to a Global Burden of Disease Study (GBDS), cryptosporidiosis accounted for approximately 100,000 deaths in children whilst amebiasis one of the common complications of *E. histolytica* was responsible for more than

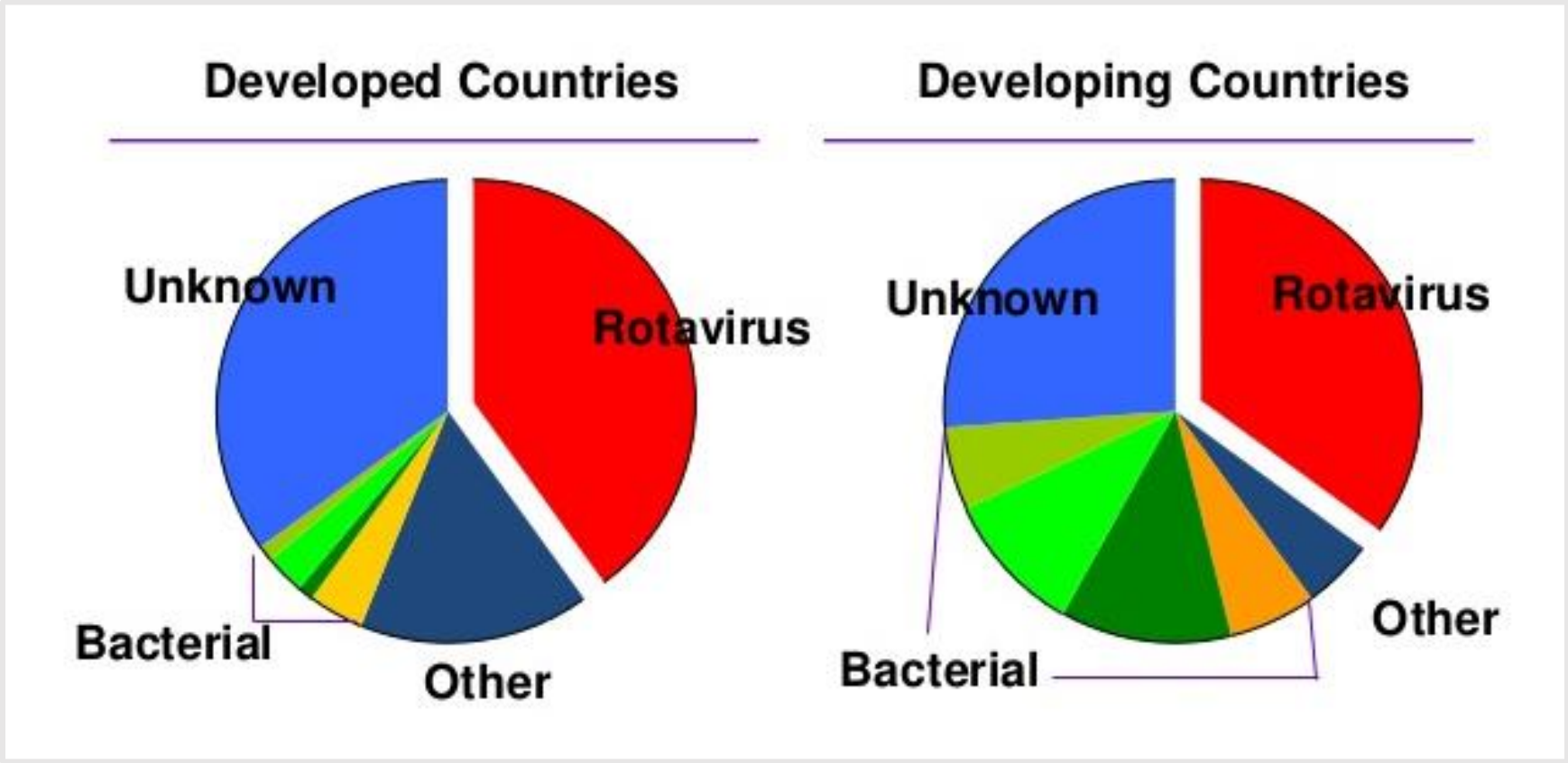


Fig 1.2: Estimate of the role of etiological agents associated with severe childhood diarrhoea both in the developed (left) and developing (right) countries.

Adapted and modified from (Kapikian AZ, 2003)

55,000 deaths globally in 2010 (Hotez, 2014). It is worthy to note that although giardiasis caused by *Giardia lamblia* does not result in deaths, it was associated with 171,100 disability-adjusted. life years (DALYs) in 2010 (Torgerson et al., 2015). In addition, *E. histolytica*, *G. lamblia*, and *C. parvum* are considered by the National Institutes of Health (NIH) and Centers for Disease Control and Prevention to be important agents for bioterrorism threats (Debnath & McKerrow, 2017).

1.0.3.3 Etiology of Viral Diarrhoea

Viral diarrhoea is usually self-limiting with recovery occurring within 2–5 days of infection (Blacklow and Greenberg, 1991). The major viral etiology of diarrhoea are the rotaviruses, noroviruses, sapoviruses, astroviruses, and human enteric adenoviruses (Green, 2013). These enteric viruses have been associated with severe diarrhoeal disease leading to hospitalizations in infants and children across the globe (Glass et al., 2001; Mokomane et al., 2018; Wilhelmi et al., 2003a). Rotavirus, norovirus, and sapovirus are the global leading cause of diarrhoea-associated morbidity and mortality in young children under five years of age (Bányai et al., 2018).

Rotavirus is a genus in the family Reoviridae and accounts for more than 258 million episodes of diarrhoeal infections, ~ 39% hospitalizations, and 128,500 deaths in children under five years of age, with 90% of these deaths occurring in the sub-Saharan African region (Troeger et al., 2018b). Rotaviruses are also known to infect and cause disease in the elderly population (Beck-Friis et al., 2019). Rotaviruses infect both humans and animals and hence have been classified into nine groups (A-I) based on their host infectivity (Bányai et al., 2017; Matthijnssens et al., 2012). It is a triple-layered double-stranded RNA virus with an outer capsid made up of two proteins, a glycoprotein (G-type) and a protease-sensitive protein (P-type) which determine the genotype of the virus. The group A rotaviruses are responsible for the majority of severe diarrhoeal illness occurring in

children less than 5 years of age with the common infective strains being genotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (Bányai et al., 2012). Rotavirus infection is seasonal peaking during the winter season in the temperate regions, and all year round with seasonal peaks seen in the dry cool months of the year in tropical countries (Armah et al., 1994; Patel et al., 2013).

Norovirus, a genus in the *Caliciviridae* family is a leading cause of acute gastroenteritis (AGE) in individuals of all age groups globally (Glass et al., 2009). Human noroviruses are mainly associated with outbreaks of diarrhoea in closed settings including hospitals, nursing homes, schools, and cruise ships. Infection is usually associated with severe disease with the greatest impact in young children, the very elderly, and immunocompromised patients (Lopman et al., 2016a). Human noroviruses have been associated with 12-24% of all AGE cases and ~ 70 000–200 000 deaths annually in children less than 5 years of age (Ahmed et al., 2014; Patel et al., 2009).

The noroviruses are non-enveloped viral particles with a 7.7 kb positive-stranded RNA genome which is comprised of three open reading frames (ORF 1-3). Classification of the norovirus is based on the antigenic characteristics of the capsid and RNA-dependent-RNA-polymerase (RdRp) proteins. Human noroviruses are classified into 5 genogroups (GI-GII, GIV, GVIII, and GIX) of which genogroup two (GII) is the most predominant globally (Chhabra et al., 2019; Vinje, 2015). The virus is further characterized into genotypes and variants based on nucleotide sequence similarities and the genogroup two-genotype four (GII.4) has been identified as the most common genotype associated with approximately 70% of severe infections requiring hospitalization (Green, 2018; Vinje, 2015). Similar to the rotaviruses, noroviruses also show a typical winter seasonal peak of infection in the temperature regions whilst in the tropics, seasonal peaks are varied and infection appears to occur all year round (Mans et al., 2016a).

Other enteric viruses that occasionally cause diarrhoea in individuals of all age groups include the sapoviruses, human astroviruses (types 1–8), and human enteric adenoviruses (serotypes HAdV40 & HAdV41) (Alcalá et al., 2018; Glass et al., 2001; Jiang et al., 2013). Together, these viruses have been reported to account for about 10% - 20% of all community diarrhoeal cases (Bányai et al., 2018). Additionally, these viruses in recent times have been reported to be the cause of long-lasting diarrhoeal disease which can sometimes be fatal in immunocompromised individuals (Bosch et al., 2014; Oka et al., 2015).

1.0.4 Clinical Forms of Diarrhoea

Based on duration and stool character, diarrhoeal disease is classified into three clinical syndromes. These include i) acute watery (Acute gastroenteritis) diarrhoea; ii) acute bloody (dysentery) diarrhoea; and iii) persistent (chronic) diarrhoea (Guerrant et al., 2001; Mathan, 1998; Nemeth & Pfleghaar, 2021).

1.0.4.1 Acute Watery Diarrhoea

Acute watery diarrhoea also referred to as acute gastroenteritis is usually associated with significant fluid loss and rapid dehydration and could last for several hours or days. A wide variety of bacterial, parasitic, and viral agents of acute diarrhea have been identified although their relative contributions tend to vary by age and geographic location (O'Ryan et al., 2005; Petri et al., 2008). Commonly isolated pathogens include rotaviruses, noroviruses, sapoviruses, diarrheagenic *Escherichia coli*, non-typhoidal Salmonella, *Giardia lamblia*, and *Cryptosporidium spp*.

1.0.4.2 Acute Bloody Diarrhoea

Acute bloody diarrhoea often referred to as dysentery is usually marked by visible or microscopic blood in the stools as a result of intestinal damage and nutrient losses. Some dehydration (rarely severe), fever, and (or) vomiting may occur (Wardlaw et al., 2010). Acute bloody diarrhoea lasts for less than 14 days and is commonly associated with pathogenic bacteria such as *Shigella, Salmonella spp., Campylobacter, Entamoeba histolytica,* and more recently *Aeromonas spp* (DeWitt, 1992).

1.0.4.3 Persistent Diarrhoea

Persistent diarrhoea is an episode of diarrhoea, with or without visible blood that lasts for at least 14 days marked by malabsorption, nutrient losses, and wasting (Behrens, 1991). Pathogens commonly implicated in persistent diarrhoea include; *Enteroaggregative E. coli* (EAEC), *Cryptosporidium parvum,* and *Giardia lamblia*.

1.0.5 Pediatric Viral Acute Gastroenteritis: A Global Issue

Viral acute gastroenteritis (AGE) is a common illness in children of all age groups and an important cause of mortality in children under the age of five years. The disease is usually characterized by fever, vomiting, diarrhoea, and severe dehydration if not properly managed (Cheng, 2011). AGE is also a common reason for a visit to the emergency department and admission to a hospital (Dekate et al., 2013). For the past four decades, impressive progress has been made in the effort to curb diarrhoeal disease globally (Liu et al., 2015; Snyder & Merson, 1982; Troeger et al., 2018a). Improved nutrition, portable water supply, and sanitation programs (Darvesh et al., 2017; King et al., 2003) as well as the improvement in case management with the use of oral rehydration therapies (Das et al., 2014; Victora et al., 2000) in children seem to have impacted on the bacterial

and other non-viral etiology of diarrhoea but have so far had limited effect on diarrhoea of viral etiology. Vaccination has been proposed as an effective method that could be used to combat pediatric diarrhoea of viral origin (Kotloff, 2017; O'Ryan et al., 2015).

Rotavirus has been identified as the single most important cause of fatal pediatric diarrhoea globally and is responsible for approximately 40% of all mortality due to diarrhoea in children younger than 5 years (Tate et al., 2016). Currently, there are two rotavirus vaccines: Rotarix® (RV 1; GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq™ (RV 5; Merck Vaccines, Whitehouse Station, NJ) which have been pre-qualified by the World Health Organisation and recommended for use in the immunization programs of both developed and developing countries (WHO, 2019). In 2018, two new rotavirus vaccines, ROTAVAC® (Bharat Biotech Ltd.) and ROTSIL (Serum Institute, India) also qualified for use globally (Chandola et al., 2017; WHO, 2019). To date, 96 countries have included rotavirus vaccines into their national immunization programs with 37 of these in sub-Saharan Africa (ROTACouncil, 2019).

Rotarix® is an oral live-attenuated monovalent vaccine derived from the wild-type of the most commonly circulating rotavirus G1PA[8] human strain. Rotarix® is administered to infants 6 weeks of age in 2 or 3 doses with an interval of at least 4 weeks between doses. Vaccine administration must be completed by the age of 24 weeks (Soares-Weiser et al., 2019).

RotaTeq™ is also an oral, live-attenuated pentavalent rotavirus vaccine of five human-bovine reassortant rotaviruses consisting of a bovine (WC3) backbone and human rotavirus surface proteins of G1, G2, G3, G4, and P1A[8] (Ciarlet & Schödel, 2009). The vaccine is administered in three doses to infants aged between 6 to 32 weeks at an interval of 4 to 10 weeks after the first

dose. The third dose of the vaccine is however administered before the child is 32 weeks of age (MerckVaccines, 2019).

ROTAVAC® an oral live-attenuated monovalent vaccine derived from a naturally-occurring reassortant strain G9P[11] was licensed for use in India in 2014. This vaccine is given in three doses at 6, 10, and 14 weeks of age (Chandola et al., 2017).

ROTASIIL, also a heat-stable oral live-attenuated pentavalent vaccine is administered to infants in a three-dose course at 6, 10, and 14 weeks of age. The ROTASIIL vaccine contains bovine-human reassortant rotaviruses against the most common rotavirus genotypes (G1, G2, G3, G4, and G9) (Deen et al., 2018). The vaccine formulation is lyophilized and packaged in single-dose vials alongside a diluent.

The recent introduction of these rotavirus vaccines into the national immunization program of most countries has impacted greatly on the incidence of rotavirus-associated diarrhoea causing a dramatic decrease in the burden of disease by ~70% (Armah et al., 2016; Das et al., 2014). A similar trend was also observed in Ghana where the incidence of rotavirus infections decreased substantially following the introduction of the Rotarix rotavirus vaccine into its EPI program in April 2012 [Fig 1.2; (Enweronu-Laryea et al., 2018)].

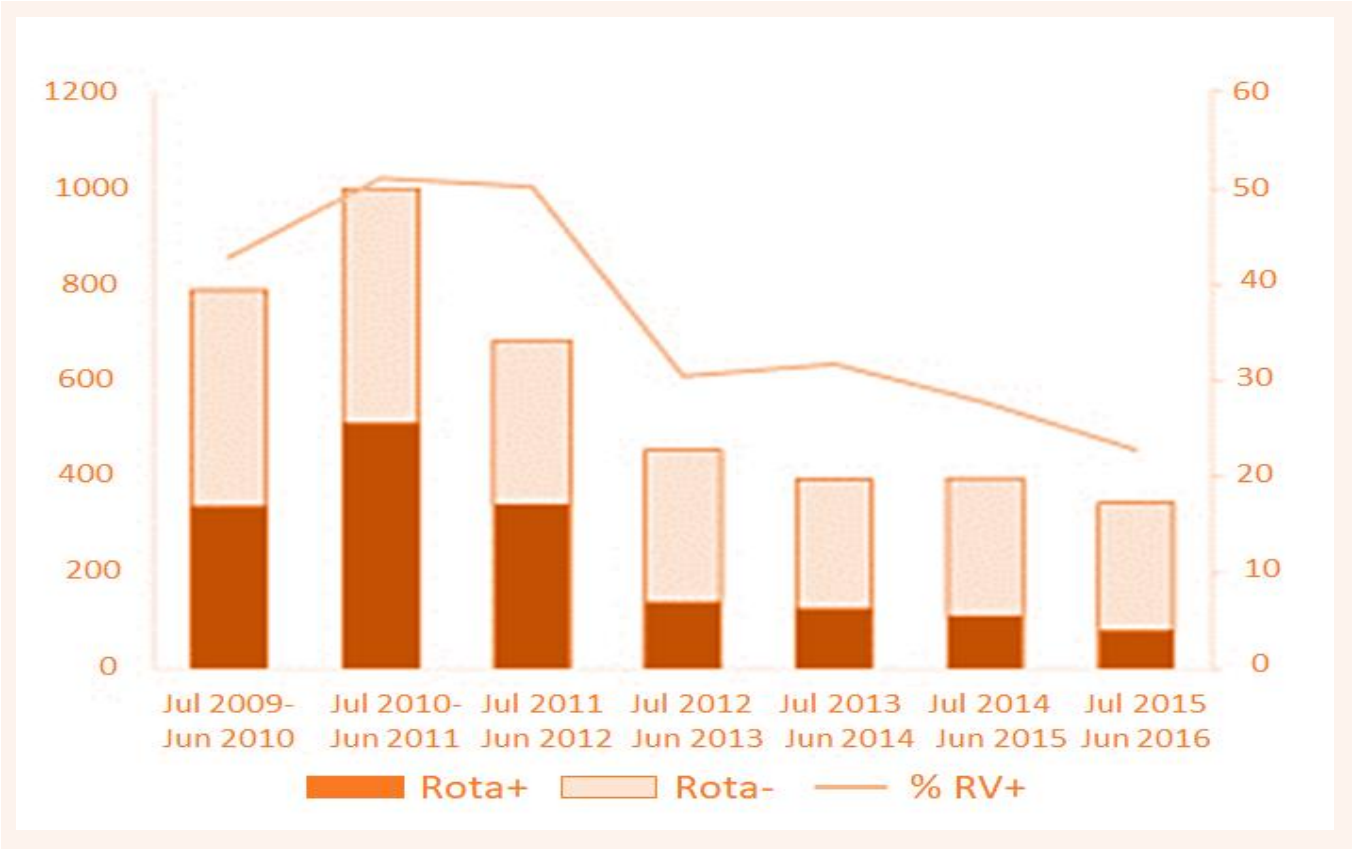


Fig 1.3: Trends in rotavirus detection in Ghana between 2009 and 2016

The graph shows yearly trends of total diarrheic stool specimen collected from children <5 years of age hospitalized with AGE in 2 major referral hospitals in Ghana. The rotavirus vaccine was introduced into the national immunization programme in April, 2012. To capture the entire Ghanaian rotavirus season, year-long periods were defined from July of one year to June of the following year. The pre-rotavirus vaccine era was defined as July 2009 to June 2012 whilst the post-vaccine period was defined as July 2012 to June 2016.

Adapted from (Enweronu-Laryea et al., 2018)

Regardless of this progress, diarrhoeal disease remains the second most common cause of morbidity (1.7 billion cases/year) and the fourth leading cause of mortality (446,000 deaths/year) in children under five years of age worldwide, with a disproportionate number of affected children coming from the low- and middle-income countries (Troeger et al., 2018a). This is probably because of the lack of access to good health care facilities and not fully understanding the etiology of diarrhoeal diseases to help plan intervention and management programs. A large variety of bacteria, parasites, and viruses have been associated with diarrhoea but their relative contribution to the burden of human disease remains unknown (Kotloff, 2017; Krumkamp et al., 2015).

1.0.6 The Role of Noroviruses in Pediatric Acute Gastroenteritis

Since its identification in 1972, the human noroviruses have been cited as a major cause of non-bacterial acute gastroenteritis in persons of all age groups and a second leading cause of childhood diarrhoeal disease (Lopman et al., 2016a) responsible for approximately 200,000 deaths in children yearly (Ahmed et al., 2014). Approximately 70,000 of these 200,000 deaths occur in children from the developing world (Lopman et al., 2016a). Noroviruses are highly contagious and efficiently transmissible with only a few viral particles needed to cause an infection (Karst, 2010). Transmission occurs primarily through the fecal-oral route upon exposure of an individual to contaminated food and water sources or through aerosolized vomitus of infected individuals (Robilotti et al., 2015). The easy transmission and high infectivity of noroviruses make it feasible for the easy spread of the virus including newly emerging strains and variants to cause global epidemics and pandemics (Parra et al., 2017a). In healthy individuals, norovirus gastroenteritis is usually self-limiting. However, the very young, elderly, and immunocompromised individuals may experience more severe symptoms and extended duration of illness (Patel et al., 2009; Widdowson et al., 2005). Such individuals are at a higher risk of being dehydrated resulting in

electrolyte imbalance that may require hospitalization (de Wit et al., 2003; Patel et al., 2009). Host innate factors and acquired immunity have long been considered to play a role in an individual's ability to fight norovirus infection, though this is not completely understood (Parrino et al., 1977). The susceptibility of an individual to norovirus infection is largely dependent on the type of histo blood group antigen (HBGA) an individual expresses (Hutson et al., 2002). Individuals may either possess a functional α1, 2 fucosyltransferase 2 (FUT2) enzyme that regulates the expression of HBGAs including the ABH and Lewis antigens. These HBGAs have been proposed to act as receptors for the virus attachment, or a non-functional FUT2 enzyme which do not support viral attachment (Hutson et al., 2002). Individuals with the functional FUT2 enzyme are referred to as "secretors" and are susceptible to norovirus infection (Hutson et al., 2002). Those with non-functional FUT2 enzymes are "non-secretors" and are usually resistant to norovirus infection because of the lack of expression of HBGAs on the surface of their mucosal cell surfaces (Hutson et al., 2002; Lindesmith et al., 2003; Murakami et al., 2013). Previous human challenge studies have shown non-secretors are largely protected from the globally dominant GII.4 norovirus strain which appears to be a secretor-dependent strain. They are however susceptible to other non-GII.4 strains (Nordgren et al., 2016b). For susceptible individuals, immunity to norovirus infection is short-lived and not cross-protective. Reinfection can occur throughout life with the diverse strains of the virus in circulation. Currently, there are neither antivirals for the treatment of norovirus infection nor vaccines for protection against infection (Kaufman et al., 2014a; Kocher & Yuan, 2015). Norovirus vaccine development has been faced with many difficulties including limited knowledge on the viral replicative cycle, immunology of infection, genetic and antigenic diversity of the virus as well as lack of permissive culture systems for cultivation of the virus (Riddle & Walker, 2016).

During the past decade, rotavirus vaccines have been deployed broadly and worldwide. We have witnessed a dramatic reduction in >50% of most cases in hospitalizations due to rotavirus disease burden. It is conceivable to consider norovirus as likely to replace rotavirus as the leading cause of acute gastroenteritis in children. This hypothesis has been difficult to prove with a well-characterized molecular epidemiologic and robust burden of norovirus-associated disease data. There remains huge uncertainty as well as dissension in defining the exact role of noroviruses in severe pediatric diarrhoea (Lopman & Grassly, 2016).

The other challenge is the difficulty in distinguishing between symptomatic and asymptomatic infections because of the prolonged virus shedding even after resolution of the symptom as was observed in the GEMS and MAL-ED studies (Kotloff et al., 2013; Platts-Mills et al., 2014). Since norovirus disease presents with several non-specific syndromes similar to other enteric viral agents of AGE laboratory confirmation of norovirus infection in AGE cases is of paramount importance. Lack of resources and capability in most developing countries, where the infection is highest, has had a big impact on the introduction of routine testing for norovirus infection in their health systems. The current detection methods rely on molecular techniques and capacity that are presently available in research reference laboratories with existing surveillance platforms usually activated only during outbreaks (Vega et al., 2011; Yen & Hall, 2013). As such, there is under-reporting of laboratory-confirmed cases of norovirus infection, further complicating the estimation of the norovirus-associated gastroenteritis burden of disease. Collecting evidence-based molecular epidemiological data and establishing accurate estimates for norovirus-associated disease burden will be crucial for the launching and utilization of any public health interventions such as vaccines that may be available in the future.

1.1 Scope of Ph.D. Study

In Ghana, diarrhoeal disease continues to be a common cause of mortality, morbidity, and hospitalization in children under five years (Anyorikeya et al., 2016). Rotaviruses have been recognized as one of the major causes of hospitalization due to AGE for the last 25 years (Armah et al., 1994; Asmah et al., 2001; Binka et al., 2003; Enweronu-Laryea et al., 2012; Reither et al., 2007). The relative contribution of noroviruses, one of the viral etiological agents of acute gastroenteritis, in children <5years of age as well as their complex molecular epidemiology strain/genotype distribution however remains unknown except for a few sentinel surveillance sites. The development of a norovirus vaccine as a public health tool to help reduce the burden of norovirus-associated diarrhoeal disease in children is far advanced. The generation of baseline data would be crucial in evaluating these vaccines when they become available. These evidence-based data will be crucial in guiding policy decisions on the introduction of norovirus vaccines in Ghana. Uniquely, the Department of Electron Microscopy and Histopathology has been conducting rotavirus diarrhoea surveillance for the past 4 decades with various established sentinel sites across the country and has a well-characterized repository of stool samples collected from children with severe watery diarrhoea. This provides a unique opportunity to conduct retrospective studies to better understand the burden of disease and molecular epidemiology of pediatric norovirus infection in Ghanaian children using samples collected during active surveillance for diarrhoea in children over the past 10-years. It is hypothesized that (1) the prevalence of norovirus-associated diarrhoeal disease in children will be high, (2) the prevalence of hospitalization of children less than five years due to norovirus will increase as the burden of rotavirus associated diarrhoea disease decreases as a consequence of the introduction of rotavirus vaccines in the immunization program in Ghana in April 2012.

1.1.1 Overarching Study Aim

Generally, the study aimed to employ improved molecular techniques to establish baseline data on the prevalence of norovirus-associated diarrhoeal disease in Ghana and to describe completely the molecular epidemiology of noroviruses-associated diarrhoea in children less than five years of age. To achieve this, several studies were carried out to address the specific objectives as described below.

1.1.2 Study Specific Objectives

Objective 1: To determine the prevalence of norovirus-associated diarrhoeal disease in children under five years of age, the seasonality and severity of norovirus-associated diarrhoea

Objective 2: To determine the molecular epidemiology, transmission dynamics, and evolution of noroviruses in Ghana.

Objective 3: To evaluate the secretor status profile in norovirus-infected children in Ghana and investigate potential associations if any between norovirus genotypes and secretor status.

1.1.3 Organisation of Thesis

This thesis consists of six (6) chapters. **Chapters 1 and 2** provide a general overview, background, and theoretical framework to the study. The actual research work is presented in the next three chapters (**Chapters 3-5**) with each chapter prepared as a separate manuscript with an abstract, aim, introduction, methods, results and discussion. **Chapter 3** examined the prevalence, clinical severity, and seasonality of norovirus-associated diarrhoeal disease in Ghanaian children under 5 years of age. **Chapter 4** investigated the molecular epidemiology, transmission dynamics, and evolution of noroviruses in Ghana. **Chapter 5** sought to evaluate the secretor status profile in norovirus-infected children in Ghana and investigate potential associations if any between

norovirus genotypes and secretor status. The thesis concludes with a general discussion (**Chapter 6**) on all the findings from the three examined objectives, their relevance, and draws conclusions outlining possible future directions for norovirus research in Ghana.

CHAPTER TWO

Literature Review

2.0 Historical Background

2.1. The Emerging Importance of Noroviruses

The first instance of norovirus infection was recorded in the late 1920s by a pediatrician named Dr. J. Zahorsky. Subsequently, he wrote an account of sporadic cases of the sudden onset of self-limited vomiting and watery diarrhoea among his patients that peaked during the winter months of November to March. He was, however, unable to cultivate nor identify the causative agent and hence coined the term “hyperemesis hemis” or “winter vomiting illness” to describe the disease (Zahorsk, 1929). Thereafter, a significant number of this non-bacterial enteric illness was observed to predominantly occur between September and April (Adler & Zickl, 1969; Badger et al., 1956; Gordon et al., 1947; Hodges et al., 1956). In 1972, Kapikian and colleagues became the first scientists to visualize a causative agent for a non-bacterial gastroenteritis outbreak. Using the immune-electron microscopy technique they were able to identify the norovirus particles in stools of volunteers challenged with stool filtrates from an outbreak of gastroenteritis that occurred in an elementary school in the town of Norwalk, Ohio in 1968 (Kapikian et al., 1972). The norovirus was therefore originally named the Norwalk virus agent. This was also the first time a virus causing gastroenteritis had been isolated and it was an ever-changing discovery because the existing dogma had been that gastroenteritis was only caused by bacterial pathogens. Later, other enteric viruses including rotaviruses (Bishop et al., 1973), astroviruses (Appleton & Higgins, 1975), sapoviruses (Madeley & Cosgrove, 1976), and enteric adenoviruses (Whitelaw et al., 1977) were discovered. Following the discovery of noroviruses, Greenberg and colleagues developed a methodology using sera from convalescent hosts to elucidate its epidemiology (Greenberg & Kapikian, 1978). Later

seroprevalence studies showed, norovirus infection to be prevalent in individuals of all age groups and across different geographical locations (Blacklow et al., 1979; Greenberg et al., 1979). To further help with the easy identification and differentiation of outbreaks of norovirus-associated gastroenteritis from other pathogens, Kaplan and colleagues in 1982 developed a set of epidemiological criteria that distinguished norovirus outbreaks from outbreaks of bacterial origin (Kaplan et al., 1982). These criteria included: i) absence of a bacterial pathogen in stool cultures of infected individuals; ii) projectile vomiting in more than half of the affected persons; iii) a mean incubation period of 24-48 hours; iv) duration of illness lasting 12-60 hrs. Erstwhile, the noroviruses were only linked to and recognized as the principal cause of water and foodborne non-bacterial gastroenteritis outbreaks accounting for approximately 90% of all outbreak cases at a significant healthcare cost (Hedberg & Osterholm, 1993; Olsen et al., 2000). Complete sequencing and characterization of the Norwalk virus genome (Xi et al., 1990) two decades later was a milestone achievement as it allowed for further examination of the viral taxonomy which led to the development of various contemporary diagnostic tools for the identification of noroviruses (Costantini et al., 2010; Jiang et al., 1992; Moe et al., 1994). Further radical improvement in molecular diagnostic methods, helped to show the clinical importance of the norovirus with a dramatic increase in the number of reported cases (Belliot et al., 2014; Platts-Mills et al., 2012). Currently, the noroviruses are said to be responsible for nearly 12% of all sporadic cases of gastroenteritis globally (Ahmed et al., 2014; Patel et al., 2008). Each year, the burden of norovirus-associated disease is estimated to result in approximately $4.2 billion direct health system costs and more than $60 billion in societal costs (Bartsch et al., 2016). Intensive research over the past few years has led to the recognition of the noroviruses as the second most common etiologic agent of severe childhood diarrhoea accounting for 10–20% of associated hospitalizations and emergency room visits in the middle/high income countries (Kowalzik et al., 2015b; O'Ryan et al.,

2017) and 1–15% hospitalizations in low income countries (Kotloff et al., 2013; Liu et al., 2016; Moyo et al., 2011).

2.2. Norovirus Classification

2.2.1 Norovirus: a genus in the *Caliciviridae* family

Caliciviridae is a family of viruses containing both human and non-human pathogenic strains as members and belongs to class IV of the Baltimore classification scheme. Its five distinct genera (Fig 2.1) approved by the International Committee on Taxonomy of Viruses (ICTV) include *Lagovirus*, *Nebovirus*, *Norovirus*, *Sapovirus*, and *Vesivirus* (Green, 2013). *Recovirus*, *Valovirus*, *Bavovirus*, *Nacovirus*, *Minovirus*, and *Salovirus* comprise the six other proposed genera yet to be approved by the ICTV (ICTV, 2019). Figure 2.1 shows the phylogenetic relationship between the genera of the *Caliciviridae* family based on full-length amino acid VP1 sequences of representative strains from each genus within the family.

Diseases associated with members within the *Caliciviridae* family include gastroenteritis, respiratory and fatal hemorrhagic disease (Desselberger, 2019). The genera *Norovirus* and *Sapovirus* constitute the major human pathogens within the family whilst the *Vesiviruses* including the feline calicivirus (FCV), *Neboviruses* with the Newbury-1 virus, and *Lagoviruses* containing the rabbit hemorrhagic disease virus (RHDV) are considered important veterinary pathogens (ICTV, 2012). FCV and RHDV cause respiratory disease in cats and deadly hemorrhagic disease in rabbits respectively (Green, 2013). Newbury-1 is an enteric virus known to infect bovines (Oliver et al., 2006). Table 2.1 provides a comprehensive list of genera (both established and proposed) within the *Caliciviridae* family, type species, and the hosts they have been found to infect.

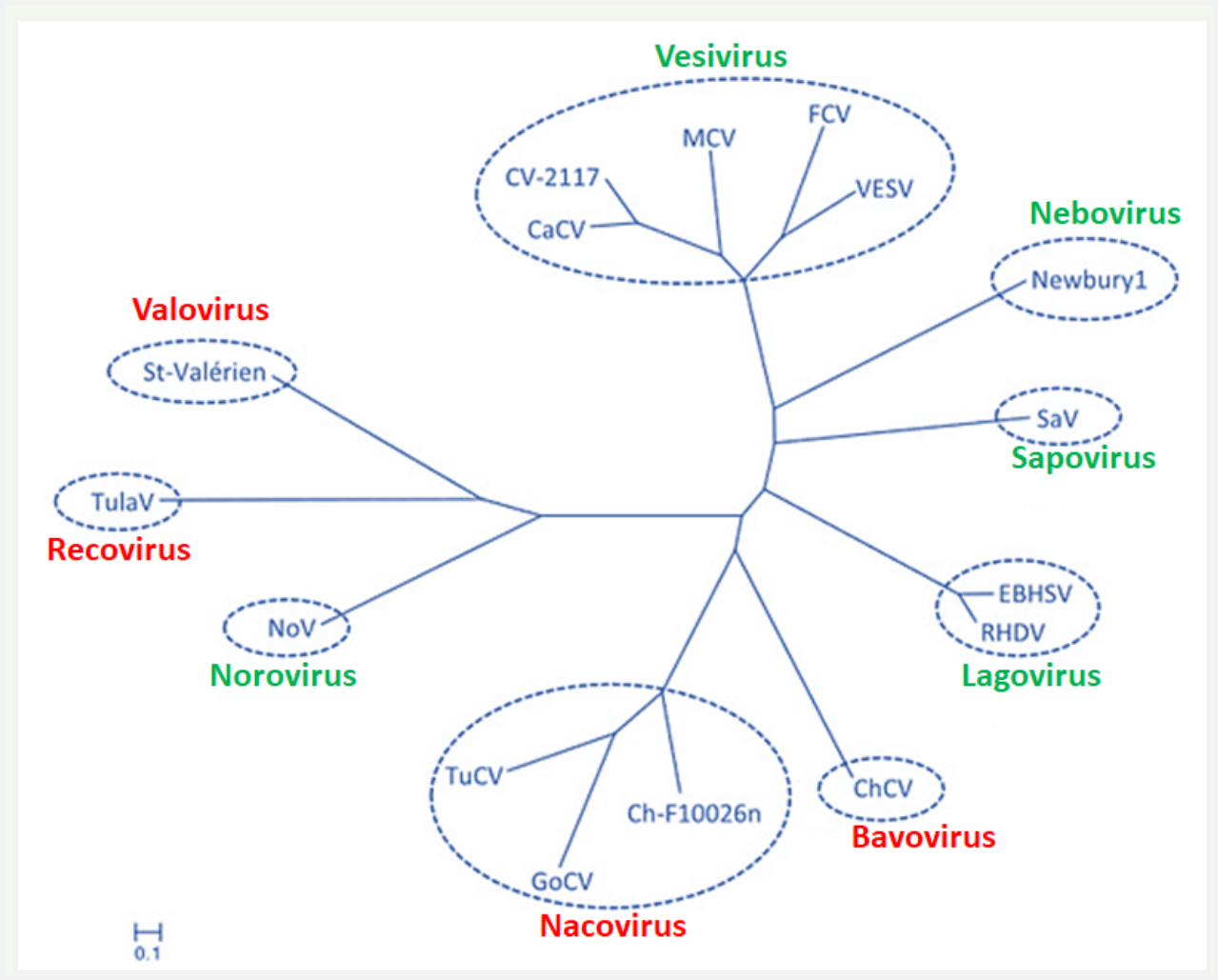


Fig 2.1: Phylogenetic relationship of members within the family *Caliciviridae*

Full-length capsid (VP1) amino acid sequences of representative strains from each genus in the family were used for the analysis. Established genus are shown in green bold fonts whilst proposed genera are shown in red bold fonts. The calibration bar shows genetic distance.

Abbreviations in clockwise direction: CaCv - Canine calicivirus; CV-2117 - Calicivirus 2117; MCV - Mink calicivirus; FCV - Feline calicivirus; VESV - Vesicular exanthema of swine virus; SaV – Sapporo virus; EBHSV - European brown hare syndrome virus; RHDV - rabbit haemorrhagic disease virus; ChCV - Chicken calicivirus Bavaria; Ch-F10026n - Chicken calicivirus F10026n; GoCV - Goose calicivirus; TuCV - Turkey calicivirus; NoV – Norwalk virus; TulaV - Tulane virus.

Image adapted from (Desselberger, 2019)

Table 2.1: Classification of the Caliciviridae family including genera and typed species

Genus	Typed Species	Host	Disease	References
Norovirus	Norwalk-like virus (NV)	**Human**, porcine, murine, bovine, ovine, feline, canine	Gastroenteritis	(Kapikian et al., 1972)
Sapovirus	Sapporo virus (SV)	**Human**, Swine, Mink	Gastroenteritis	(Chiba et al., 1979)
Nebovirus	Newbury-1 virus (NBV)	Cattle	Gastroenteritis	(Woode & Bridger, 1978) (Oliver et al., 2006)
Lagovirus	Rabbit hemorrhagic disease virus (RHDV) European brown hare syndrome virus (EBHSV)	Leporidae (Rabbit, Hare)	Hemorrhagic disease	(Liu et al., 1984) (Meyers et al., 1991)
Vesivirus	Vesicular exanthema of swine virus (VESV)	Swine	Vesicular syndrome	(Chen et al., 2006)
	San Miguel sea lion virus (SMSV)	Seals, Sea Lions	Vesicular syndrome	(Smith et al., 1973)
	Feline calicivirus (FCV)	Feline	Upper Respiratory Tract Infection	(Chen et al., 2006)
Recovirus	Tulane virus	Simian (Rhesus macaque)	Gastroenteritis	(Farkas et al., 2008)
Valovirus	St Valérian virus	Porcine	Gastroenteritis	(L'Homme et al., 2009)
Bavovirus	Bayern (Bavaria) virus	Avian (Chicken)	Yet to be determined	(Wolf et al., 2011)
Nacovirus	Nacovirus A	Avian (Chicken, turkey, goose)	Yet to be determined	(Wolf et al., 2012) (Liao et al., 2014)
Salovirus	Nordland virus	Fish (Atlantic salmon)	Systemic Infections	(Mikalsen et al., 2014)
Minovirus	Minovirus A	Fish (Fathead minnow)	Yet to be determined	(Mor et al., 2017)

Classification shows both established (green bold faced fonts) and proposed (red bold faced fonts) genera.
Table adapted and modified from (Desselberger, 2019) and (ICTV, 2019)

2.3. Norovirus Morphology and Genome Organisation

2.3.1. Morphology and structure of the noroviruses

Members of the calicivirus family display an atypical 32 distinct cup-shaped depressions on their surfaces (Fig 2.2a) and it is from this feature that the *Caliciviridae* family derived its characteristic name, where "calici" means "cup" and is derived from the Latin word "calyx" (Clarke & Lambden, 1997). The noroviruses are non-enveloped with icosahedral symmetry. Electron micrographs show the noroviruses display small amorphous structures (27 to 40 nm in diameter) with distinct surface morphologies and feathery ragged outer edges (Fig 2.2b).

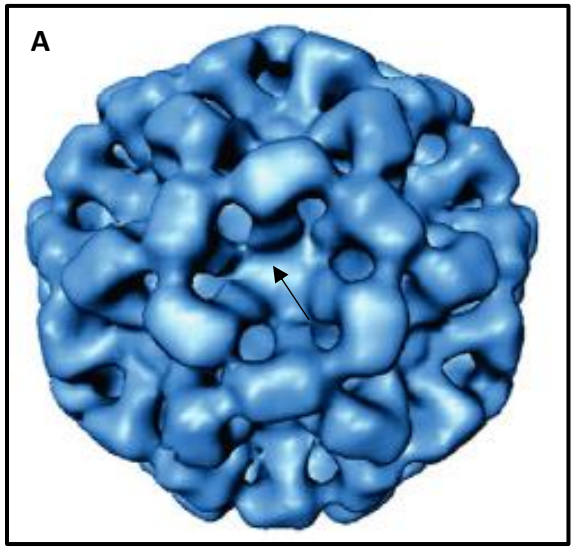


Fig 2.2a: Surface representations of the three-dimensional structure of the norovirus capsid

The surface representations are exemplified by cryo-image showing the atypical cup-shaped depressions (Shown by black arrow) viewed along the icosahedral threefold axis. Adapted from (Prasad et al., 1994).

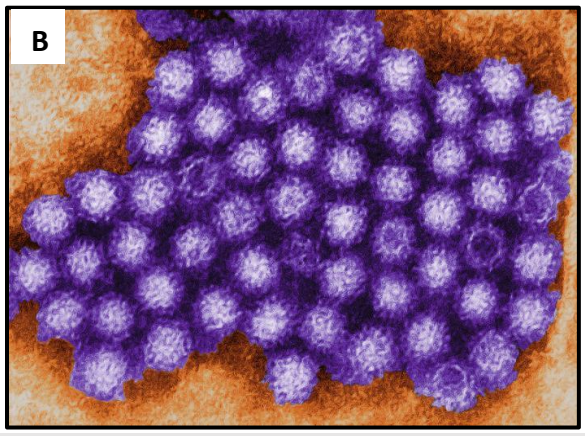


Fig 2.2b: Norwalk Virus

Digitalized Transmission electron micrograph of a cluster of human norovirus virions. Source: CDC/Charles D. Humphrey. CDC Public Health Image Library ID 10708

2.3.2. Norovirus Genome Organisation

The norovirus genome consists of a linear, positive-sense, single-stranded (ss)RNA molecule of 7.3 to 8.5 kilobase pairs (Kb) in length (Green, 2013). While the 5' end of the messenger (m)RNAs of eukaryotes display a 7^{Me}-GpppG cap structure, that of the noroviral genome is covalently linked to a virion-genome-linked protein (VPg) which serves as a 5' cap structure (Fig 2.3A). Similar to the eukaryotic mRNA, the 3' end of the norovirus genome is also polyadenylated (Thorne & Goodfellow, 2014a). The noroviral genome is also organized into three overlapping open reading frames (ORFs): ORF1- which encodes six non-structural proteins (NS1-NS7) and ORF2 – ORF3 which encodes for two structural proteins VP1 and VP2 of the virus respectively (Donaldson et al., 2010). A subgenomic (sg)RNA (consisting of ORF-1 and -2) of approximately 2.2 – 2.4 kb is also intracellularly synthesized and VPg-linked (Fig 2.3B). The sgRNA is produced alongside the genomic (g)RNA during viral replication and serves as a template for the production of structural proteins similar to the gRNA. Like the gRNA, the sgRNA is also capped at the 5' end with the VPg-linked protein and polyadenylated at the 3' end.

2.3.3. Norovirus Non-Structural Proteins

2.3.3.1 Open reading frame 1

Open reading frame one (ORF-1) is approximately 5.1 kb and located at the 5' proximal region of the viral genome. It encodes a single polypeptide protein that is sequentially cleaved into six or seven non-structural proteins (Fig 2.3A) when matured. These non-structural proteins include N-terminal protein (p48 (NS1-2), Nucleoside triphosphatase (NTPase (NS3), p22 (NS4), Virion protein-genome-linked protein (VPg (NS5), Viral Protease (Pro (NS6) and an RNA-dependent RNA polymerase (RdRp (NS7) (Fukushi et al., 2004; Liu et al., 1996; Pfister & Wimmer, 2001;

Rohayem et al., 2006a). The non-structural proteins are essential for the production of new viruses within an infected host cell. Non-structural proteins NS3, NS5, NS6, and NS7 have defined roles they perform during viral replication and packaging (Hardy, 2005; Thorne & Goodfellow, 2014a). The functions of NS1-2 and NS4 to date remain speculated (Green, 2013).

2.3.3.1.1 N- Terminal Protein (p48 / NS 1-2)

The N – terminal protein translates into the non-structural proteins one and two (NS1-2) of the norovirus and consists of approximately 398 amino acid sequences of the viral genome (Hardy, 2005). The protein tends to vary in length and sequence among the different genogroups of the virus, however, amino acid sequence conservation increases toward the C terminus of the protein (Hardy, 2005). It is speculated that the N-terminal protein may play a role in norovirus replication complex formation (Hyde & Mackenzie, 2010) and can co-localize with Golgi complexes formed in transfected cells to induce rearrangement of the Golgi membrane (Ettayebi & Hardy, 2003; Fernandez-Vega et al., 2004). It has also been reported to be involved in disrupting intracellular host protein trafficking by interacting with a host protein involved in regulating vesicle transport and can inhibit cell surface expression of these host proteins (Ettayebi & Hardy, 2003; Karst, 2010).

2.3.3.1.2 Nucleoside triphosphatase (NTPase (NS3)

NS3 is putatively classified into the superfamily 3 of RNA helicases but has no helicase activity hence cannot unwind DNA during replication (Hardy, 2005). It has however been shown to have NTPase activity and can bind and hydrolyze NTPs during virus replication (Clarke & Lambden, 2000; Donaldson et al., 2008; Green, 2013; Pfister & Wimmer, 2001).

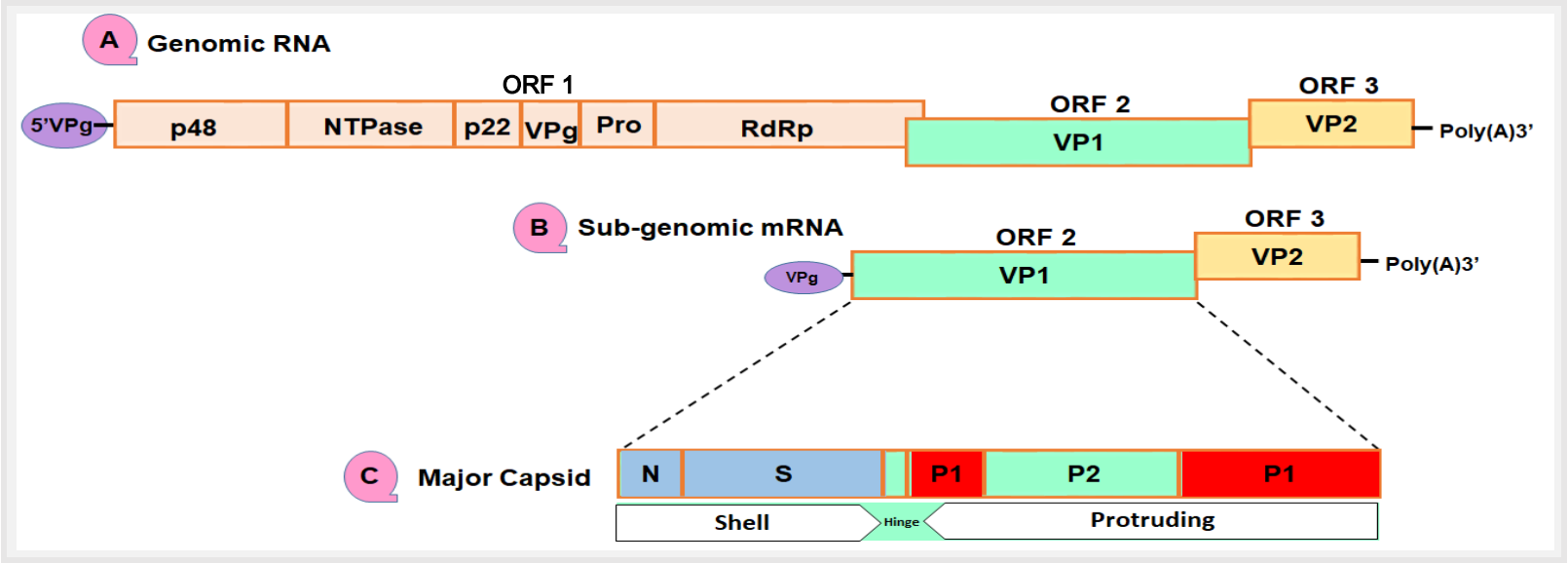


Fig. 2.3: Norovirus genome structure and organisation

A| The human norovirus genome consists of three open readings frames (ORF 1-3).
ORF-1 encodes a large polyprotein (coloured pink) that undergoes post-translational cleavage to release seven (7) non-structural precursor proteins (also shown in pink colour). ORF 2 encodes a major structural capsid protein, VP1 (shown in green colour) while ORF 3 encodes a minor structural capsid protein, VP2 (coloured Orange). A virion-genome-linked protein (VPg), depicted as a purple oval is linked to both genomic (A) and sub-genomic (B) RNAs.

B| ORF 2 and ORF 3 forms a sub-genomic mRNA. The sub-genomic mRNA is also capped at the 5' end with the VPg-linked protein (purple oval) and polyadenylated at the 3' end.

C| The major capsid protein is further divided into a conserved shell (S) and a more variable protruding (P) domains (shown in blue and red respectively) connected by a Hinge region. The P domain subsequently divides into two different sub-domains, P1 and P2 (Shown in red and green respectively).

Adapted and modified from (Prasad et al., 1999).

2.3.3.1.3 3A-Like Protein (p22/p20 (NS4)

The protein p22 is found in norovirus genogroup I while p20 is found in genogroup II. The main function of this protein remains unknown. Studies by Sharp et al. suggests NS4 tends to disrupt endoplasmic reticulum (ER) to Golgi trafficking resulting in Golgi disassembly during norovirus replication (Sharp et al., 2010b). The NS4 protein is also believed to be involved in virus replication complex formation on host intracellular membranes (Hyde & Mackenzie, 2010).

2.3.3.1.4 Virion protein-genome-linked protein (VPg (NS5)

NS5**,** a 15 kDa VPg protein is covalently linked to the 5' end of the viral RNA genome and severs as a 5'-cap protecting the genome (Burroughs & Brown, 1978; Herbert et al., 1997). During viral RNA replication, it functions as a primer for RNA synthesis (Belliot et al., 2008; Rohayem et al., 2006b). It is also believed to be involved in the initiation of translation by recruiting host cell transcription factors (Chaudhry et al., 2006; Daughenbaugh et al., 2003; Karst, 2010).

2.3.3.1.5 Viral Protease (Pro (NS6)

NS6 is thought to belong to a group of trypsin-like proteases (Elbashir et al., 2002) and is responsible for the processing of the ORF1 polyprotein into the non-structural proteins (Hardy et al., 2002; Liu et al., 1996). It is also speculated that this proteinase may also play a role in the host cellular translation by cleavage of poly (A)-binding protein (PABP) (Kuyumcu-Martinez et al., 2004).

2.3.3.1.5 RNA-dependent RNA polymerase (RdRp (NS7)

NS7 an RNA-dependent-RNA polymerase (RdRp) protein is encoded by the 3D region of the viral genome and has a molecular weight of approximately 57kDa. Like all caliciviral RdRp, the NS7 protein possesses a conserved amino acid motif, glycine-aspartic acid-aspartic acid (YGDD) that

is involved in the catalytic activity of the enzyme (Koonin, 1991; Vazquez et al., 2000). This conserved region is thus usually targeted for primers meant for the detection of noroviruses (Jing et al., 2000). The RdRp protein also possesses a "right hand" structure of a rigid finger-palm domain and a flexible thumb domain which can assume an 'opened or closed' conformation (Green et al., 2013). By its conformation, the RdRp can form a primer-template RNA duplex considered essential for the initiation of RNA synthesis and viral replication (Green, 2013; Thorne & Goodfellow, 2014b).

2.3.4 Norovirus Structural Proteins

2.3.4.1 Open reading frames 2 and 3 Encodes the Viral Structural Proteins

Towards the 3' end is a sub-genomic like RNA (Fig 2.3B) with open reading frames 2 and 3 which encodes the major structural protein - Viral protein 1 (VP1) or capsid protein) and the minor structural protein - Viral protein 2 (VP2) of the virus respectively (Hardy, 2005).

2.3.4.1.1 Structural Viral Protein One (VP1)

VP1 is an approximately 60kDa protein made up of about 530-555 amino acids arranged as 90 dimers of 180 copies (Chen et al., 2004). The capsid protein (VP1) of norovirus is well organized into a conserved N-terminal internal shell (S) domain, an intermediate shell (IS) domain and a variable surface-exposed protruding (P) domain (Fig 2.4) linked to the S-domain by a flexible hinge (Green, 2013; Prasad et al., 1999). The S-domain is involved in the formation of the interior icosahedral shell of the capsid surrounding the viral RNA while the IS-domain is responsible for controlling the size of the capsid. The P-domain forming protrusions that project out of the S domain, interacts in dimeric contacts that increase the stability of the capsid (Bertolotti-Ciarlet et al., 2002; Hardy, 2005). In addition, the P-domain of the viral capsid is thought to contain the key

determinants of antigenicity and genotype specificity of the virus (Vinjé et al., 2004). This domain further subdivides into P1 and P2 subdomains (Fig 2.3C) of which the P1 subdomain forms the sides of the arches whilst the P2 subdomain is located exposed on the tops of the arches (Karst, 2010). Being situated at the outermost surface of the viral capsid, the P2 subdomain is the most exposed antigenic site displaying a hypervariable region involved in immune recognition and receptor binding (Bu et al., 2008; Cao et al., 2007; Tan et al., 2003). The slow accumulation of mutations within this exposed hypervariable P2 subdomain has been shown to lead to the evolution of novel norovirus strains with the potential to escape herd immunity because of altered antigenicity and receptor binding properties (Debbink et al., 2012; Karst, 2010; Nilsson et al., 2003).

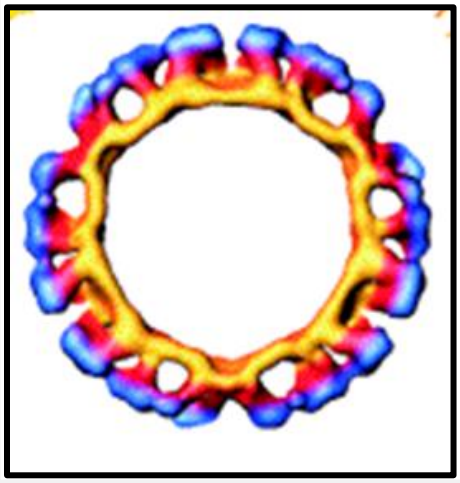


Fig 2.4: A schematic cross section representation of the structural organisation of the Norovirus particle

- The Shell (S) domain (yellow) forms the continuous surface of the virus whilst the Protruding (P) domains (red and blue) constitute the arch-like surface of the virus.
- The P-domain further subdivides into the P1 (red) and P2 (blue) domain with the later domain implicated in virus-host interactions

Source (Chen et al., 2004)

2.3.4.1.1 Structural Viral Protein Two (VP2)

VP2 is a small basic protein with a predicted molecular weight of approximately 29kDa. It forms the minor structural protein of the virus (Hardy, 2005). Though the main function of this protein is not yet known, extensive variability in its 268 amino acid sequences has been observed to exist between the different norovirus strains (Hogbom et al., 2009; Seah et al., 1999). Since the existence of ORF3 is conserved among the caliciviruses, it is presumed that this protein may play a role in viral replication or assembly (Fout et al., 2003; Green, 2013). Also, the basic nature, chemistry, and high isoelectric point of the viral protein led Fretz et al., to conclude that, the protein may function as an RNA binding protein that might be involved in RNA genome packing (Fretz et al., 2003). VP2 has also been reported in regulatory functions for the expression and stability of VP1 (Benedict et al., 2002; Doedens et al., 1997).

2.4. Norovirus Replication

2.4.1 Viral Attachment and Entry

Replication of the noroviruses is yet to be fully expounded. Current knowledge of the virus replicative cycle is drawn by likeness with other single-stranded positive-sense RNA viruses and studies from other culturable animal caliciviruses including the murine and feline caliciviruses (Karst, 2010; Thorne & Goodfellow, 2014a). The noroviruses exhibit a simple life cycle (Fig 2.5). Similar to other positive single-stranded RNA viruses, replication of the noroviruses occur within the host cell cytoplasm (de Graaf et al., 2016a). Its genome functions directly as the mRNA beginning the infectious cycle with the synthesis of a precursor polyprotein producing non-structural (NS) proteins and a sub-genomic mRNA which encodes for the structural proteins (de Graaf et al., 2016a). Recent studies using mouse models have shown the virus can infect and

replicate in its host immune cells including macrophages, dendritic, and B-cells in addition to intestinal epithelial cells (Jones et al., 2014a; Karst & Tibbetts, 2016; Wobus et al., 2004). The P2 domain of the viral capsid binds to host histo blood group antigens (HBGAs) found on the mucosal surfaces of the small intestines and are thought to serve either as receptors or co-receptors (Hutson et al., 2002; Marionneau et al., 2002; Rydell et al., 2009). The Noroviruses have also been shown to bind sialic acid (Stuart & Brown, 2007; Taube et al., 2009) or heparan sulfate (Tamura et al., 2004) moieties. The mechanism by which the virus enters the host cell after receptor-binding remains unknown but is thought to be dependent on dynamin II or cholesterol as shown in the murine noroviruses (Gerondopoulos et al., 2010; Perry & Wobus, 2010). Work done by Perry and colleagues suggests uncoating of the infecting norovirus is pH-independent and occurs with no conformational change in the capsid protein (Perry et al., 2009).

2.4.2 Viral Replication, Assembly and Release

After uncoating and disassembling, the virion protein VPg which covalently binds the 5' end of the viral RNA genome recruits host translation factors to begin the translational processing of a large polyprotein encoded for by the viral ORF1 (Daughenbaugh et al., 2003; Daughenbaugh et al., 2006). Virus encoded protease (Pro) is then auto-catalytically released from the encoded polyprotein precursor (Sosnovtsev et al., 2006). As part of post-translational processing, the released protease then cleaves the remaining polyprotein for the release of the rest of the NS proteins (Fig 2.5) and their precursors to facilitate further rounds of replication (Sosnovtsev et al., 2006). Through modification of the host secretory pathway, NS1-2 and NS4 are thought to induce replication of the viral genome by the formation of a replication complex consisting of intracellular membranous structures containing all NS proteins, needed host proteins, viral intermediate RNA,

and ssRNA (Denison, 2008; Hyde & Mackenzie, 2010; Sharp et al., 2010a). After assembly of the replication complex, the VPg protein serving as a primer gets uridylated to initiate the synthesis of a negative sense (antigenomic) viral RNA strand from the positive sense viral genomic RNA template by the RdRp (Rohayem et al., 2006a; Rohayem et al., 2006b). This antigenomic RNA is then used as a template for the synthesis of new genomic and subgenomic RNA of the virus (de Graaf et al., 2016a). The newly synthesized genomic RNA is either translated as a polyprotein precursor or used for packaging in the assembled viral protein core whilst the subgenomic RNA containing only ORF 2 and ORF 3 is used for the production of structural proteins, VP1 and VP2 (Fig 2.5). After the structural proteins are assembled and the genomic RNA packaged, the mature virion is finally released from the infected host cell. Though the mechanism is not completely understood, it is presumed that the infected host cell undergoes lysis and it is during this time that the viruses are released (Green, 2013).

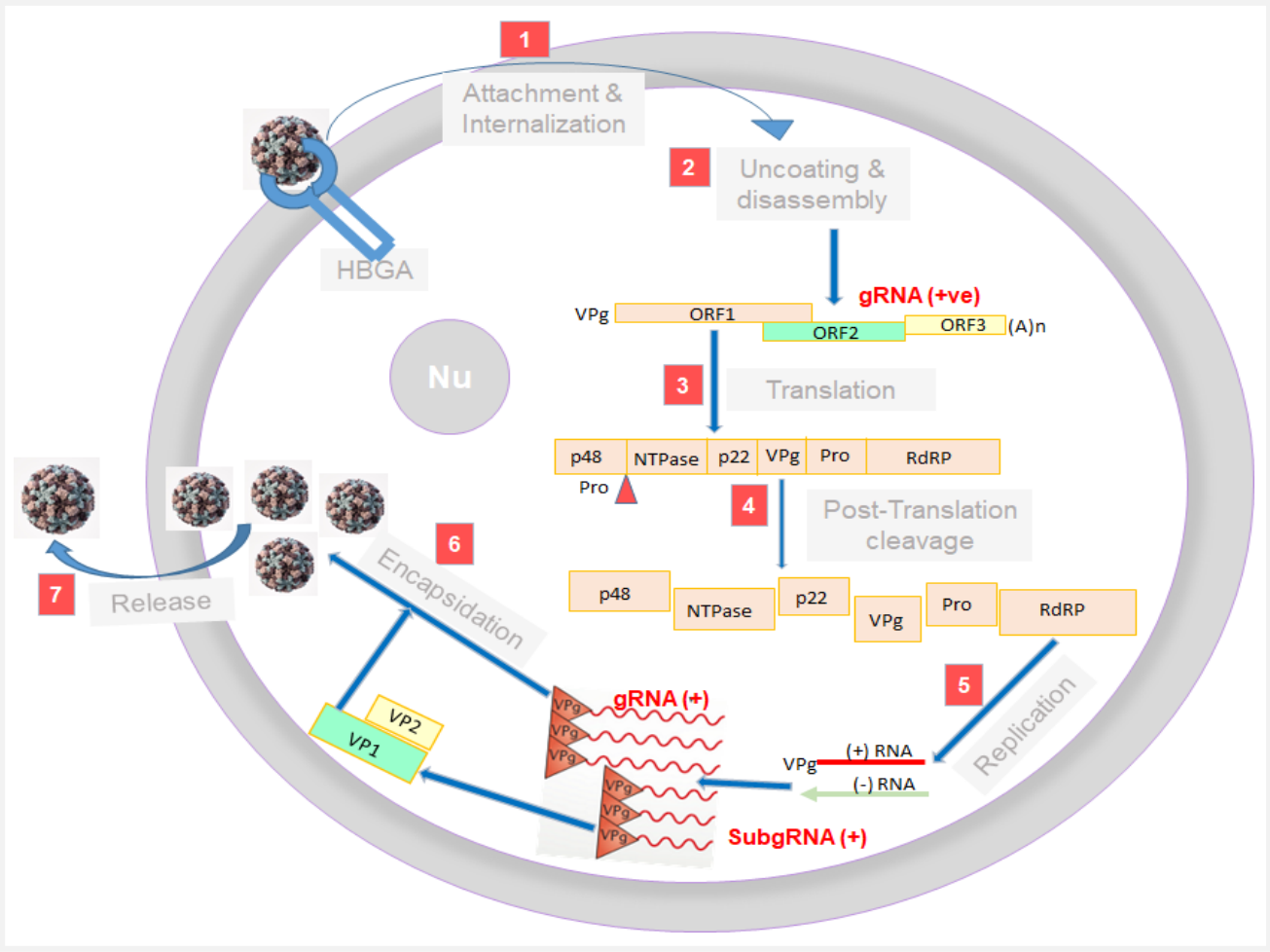


Fig. 2.5: The life cycle of human noroviruses

The replication of the norovirus has been schematically subdivided into seven steps (**Step 1 - Step 7**).
Nu: Host cell Nucleus; **gRNA**: Viral Genomic RNA **subgRNA**: Viral sub-genomic mRNA.
Image adapted and modified from (de Graaf et al., 2016a)

2.5 Host Susceptibility and Immune Response to Norovirus Infection

2.5.1 Host Susceptibility

After the identification of the Norwalk virus as the cause of the numerous winter gastroenteritis outbreaks, several volunteered challenge studies were performed in the 1970s to better understand the immunobiology of the virus (Agus et al., 1973; Dolin et al., 1971; Parrino et al., 1977; Schreiber et al., 1974; Wyatt et al., 1974). In one of these challenge studies, Wyatt and co were able to show the absence of heterotypic immunity to infection with different norovirus strains when they compared the infection patterns of 3 agents known to have caused non-bacterial gastroenteritis outbreaks in cross-challenged studies in healthy volunteers (Wyatt et al., 1974). These 3 agents were later identified as serotypically distinct Norwalk (GI), Snow mountain (GII), and Hawaii (GII) prototype strains of noroviruses (Lew et al., 1994). Later studies performed by Parrino and colleagues who challenged 12 healthy volunteers with the first isolated Norwalk viral strain highlighted the heterogeneous response of individuals to norovirus infection (Parrino et al., 1977). Interestingly 50% (6/12) of these volunteers remained asymptomatic whilst the rest developed symptoms of infection. The same outcome was observed when these volunteers were challenged again 27 and 42 months later. In a subsequent re-challenge study, this time, on just the symptomatic volunteers performed 4-8 weeks later, only one volunteer developed symptoms while the rest showed high titers of serum antibodies after each challenge (Parrino et al., 1977). The conclusion drawn from these challenge studies was that Norwalk virus infections conferred only short-term immunity to reinfection with the same strain of the virus. They also noted that other factors, other than serum antibodies were involved in mediating resistance to infection (Parrino et al., 1977). Later, other volunteer challenge studies also came to the same conclusions as drawn by Parrino et al., (Gary et al., 1987; Johnson et al., 1990; Matsui & Greenberg, 2000). The other factors

mediating resistance, however, remained elusive from scientists for over 2 decades until 2002 when Hutson et al., found a correlation between an individual's ABO (H) histo-blood type and the risk to developing symptomatic norovirus infection. In their study, they were able to show that among the blood group types, people with blood group type O were the most susceptible to norovirus infection (Hutson et al., 2002). In that same year, Marionneau et al., also found that Norwalk virus-like particles bound to gastroduodenal epithelial cells from the so-called secretor positive individuals who express histo-blood group antigens (HBGA) in their saliva and mucosal surfaces (Marionneau et al., 2002). Then in 2003, Lindesmith et al. found a correlation between the G428A nonsense mutation in the FUT2 enzyme and resistance to symptomatic norovirus infection (Lindesmith et al., 2003). All this evidence put together, paved the way for earnest investigations into the genetic determinants of susceptibility to norovirus infections.

2.5.2 Diversity in Norovirus and Host Glycan Interactions

2.5.2.1 FUT2 and Secretor Status

Interaction with host cellular glycans is a critical initial step in the pathogenesis of many infectious agents (Ramani et al., 2016). Histo-blood group antigens (HBGA), which includes the ABH and Lewis antigens have been identified as one of these cellular glycans that serve as the binding sites for some intestinal microbes including the noroviruses (Hutson et al., 2002; Johnson et al., 1990; Koopman et al., 1982; Parrino et al., 1977). These HBGAs are complex carbohydrates that determine human blood types and are expressed mainly on mucosal epithelium of the digestive, respiratory and genitourinary tracts as well as in exocrine secretions (de Mattos, 2016). Their synthesis is regulated by the fucosyltransferase enzymes encoded for by α1,2 fucosyltransferase 2 (*FUT2* (secretor) and α1-3/4-fucosyltransferase (*FUT3* (Lewis) genes. Within the human

population, these genes are highly polymorphic (de Mattos, 2016; Guo et al., 2017; Previato et al., 2015). Individuals possessing at least one functional FUT2 allele are able to express functional α1,2 fucosyltransferase 2 (FUT2) enzyme which works on precursor chains to express the A and B blood group antigens as well as H-type 1 precursor substrate (Fig 2.5). The most common FUT2 single nucleotide polymorphism (SNP) in the European, Iranian, and African populations is a non-sense mutation (G482A) which creates a stop codon at the amino acid 143 (Trp143Ter) hence the expression of an inactive FUT2 enzyme responsible for the ABH non-secretor phenotype (Ferrer-Admetlla et al., 2009; Kelly et al., 1995; Liu et al., 1998). A homozygous A385T nucleotide substitution resulting in a missense mutation at codon 129 (Ile129Phe) is the primary mutation responsible for the non-secretor phenotype within the Asian population (Kudo et al., 1996; Soejima et al., 2007).

2.5.2.2 FUT3 and Lewis Status

The Lewis A (Le^{a+}) and Lewis B (Le^{b+}) antigens are synthesized directly by the α1-3/4-fucosyltransferase (FUT3) enzyme. The synthesis of Le^{b+} antigens however first requires the action of the FUT2 enzyme to make the H-type 1 precursor (Fig 2.5). The absence of a functional FUT3 enzyme stops the synthesis of either the Le^{a+} or Le^{b+} antigens resulting in the Lewis-null phenotype [Le^{a-b-}] (Henry et al., 1995). Whilst the expression of Le^{a+} and Le^{b+} phenotypes correlate with an individual's secretor status, the expression of Le^{a-b-} remains independent of the determined secretor status (Fig 2.6). A high number of *FUT3* gene mutations have been identified in different populations across the world. The commonly determined *FUT3* gene mutations include 59T>G, 202T>C, 314C>T, 508G>A, and 1067T>A. Substitution of amino acids caused by mutations T202C, C314T, G508A, T1067A, G484A, G667A, and G808A are known to lead to inactivation of the FUT3 enzyme (Pang et al., 1998b; Pang et al., 1998a) while mutation T59G reduces the

availability of the enzyme (Corvelo et al., 2013). In the Ghanaian settings SNPs A858G, G179A, C548T have also been reported (Soejima et al., 2009).

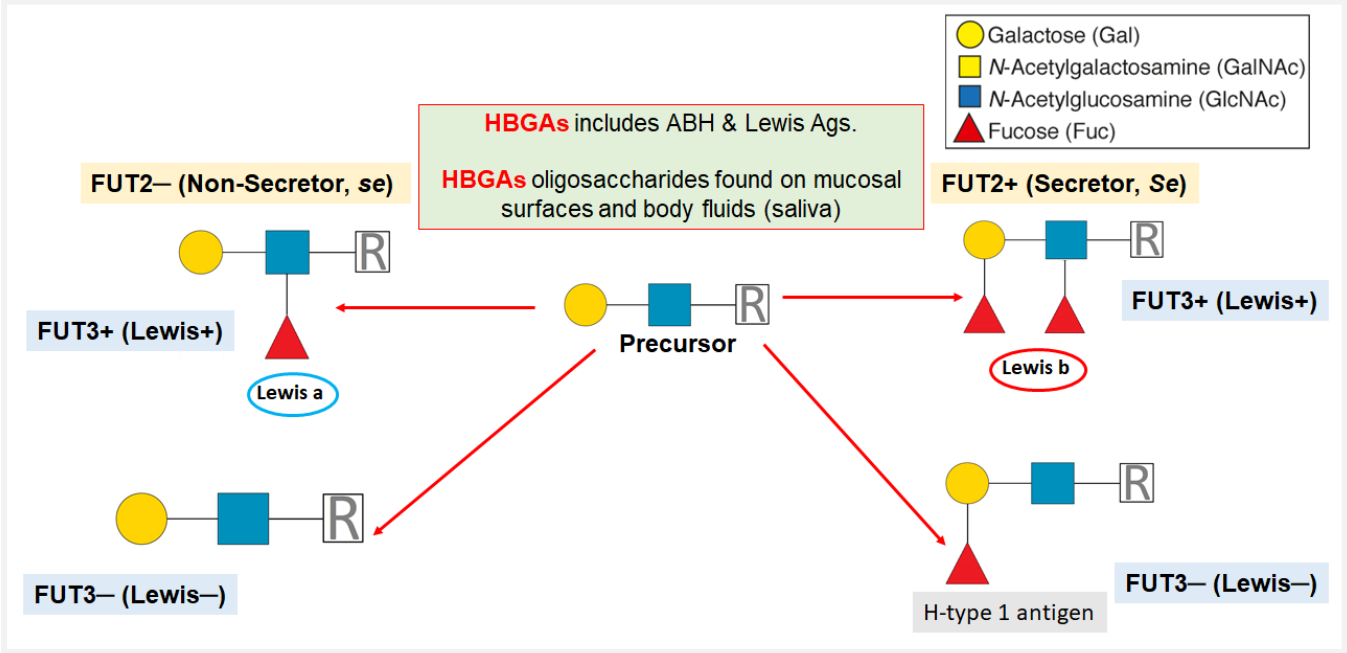


Fig 2.6: Simplified schematic overview of Lewis and Secretor status HBGA systems

Final antigen phenotype is determined by the combined action of the fucosyltransferases encoded by the *FUT2* (secretor) and *FUT3* (Lewis) genes upon Type 1 or 2 oligosaccharide precursor glycans. *FUT2* controls the secretion of ABO HBGAs on the mucosal epithelial surfaces and in body secretions and are synthesized from the H type 1 antigen precursor. Non-secretors, having an inactivated FUT2 enzyme are unable to synthesize the H type 1 antigen from its precursor hence do not express the ABO HBGAs.

Adapted from (Lee et al., 2018)

2.5.2.3 Norovirus Strain Diversity and their Interactions with HBGAs

Globally, about 70-80% of all individuals are secretors whilst the remaining 20-30% are non-secretors (Ferrer-Admetlla et al., 2009). However, in certain populations such as the Meso-Americans, non-secretor status can be as low as 5% (Nordgren et al., 2016b). When it comes to norovirus infection, secretor status giving rise to the ABH and Lewis antigens are important as they serve either as ligands or restriction factors (Karst, 2010; Nordgren & Svensson, 2019). This has been shown in many challenge studies where non-secretors always demonstrated resistance to specific norovirus strains including GII.4 and GI.1 developing no symptoms to infection (Frenck et al., 2012a; Lindesmith et al., 2003; Parrino et al., 1977) whilst showing susceptibility to other strains such as GII.3, GII.2, GI.3 (Nordgren et al., 2010; Van Trang et al., 2014b). Also, individuals expressing O-type HBGAs were found to be more susceptible to GI.1 norovirus infection compared to those expressing A and B-type HBGAs (Hutson et al., 2002; Lindesmith et al., 2003). Subsequent studies by Tan and Jiang demonstrated the ability of numerous other norovirus strains to bind to a variety of different HBGA types, generally in a genotype- or strain-specific manner (Huang et al., 2005b; Tan & Jiang, 2005b, 2008). Currently, two major binding profiles (Table 2.2) have been described for the noroviruses: those that bind A or B and H antigens and those that bind Lewis or H antigens (Harrington et al., 2002; Huang et al., 2005b). Since the proportions of secretor, ABO (H) and Lewis HBGA types differ across geographical populations (Ferrer-Admetlla et al., 2009), it has raised questions as to whether the prevalence and circulating norovirus strains would also vary as well as whether the percentage of secretors within a population would influence the prevalence of specific norovirus genotypes as a function of their ability to infect secretors compared to non-secretors. Answers to these questions would be important as they would inform on what other norovirus strain types would be important to include in vaccine formulations.

Table 2.2: Potential HBGA targets for binding of specific Norovirus strains

Genogroup	Strain (Genotype)	HBGA Binding Profile		
		AB	H	Le
GI	Norwalk (GI.1)	+++	++	-
	C59 (GI.2)	+++	++	-
	Boxer (GI.8)	-	++	++
	VA115 (GI.3)	-	-	-
GII	Hawaii (GII.1)	++	+	-
	BUDS (GII.2)	+++	-	-
	Mexico (GII.3)	+++	++	-
	PiV (GII.3)	+++	++	-
	Grimsby (GII.4)	+++	++	-
	VA387 (GII.4)	+++	++	-
	MOH (GII.5)	+++	-	-
	VA207 (GII.9)	-	+	++
	OIF (GII.21)	-	-	++

Binding studies show potential HBGA target of some norovirus strain. The binding result of each norovirus strain was assigned based on reactions in assays with saliva and/or oligosaccharide conjugates.

+: is indicative of observed positive binding; **++, +++:** indicative of higher binding signals; −: indicates no binding.

Table was adapted and modified from (Huang et al., 2005b)

2.6. Pathogenesis and Clinical Manifestations of Norovirus Infection

2.6.1 Pathogenesis

Although some progress has been made in norovirus research, the pathogenesis leading to disease conditions is still not fully understood. Most information on the viral pathogenesis has come primarily from physical, histological, and biochemical studies that have been carried out by different groups of researchers on human volunteers infected with the virus (Karst, 2010; Schreiber et al., 1974; Teunis et al., 2008; Wilhelmi et al., 2003b). It has been inferred that the small intestinal enterocytes seem to be the primary site for norovirus infection (Schreiber et al., 1974; Wilhelmi et al., 2003b). Huang and colleagues also suggested that human histo-blood group antigens present on the gastro-duodenal epithelium of individuals may serve as ligands for the attachment of the Norwalk virus (Huang et al., 2005b). Histopathological changes observed in the small intestines of infected humans include expansion of the villi, shortening of the microvilli, and crypt hypertrophy in the proximal duodenum (Wilhelmi et al., 2003b). These changes were also noted to be associated with a decrease in the activity of small intestinal brush border enzymes, leading to malabsorption (Bonani et al., 2017; Glass et al., 2009). Though the mechanism of the onset of diarrhoea is not well understood, it is believed that the development of patchy lesions on the mucosa and the delay in gastric emptying may play a role in its development (Thornton et al., 2004; Wilhelmi et al., 2003b). In symptomatic volunteers, it has been observed that delayed gastric emptying and (or) the transient structural damage of the intestinal villi may be associated with nausea and vomiting associated with infection (Meeroff et al., 1980).

2.6.2 Clinical Symptoms

Symptomatic illness occurs only in a proportion of infected individuals (Atmar et al., 2018). Asymptomatic carriage of noroviruses can be as high as 50% in some populations (Bucardo et al., 2010; Garcia et al., 2006; Huynen et al., 2013). The average incubation period for the virus ranges between 18-48 hours after exposure (Estes et al., 2006), and the illness caused is characterized by the onset of nausea, vomiting, abdominal cramps, and diarrhoea (Clark & McKendrick, 2004). Projectile vomiting is said to be one of the classic symptoms of norovirus infection (Makison Booth, 2014) supporting and promoting the rapid spread of the virus within the population. Other accompanying symptoms may include fever, chills, headache, malaise, and fatigue (Arness et al., 2000). Vomiting is a relatively common symptom in children more than one year of age, while diarrhoea is most commonly observed in children less than one year of age (Patel et al., 2009). In healthy individuals, norovirus infection is usually mild and self-limiting, lasting between 1-3 days, whilst in immune-compromised persons, the elderly and young children infection may last longer and symptoms may be severer. Work done by Murata and colleagues shows that the duration of illness is prolonged in children under the age of two (2) years compared to children between the ages of two (2) and five (5) years (Murata et al., 2007). The often observed complications of norovirus infection include electrolyte abnormalities, renal insufficiency, malnutrition (Green, 2013), and afebrile convulsions in young children (Higuchi et al., 2017; Kim et al., 2018a).

2.7. Treatment

Strategies for the prevention and treatment of norovirus disease include infection control, antiviral treatments, immunotherapy and vaccination. Generally, infection induced by the virus is mild and self-limited gastroenteritis that resolves without any complications (Mattner et al., 2006; Trivedi et al., 2013). For risk groups such as the elderly, young children, and immunocompromised individuals within the population, oral rehydration, and electrolyte replacement therapy may have to be given upon hospitalization with severe dehydration (Mattner et al., 2006). Parenteral administration of fluids may also be necessary if severe vomiting and diarrhoea occur. The use of bismuth subsalicylate (Kaufman et al., 2014b) is effective in reducing significantly the severity and duration of abdominal cramping in adults (Robilotti et al., 2015; Simons et al., 2016; Steinhoff et al., 1980) but not in children (AAP, 1996).

2.7.1 Antiviral Treatment

Because norovirus disease was previously perceived as brief and self-limiting, the development of specific antiviral medications has lagged (Kaufman et al., 2014b). There are currently no antivirals effective against norovirus approved for human use (Netzler et al., 2018; Woodward et al., 2017). Drugs including nitazoxanide, ribavirin, and favipiravir are well-studied agents that have been shown to inhibit norovirus replication either in mouse models or infected individuals (Ghusson & Vasquez, 2018; Siddiq et al., 2011; Woodward et al., 2017). Nitazoxanide exhibits broad-spectrum antimicrobial activity and is the only anti-noroviral agent to be going through a phase II randomized placebo-controlled clinical trial for the treatment of gastroenteritis in transplant patients (NCT03395405) and children (Waddington et al., 2018). Both trails sort to investigate if

nitazoxanide will be clinically beneficial for the empirical treatment and management of norovirus-associated AGE in children and transplant patients respectively.

2.7.2 Immunotherapy

Because of their ability to induce a strong host response against foreign intracellular agents, immunomodulators have been considered an excellent therapeutic option for viral infections. Studies have shown that type I and II interferons protect against both human and murine norovirus infections (Chang & George, 2007; Changotra et al., 2009; Maloney et al., 2012; Thackray et al., 2012).

2.7.3 Norovirus Vaccines

Licensed vaccines for norovirus prevention are not yet available, however, some vaccine candidates have completed either Phase I and (or) Phase II clinical trials (NCT02153112, NCT02661490, NCT02669121, NCT03039790, NCT02038907) (Debbink et al., 2014; Lucero et al., 2018). Formulations of these vaccine candidates were based on adjuvanted norovirus virus-like particle (VLP) antigens of the GI.1 genotype for a monovalent vaccine (Lucero et al., 2018; Ramirez et al., 2012) or in the case of bivalent vaccine, GI.1-derived VLPs and VLPs of GII.4 consensus strain made from GII.4 variants (Leroux-Roels et al., 2018; Treanor et al., 2014). Results from these clinical trials showed there was stimulation of antibody response regardless of the route of administration (Parra et al., 2012; Ramirez et al., 2012; Sundararajan et al., 2015; Treanor et al., 2014). A challenge study by Bernstein et al. in healthy individual volunteers showed that intramuscular vaccination with the bivalent VLPs did not significantly reduce the incidence of norovirus-associated illness after challenge with a GII.4 strain of norovirus (Bernstein et al., 2015). However, this vaccination was able to reduce the frequency and severity of vomiting and

diarrhoea (Bernstein et al., 2015). A recent safety and immunogenicity clinical trial by Leroux-Roels and colleagues on different formulations of a bivalent virus-like particle (VLP) norovirus vaccine candidates also showed encouraging results in both younger and older age cohorts. Reports from the trials showed all candidate vaccine formulations were well tolerated, displaying similar immune responses across the different age categories (Leroux-Roels et al., 2018). Future studies will also be needed to address the issue of whether these current vaccines will provide heterotypic cross-protection against other norovirus strains not included in the vaccine formulations. Summaries of the different norovirus vaccine candidates under development and their stage of development are shown in Table 2.3.

Table 2.3: Norovirus Vaccine Candidates under development

Investigators	Stage of development	Vaccine Type	Nov Genotype in Vaccine Formulation	Route of administration	Comments (References)
Takeda Vaccines, Inc	Clinical Phase 2b	Norovirus VLP	GI.1, GII.4	Intramuscular	(Leroux-Roels et al., 2018) NCT02153112 NCT02669121
Vaxart, Inc	Clinical Phase 1	Recombinant Adenovirus expressing Nov VP1	GI.1	Oral	(Kim et al., 2018b) NCT02868073
Ology Bioservices	Preclinical	Norovirus VLP	GI.1. GII.4	Intranasal	(Ball et al., 2017)
Vesikari and colleagues	Preclinical	Norovirus VLP	GI.3, GII.4, RV VP6	Intradermal /Intramuscular	(Malm et al., 2016)
Jiang and colleagues	Preclinical	Norovirus P-particle	GII.4	Intranasal	(Verma et al., 2016)
Takeda Vaccines, Inc	Clinical Phase 2a*	Norovirus VLP	GI.1	Intranasal	(Atmar et al., 2011)

*: Vaccine development discontinued **VLP:** virus-like particle

2.8 Diagnosis and Detection

The clinical symptoms related to norovirus-associated gastroenteritis are too non-specific to be used effectively to discriminate between infections caused by norovirus and other enteric pathogens, laboratory confirmation is therefore required. Human noroviruses could be detected from rectal swabs, vomitus, blood (serum), food, water, and other environmental samples. Whole-stool samples are however preferred as they contain higher concentrations of the virus (CDC, 2018a). With time the diagnostic techniques for the detection of norovirus progressed from microscopy to molecular methods (Fig 2.7). Between its identification in 1972 to 1990, norovirus detection depended upon the visualization of the viral morphological features under the electron microscope (Kapikian et al., 1972; Lewis et al., 1995; Okada et al., 1990). This method was however expensive, time-consuming, relatively insensitive especially in specimen with low viral concentrations (less than 10^6 - 10^7), and not readily available for routine testing in most diagnostic laboratories (Glass et al., 2000).

The cloning and sequencing of the viral genome in the early 1990s (Lambden et al., 1993; Xi et al., 1990) accelerated the development of various molecular diagnostic methods for norovirus detection (Fig 2.7). Reverse transcriptase-polymerase chain reaction (RT-PCR) assay was one of the first molecular methods employed for the detection of norovirus RNA in stool specimen (Green et al., 1993; Jiang et al., 1992; Kageyama et al., 2004; Khan et al., 1994). This assay employs the use of a reverse transcriptase enzyme to reverse transcribe the extracted viral RNA into complementary DNA (cDNA) which are then subsequently amplified with broadly reactive genogroup-specific primers (Katayama et al., 2002; Kojima et al., 2002; Vinjé et al., 2004). Due to the high sequence variability among the different norovirus strains most primers used target relatively conserved regions (Fig 2.8) in the ORF1 coding region for the viral RdRp

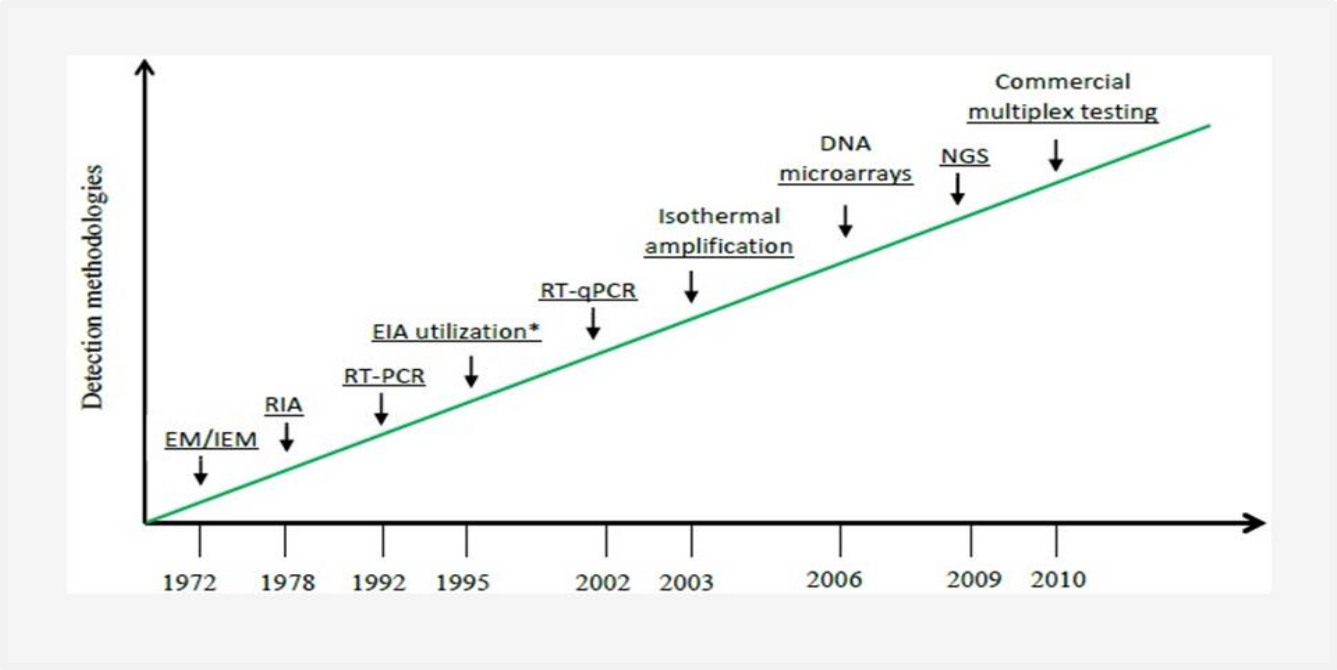


Fig 2.7: Evolving stages of molecular diagnostic methods for the detection of human noroviruses
Source: (Chen & Hu, 2016a)

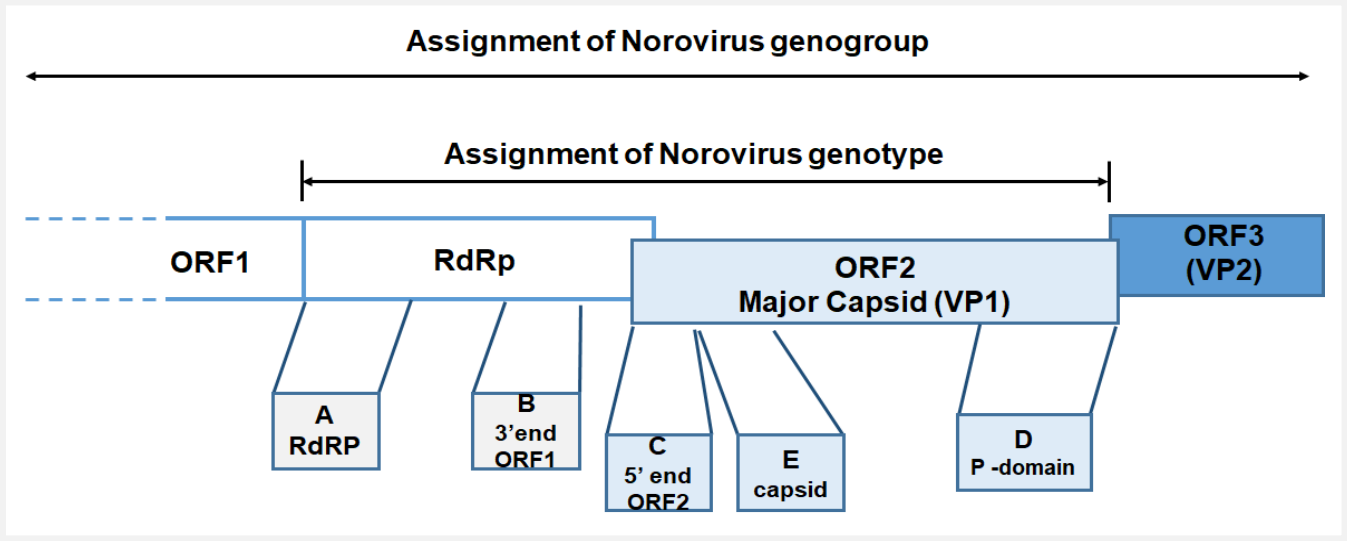


Fig 2.8: Schematic representation of the locations of the genomic regions of norovirus used for genogrouping and genotyping

Overlap junction between Regions B and C were used for analyses in this study. **ORF:** open reading frame
RdRp: RNA-dependent RNA polymerase **VP:** Viral Protein
Source: (Vinjé et al., 2004)

(Girish et al., 2002; Green et al., 1995; Rachakonda et al., 2008; Vennema et al., 2002) or the conserved region in the ORF1-ORF2 overlap region (Kageyama et al., 2003; Vinjé et al., 2004) which is most preferred in recent times (Kroneman et al., 2013). For subsequent confirmation and genotyping, sequencing is performed.

More recently, real-time (RT)-quantitative PCR (RT-qPCR) assays have been developed with increased sensitivity and specificity (Chen & Hu, 2016b; Vinje, 2015). These RT-qPCR assays do not require agarose gel analysis and require less sample handling as both reverse transcription and cDNA amplification is performed in a single reaction hence reduces the risk of cross-contamination. Most reported norovirus RT-qPCR assays employ the use of genogroup-specific oligonucleotide primer-probes targeting the ORF1-ORF2 overlap region (Kageyama et al., 2003; Vega & Vinje, 2011).

Enzyme Immunoassay commercial kits including IDEIA Norovirus (Thermo Fisher Scientific, UK), RIDASCREEN Norovirus (R-Biopharm, Germany) and SRSV (II)-AD (Denka Seiken Co. Ltd., Japan) are now available to detect norovirus antigen in stool samples. These EIA tests use a combination of both GI and GII specific monoclonal and polyclonal antibodies in a solid phase sandwich enzyme immunoassay to detect GI and GII noroviruses. These kits usually have a low sensitivity of less than 70%, while their specificity is highly variable and ranges between 57% - 90% (Burton-MacLeod et al., 2004; Dimitriadis et al., 2006; Dimitriadis & Marshall, 2005; Gray et al., 2007; Siqueira et al., 2011). The EIA assays compared to the RT-PCR or RT-qPCR methods are simple and fast to perform and do not require any specialized equipment. Nevertheless, due to the low sensitivity of the kits and the high genetic diversity of the noroviruses, it is very easy to miss positive samples (Rabenau et al., 2003). All negative samples would therefore have to be confirmed with an RT-PCR assay.

2.9. Taxonomy

2.9.1 Norovirus Classification

Though members within the *Norovirus* genus are genetically and antigenically diverse they cannot be classified based on their antigens because antigenic classification lacks accuracy and reproducibility (Ando et al., 2000). The unavailability of an efficient culture system also currently makes it impossible to serotype human noroviruses by neutralization assays (Chan et al., 2017c). This makes genomic classification based on sequencing, the only means of characterizing noroviruses (Vinje, 2015; Xi et al., 1990). Previously, investigators focused on the conserved sequence region of the RdRp encoded for by ORF-1 to design primers for characterization of the virus (Jiang et al., 1999; Kageyama et al., 2004). It was however demonstrated later by phylogenetic analysis of the complete norovirus genome that the polymerase gene was not suitable for genotyping but useful for identification of recombinant strains (Bull et al., 2007; Katayama et al., 2002). Some researchers have therefore focused mainly on the partial or complete capsid sequence encoded for by ORF-2 (Green et al., 2000; Vinjé et al., 2004) while others have used both the RdRp and capsid sequence (Vinje, 2015) to classify the virus. In 2011 the "norovirus genotyping tool"- a freely accessible online resource was launched for the typing of noroviruses (Kroneman et al., 2011). This online typing tool mainly combined the classification systems as described by Zheng et al. and Vinje et al., and allowed for the creation of standard software for the genotyping of the noroviruses (Vinje et al., 2003; Zheng et al., 2006). Further work done by Kroneman et al., showed the overlap region of the ORF1 and ORF2, coding for the RdRp and capsid (VP1) genes respectively to be important for viral genome identification and characterization hence the proposal for a dual nomenclature system for norovirus classification using both VP1 and RdRp genes (Kroneman et al., 2013). Therefore a norovirus strain with a

"GII.P4_GII.3" designation implies the strain has GII.4 RdRp and GII.3 VP1 sequence. This mode of classification has allowed for the detection of strains with different VP1 and RdRp gene combinations as well as novel strains and has further standardized the norovirus naming (Classification) system.

Based on nucleotide sequence similarities of the capsid gene, the virus is genetically classified into ten phylogenetic clades designated as genogroups [GI-GX] (Vinje, 2015). Further divergence in both the RdRp and VP1 genes further clusters members within a genogroup into genotypes. Using the above criteria, more than 49 norovirus genotypes have been identified: 9 of these genotypes belong to Genogroup one (GI), 27 to GII, 3 to GIII, 2 each to GIV, GV, GVI, and 1 each to GVII, GVIII, GIX, and GX (Cortes-Penfield et al., 2017; Kroneman et al., 2013). Strains belonging to genogroups I (GI.1-GI.9), GII (GII.1-GII.10, GII.12-GII.17, GII.20-GII.27), GIV (GIV.1), GVIII (GVIII.1), and GIX (GIX.1) infect humans while strains belonging to genogroups GIII, GV, GVII, and GX infect bovine, murine, canine, and bat species respectively (Fig 2.9). Accumulation of mutations within the gene of a genotype further leads to the periodic emergence of new norovirus variant strains. At least seven variant strains (Fig 2.9) have been described for the most common circulating norovirus genotype GII.4 (Chan et al., 2017b).

Noroviruses tend to be species-specific although some human norovirus strains have been found infecting bovine and porcine species (Mattison et al., 2007). To date, no infections with animal strains have been identified in humans. However, some strains clustering within the GII (GII.11, GII.18, GII.19) have been found to infect only porcine species while some strains in GIV (GIV.2) also infect feline and canine species only (Atmar, 2010).

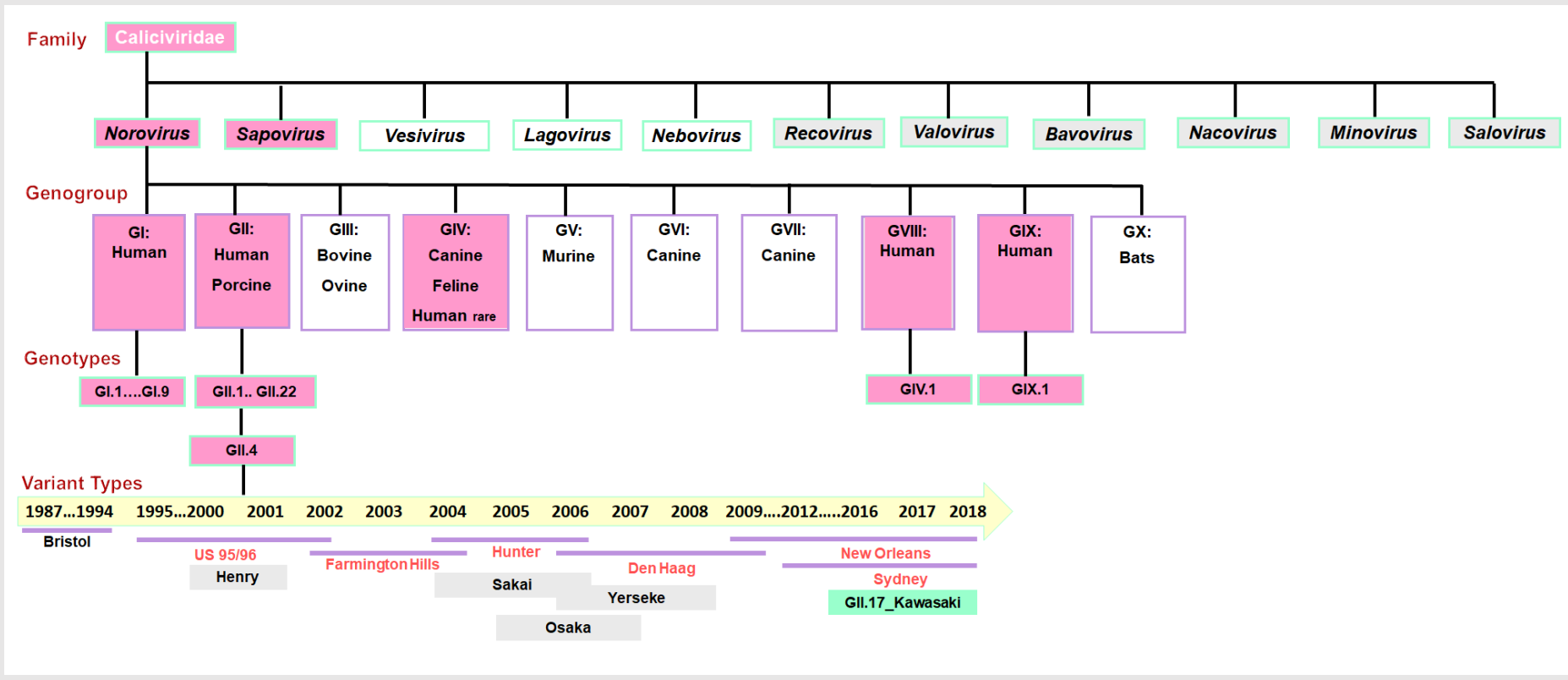


Fig 2.9: Taxonomy of the family *Caliciviridae*

Human pathogenic genera within the family are displayed in filled pink boxes whilst the recently proposed genera and genogroup yet to be approved by the ICTV are displayed in filled grey boxes. The GII.4's involved in majority of clinical cases, evolve every 2-4 years giving rise to antigenically distinct variant strains. This schematic diagram also shows the years of circulation of key GII.4 variant strains. The long yellow filled arrow shows the timelines of variant circulation while the purple lines denotes the timeframe for which the variants were in circulation. Pandemic and epidemic variants are shown in bold red fonts and filled grey boxes respectively. Since 1995 to date six Pandemic GII.4 variant strains (shown in red fonts) have been described. The year 2014 to 2016 saw the emergence and global spread of GII.17 Kawasaki (shown in green filled box) epidemic strain.

2.9.2 An adaptable virus: Norovirus genetic diversity and evolution

Noroviruses can be found in a wide range of hosts, such as humans, rodents, felines, canines, ovine, bovine, and bats (Fig 2.9). Because of their wide host range, the noroviruses tend to be genetically diverse sharing only 51-56% nucleotide sequence similarity among the genomes of the different genogroups (Donaldson et al., 2010). The diversity between these genogroups is even higher when comparing only the capsid (VP1) sequences (Donaldson et al., 2010) compared to the whole genome (Katayama et al., 2002). Antigenic drift and shift (genetic recombination) are the two main mechanisms thought to drive the continuous diversity and evolution in the noroviruses.

2.9.2.1 Antigenic Drift results in new Norovirus Variants

Antigenic drift occurs with the accumulation of mutations within the genes encoding for the antigenic regions of the virus as a result of the error proneness of the polymerase enzyme leading to antigenic variations and the emergence of novel strains (Boni, 2008). In the noroviruses, these variations have been observed to be localized to five (5) evolving blockade epitopes (A-E) found in the hypervariable P2 domain of the capsid protein involved in host cell binding (Lindesmith et al., 2008b; Siebenga et al., 2007b). These blockade motifs are potential sites of virus neutralization hence confer protection against clinical disease. Variations in amino acids within these epitopes allow for immune evasion and the continuous infection of the norovirus within the human population. Because the P2 domain of the norovirus is under constant selective pressure, there is the replacement of previously established genotype or variant at a typical interval of 2–3 years (Fig 2.7) through epochal evolution (Allen et al., 2009; Bull et al., 2010; Lindesmith et al., 2011). Most of the noroviral antigenic variations have been studied in the successful GII.4 strain (Bull et al., 2010; Cannon et al., 2017a; Lindesmith et al., 2008b; Siebenga et al., 2007a). Through the accumulation of mutations at these 5 antigenic epitopes in the capsid P2 domain, the GII.4 strains

have been able to generate six pandemic variants over the last 2 decades (Fig 2.9). Four (US 95/96, Farmington Hills_2002, Hunter_2004, Den Haag_2006b) out of these six pandemic variants evolved as a result of antigenic drift in the P2 domain (Bull & White, 2011; Donaldson et al., 2010). Interestingly the more recent variants (New Orleans_2009, and Sydney_2012) evolved as a result of both antigenic capsid variation as well as intra-genotype recombination at the ORF1-ORF2 overlap region (Eden et al., 2010; White, 2014a).

Studies have also suggested that novel variants usually circulate at low levels in the population as pre-epidemic forms before acquiring the necessary P2 mutations to escape herd immunity (White, 2014a). These pre-epidemic forms are associated with causing limited and localized outbreaks but show no global prevalence (de Graaf et al., 2015b; Eden et al., 2014; Giammanco et al., 2014; Hasing et al., 2013; Lu et al., 2016). This shows that, as done for influenza viruses, these variants could be detected and monitored long before they become pandemic strains and therefore be targeted for vaccine development. There are also speculations as to whether established genotypes such as GI.3, GII.2, and GII.3 would evolve to display variants soon hence they are also genotypes worth monitoring.

2.9.2.2 Recombination events may result in novel strains

Viral RNA recombination or antigenic shift is one of the major forces that drive viral evolution and genetic diversification leading to the emergence of novel and potential pandemic norovirus strains (Pérez-Losada et al., 2015). Studies have shown that inter-and intra-genotype recombination events of the noroviruses, mainly, occur at the overlapping junction between ORF1 and ORF2 which also serves as a transcription start site for the viral sub-genomic RNA and separates the non-structural and structural regions of the viral genome (Bull et al., 2005; Bull et

al., 2007). Recombination at this junction, therefore, allows the virus to exchange its viral capsid while retaining the region involved in viral genome replication hence aiding in the escape from herd immunity (White, 2014a). Though uncommon, recombination breakpoints have also been identified within the overlapping regions of ORF2 and/ ORF3 of the GII.4 noroviruses (Eden et al., 2013b). Despite the predominance of the GII.4 strains, several other non-GII.4 strains have also been an important cause of norovirus infections (Kwok et al., 2017; Matsushima et al., 2015). Interestingly, norovirus recombination events are more common in these non-GII.4 strains. Examples of some of the commonly detected recombinant strains include GII.21/GII.3 (GII.Pb/GII.3), GII.Pb/GII.13, GII.P16/GII.13, GII.P6/GII.7 and GII.Pg/GII.12 (Rohayem et al., 2005; Waters et al., 2007; White, 2014a). The GII.P21 formerly designated GII.Pb has been found to naturally recombine with GII.3 capsid genotype (Boon et al., 2011a; Mahar et al., 2014) and seems to be an important strain associated with childhood norovirus infections (Hoa Tran et al., 2013; Sdiri-Loulizi et al., 2009).

2.10. Norovirus Epidemiology

2.10.1 Burden of Norovirus disease

Human noroviruses are the most common cause of both sporadic and epidemic acute gastroenteritis in all age groups (Ahmed et al., 2014) worldwide. They are also the cause of outbreaks of acute gastroenteritis in virtually any setting where people are in close contact. The most common of these being nursing homes, hospitals, schools, cruise ships, and restaurants (de Wit et al., 2007; Gallimore et al., 2004; Hall et al., 2013b; Okada et al., 2006; Tam et al., 2012). Severe outcomes of norovirus gastroenteritis are also common among children less than 5 years and the elderly (>65 years) who account for the majority of the fatalities associated with norovirus infections. Although the impact of the virus in developing countries has not been well studied, estimates by Bartsch et al. suggests that noroviruses are a significant cause of morbidity and mortality in children under five years globally (Bartsch et al., 2016). Studies in some parts of Ghana showed norovirus to be responsible for approximately 10-16% of all pediatric diarrhoeal cases (Armah et al., 2006a; Chen JC et al., 2013; Reither et al., 2007). After the success of the rotavirus vaccine introduced a couple of years ago, the development of a norovirus vaccine has become a high priority for the prevention of non-bacterial acute gastroenteritis (Tan & Jiang, 2014b). The successful implementation of a norovirus vaccine is estimated to result in approximately $2.1 billion of economic savings and the prevention of more than 2.0 million cases annually in the USA alone (Bartsch et al., 2012).

2.10.2 Transmission of infection

Noroviruses are highly contagious with a basic reproduction number (the number of people one infected person can infect during its infectious period, also known as R0) of approximately 14 (Heijne et al., 2009), the noroviruses need only as little as ten viral particles to cause an infection (Karst, 2010). The virus is also listed as a category B agent in the NIH/CDC bio-defense program since its outbreaks usually cause panic and severe incapacitation (Matson & Szucs, 2003). Outside the human host, the virus is relatively stable within the environment and can withstand physical conditions such as freezing, heating up to 60 ^{O}C, and exposure to high levels of chlorine (Glass et al., 2009; Oshiro & Schaub, 2000).

Transmission of the virus occurs mainly via the fecal-oral route (Green, 2013; Glass *et. al.*, 2009). Secondary infections have been shown to occur by person-to-person contact, fomites, aerosolized vomitus, and also from infected food handlers (Fankhauser et al., 2002; Thornton et al., 2004). Low levels of transmission have also been found to occur through contaminated drinking water supplies (Leclerc et al., 2002). An infected individual remains contagious even after recovery from diarrhoea because asymptomatic viral shedding continues for up to three weeks (Hall, 2012).

2.10.3 Seasonality

The noroviruses display a complex incidence of disease with varied peak seasons globally (Lopman et al., 2016a). Two main factors control the incidence of the virus: (i) genetic factors that lead to the emergence of new epidemic and pandemic strains (ii) environmental factors including temperature, climate, human population, and behavior may contribute to give rise to seasonal peaks even without the emergence of new virus variants (Lopman et al., 2009; Rohayem, 2009). Peaks of incidence of norovirus infections tend to be higher when new pandemic strains emerge. In temperate climates, norovirus incidence is highly seasonal with yearly epidemics occurring during winter from October to April (Ahmed et al., 2013; Debbink et al., 2014; Hall et al., 2013a). In the tropical and sub-tropical climates, however, norovirus seasonality is less obvious and has been reported to occur all year round. Sub-tropical regions such as Morrocco, Lybia, and Tunisia all located in the northern hemisphere report peak of detection during the summer months of June to September (Ahmed et al., 2013; Kreidieh et al., 2017) whilst South Africa and Malawi in the southern hemisphere observe norovirus peak seasons during spring or early summer or during (or) at the end of the rainy season respectively (Mans et al., 2016a; Mans et al., 2015). Tropical countries including Ghana, Burkina Faso and Egypt have reported the peak of norovirus incidence to coincide with the peak of rotavirus detection, in the cool dry seasons (Armah et al., 2006b; Kreidieh et al., 2017; Mans et al., 2016a).

2.10.4 Global diversity in Norovirus genotypes

While the epidemiological and clinical implications of circulating norovirus genotypes are not fully understood, norovirus genotyping serves as an essential epidemiological tool (Pang & Lee, 2015) used to track strain types, their circulation, and how they change over time. Out of the 10 identified genogroups, only GI, GII, and GIV, GVIII, and GIX are known to infect humans with the majority of infections belonging to GII (Glass et al., 2009; Green, 2013; Robilotti et al., 2015) and infections with GIV, GVIII, and GIX being very rare (Karst, 2010).

Despite the large genotype diversity displayed by the noroviruses, GII.P4/GII.4 is reported as the most prevalent genotype associated with 70-80% of all human norovirus-associated gastroenteritis (Bok et al., 2009; Green, 2013; Hoa Tran et al., 2013). The GII.4 strains are also able to undergo periodic recombination events giving rise to new circulating epidemic variants every 2-4 years, hence able to induce more severe clinical symptoms (de Graaf et al., 2015b; Debbink et al., 2013; Gustavsson et al., 2017; Lindesmith et al., 2013). Studies from Asia in recent times have reported a major shift in genotype predominance from the almost-two-decade predominant GII.4 genotype to the novel GII.P17/GII.17 (GII.17 Kawasaki 2014) norovirus strain (de Graaf et al., 2015a; Fu et al., 2015; Jung et al., 2017; Lindesmith et al., 2017; Lu et al., 2015). The winter seasons of 2016-2017 saw the emergence and widespread circulation of a potential epidemic recombinant strain GII.P16/GII.2 (Lu et al., 2017; Niendorf et al., 2017; Tohma et al., 2017).

Interestingly, the prevalence of circulating norovirus strains differs between human populations, geographical locations, and route of transmission (de Graaf et al., 2017; Kroneman et al., 2008). Norovirus genotype GII.4 is most often associated with person-to-person contact transmission (de Graaf et al., 2017; Wikswo & Hall, 2012) whiles non-GII.4 genotypes including G1.3, GI.6, GI.7,

GII.3, GII.6, and GII.12 are most often associated with foodborne transmission (Lysen et al., 2009; Verhoef et al., 2015). The GI's are also more often associated with waterborne transmissions compared to the GII's (Lysen et al., 2009). Some genotypes even show the tendency to occur more frequently in children than in adults (Bruggink et al., 2017; Chan et al., 2015b; Lindell et al., 2005). GII.3 norovirus strain has been suggested to be an important cause of infantile diarrhoeal disease (Hoa Tran et al., 2013; Mahar et al., 2014; Mans et al., 2016b; Trainor et al., 2013). The reasons for this remain unclear and merit further work to investigate this observed phenomenon.

Recently it has been suggested that differences in histo-blood group antigens between African and non - African people may be related to differences in circulating norovirus strains (Nordgren et al., 2016b). Histo-blood group compatibility is probably the most important determinant for host range restriction of noroviruses and could explain why animal noroviruses do not cause infections in humans (de Graaf et al., 2016a; Tan & Jiang, 2014a). Norovirus GI is said to specifically bind to host epithelial cells with A and O blood group antigens whiles GII binds to host epithelial cells with A and B blood group antigens (Donaldson *et al.*, 2008). It is also not yet fully understood how the different norovirus strains relate to each other from one season to the next.

CHAPTER THREE

Pediatric Norovirus Diarrhoea in Ghana

Understanding Pediatric Norovirus Epidemiology: A Decade of Study among Ghanaian Children: Published in *Viruses. 2020 Nov 18; 12(11):1321. doi: 10.3390/v12111321.*

Abstract

Understanding the epidemiology of human norovirus infection in children within Ghana and the entire sub-Saharan African region, where any future norovirus vaccine would have the greatest impact, is essential. Unfortunately, little or no information exists in this region on this important topic. To address this, the prevalence, clinical features, and genotype diversity of norovirus disease were investigated in Ghanaian children less than 5 years of age in Ghana. Diarrhoeic stool samples collected as part of ongoing national rotavirus surveillance from children <5 years from January 2008 to December 2017 were tested for norovirus by RT-PCR and partial nucleotide sequencing of the polymerase and capsid genes carried out. Of the 1337 diarrhoeic samples tested, 485 (36.2%) of children were found to be shedding the virus. Norovirus infection was detected across all age groups but was most commonly detected in stools of children aged between 9 and 24 months. Three hundred and seventy-two (372 (76.7%) of the isolates detected belonged to genogroup II viruses, 70 (14.4%) isolates belonged to genogroup I, and 43 (8.9%) were mixed infections of both genogroups I & II. The most commonly isolated genotypes were GII.4 (54.1%), GII.3 (7.7%), GII.6 (5.3%), GII.17 (4.7%), and GII.5 (4.7%). Though, norovirus GII.4 remained the predominant capsid genotype throughout the study duration, a slight increase in GII.6 and GII.3 genotypes was observed in 2013 and 2014 respectively. The severity of clinical illness in children infected with GII.4 norovirus strains was similar to those caused by the non - GII.4 norovirus strains. The study confirmed the significant role that noroviruses play as an important etiological agent of acute

gastroenteritis among Ghanaian children under five of age in Ghana. Since the epidemiology of norovirus changes rapidly, the establishment of systematic surveillance within sentinel sites across the country would enhance the monitoring of circulating norovirus strains and allow us to have a continuous understanding of the current state of norovirus infection within our settings.

3.1 Introduction

Globally acute gastroenteritis (AGE), the most common presentation of diarrhoea disease remains one of the infectious diseases associated with a majority of morbidity and mortality (Mokomane et al., 2018; Troeger et al., 2018a). After birth asphyxia, pneumonia, and neonatal sepsis, diarrhoea disease remain the fifth most important cause of death in children less than five years of age (WHO, 2018). Most of these deaths occur in countries within sub-Saharan Africa and Southeast Asia where there is relatively poor access to healthcare systems, safe drinking water, and poor sanitation compared to other parts of the world (Troeger et al., 2018a). Diarrhoeal deaths are rarely seen in developed countries, although those countries still record a high number of cases and hospital visitations due to the illness (Mokdad, 2017). As a result, the diarrhoeal disease burden in terms of morbidity, health care use, and expenses on disease management are quite high in the developed countries compared to underdeveloped countries (Rheingans et al., 2009). Until recently, rotavirus gastroenteritis was one of the most common reasons for pediatric hospitalizations, nonetheless, in countries where the rotavirus vaccines have been adopted for routine use in their expanded immunisation programs (Troeger et al., 2017b), the magnitude of rotavirus-associated diarrhoea inpatient admissions and deaths has reduced greatly (Aliabadi et al., 2019; Burnett et al., 2017). It has been suggested that, if the remaining burden associated with other causative agents of AGE is minimized or prevented, an enormous amount of human suffering and cost to the health systems of countries could be averted (Hallowell et al., 2019).

The importance of human norovirus disease is a picture that is slowly coming into focus. Previous efforts to detect and characterize noroviruses were faced with many obstacles with the major one being inadequate and sufficiently sensitive detection methods (Lopman, 2015). Recent advances in the use of highly sensitive RT-PCR assays have made the detection of noroviruses to be more

frequent in diarrhoeic samples (Chen & Hu, 2016a; Vinje, 2015). Human noroviruses are now being recognized as an important cause of both sporadic and epidemic forms of AGE in persons of all age groups and are now considered the second leading cause of viral gastroenteritis in children less than five years of age after rotaviruses (Oude Munnink & van der Hoek, 2016). Noroviruses, usually the cause of major outbreaks of gastroenteritis cases have been suggested to be responsible for sustaining the morbidity and mortality rates of diarrhoeal disease globally after the introduction of rotavirus vaccine (Capece & Gignac, 2018; Stuempfig & Seroy, 2019), as well as, ranking as the most common cause of gastroenteritis among pediatric populations (Koo et al., 2013; Lopman & Grassly, 2016). Currently, noroviruses are estimated to be the cause of about one-fifth of all gastroenteritis cases in children less than five years worldwide, and translating into approximately 685 million episodes of diarrhoea (CDC, 2018b) and 212,000 deaths annually (Mans, 2019; Pires et al., 2015). Nearly 99% of these deaths occur in middle- and low-income countries (Pires et al., 2015). Studies by others have also shown that norovirus disease exerts a global economic burden of $4.2 billion on health system costs (Bartsch et al., 2016).

Recent systematic reviews, using data generated from the USA, Europe, and Asia, to evaluate the role of noroviruses in AGE globally, pegged the incidence of norovirus disease at an estimated rate of 18% with a winter seasonality (Ahmed et al., 2014; Lopman et al., 2016b; Patel et al., 2009). More prominently, however, these reviews, as well as other reviews from within the less developed countries, highlighted the lack of data from the regions believed to record high numbers of mortality rates; the middle- and low-income countries (Kreidieh et al., 2017; Lopman et al., 2016b; Mans, 2019). These reviews also highlighted the need for more studies from the less endowed regions to better understand the real contribution of noroviruses to the burden of diarrhoeal diseases.

Current breakthroughs in the development of *in vitro* culture systems for human norovirus (Ettayebi et al., 2016; Jones et al., 2014a) have now made it possible for targeted antivirals, as well as potent multivalent norovirus vaccine development. Suffice to add that a norovirus vaccine is currently advancing in Phase 2b clinical trials (Leroux-Roels et al., 2017).

Understanding human norovirus epidemiology in children within sub-Saharan Africa, where any future vaccines would have the greatest impact, is therefore essential. In this study, the prevalence, clinical features, and genotype diversity of norovirus disease over a 10-year-period was investigated in children less than 5 years of age in Ghana, a country that implemented infant rotavirus vaccination in April 2012, utilizing an already established rotavirus-sentinel surveillance system.

3.2 Methods

3.2.1 Study Design

This was both a retrospective and prospective cross-sectional study of the norovirus disease incidence in hospitalized children.

3.2.2 Study Sites

Samples used in this study were obtained from children under the age of five years as part of an ongoing National Rotavirus Surveillance study. Samples were obtained from the Navrongo War Memorial Hospital (WMH), Paga, and Kassena East Health Centers in the Kassena Nankana District in the Northern Region of Ghana and Agogo Presbyterian Hospital in the Ashanti region of Ghana. Other sites were the Ho Municipal and Volta Regional Hospitals in the Volta region and the Korle-Bu Teaching and Princess Marie Louise Children's Hospitals in the Accra Metropolitan district of the Greater Accra Region of Ghana. These study sites represent the northern savannah, middle forest, and southern coastal ecological zones of Ghana respectively (Fig 3.1).

3.2.3 Study Sites description

Kassena Nankana District, one of the nine Districts in the Upper East Region which covers about 1,674 square kilometers of the Sahelian savannah and borders with Burkina Faso, is of a rural setting with a population of about 142,000 inhabitants, almost all of who are engaged in subsistence farming of millet and livestock. The District gets two main climatic conditions – the wet months that occur in June to September with the rest of the months being relatively dry. The District has one hospital and four Health Centers. Each Health Center serves a population of about 30,000 with the District Hospital serving as a referral facility to all the Health Centers.

Agogo, on the other hand, is a peri-urban town in the Asante-Akim North municipality with a population of about 170.000, who engage in crop farming and business trading as the main economic activities. The municipality is approximately 80 km to the east of Kumasi, the capital of the Ashanti Region of Ghana, and houses the Agogo Presbyterian Hospital (APH) which is the second-largest hospital of the Ashanti Region. The hospital has a 250-bed capacity which serves the inhabitants and patients from neighboring countries of Togo, La Cote d'Ivoire, and Burkina Faso. Among its facilities are a children's Outpatient Department (OPD) and a pediatric ward.

Ho, the capital city of the Volta Region of Ghana lies east of the Volta Lake with a savannah woodland vegetation and has two rainy seasons which are referred to as major and minor rainfall seasons from March to June and July to November respectively. This capital city houses the Volta Regional Hospital (VRH), which was recently upgraded to a Teaching Hospital, and the Ho Municipal Hospital (HMH), which is a district public hospital with a 150-bed capacity serving a population of about 265,046 inhabitants of the Ho municipality and its environs.

Accra is the capital and the largest city of Ghana and lies within the savanna zone. It is the administrative and economic hub of Ghana and has a population of about 1,658,937 people. The Korle – Bu Teaching Hospital is the leading national referral medical center in Ghana with over 2,000 beds whilst Princess Marie Louise Children's Hospital severs as one of the few specialist children's hospitals in the West African sub-region working in collaboration with UNICEF to combat the high level of child malnutrition in Africa. The two hospitals serve the surrounding urban population, as well as referral cases from other Regions of Ghana and other countries in the West African sub-region. Together, both hospitals have the capacity for over 250 pediatric beds and provide care for about 105,000 outpatients and 10,000 in-patients annually.

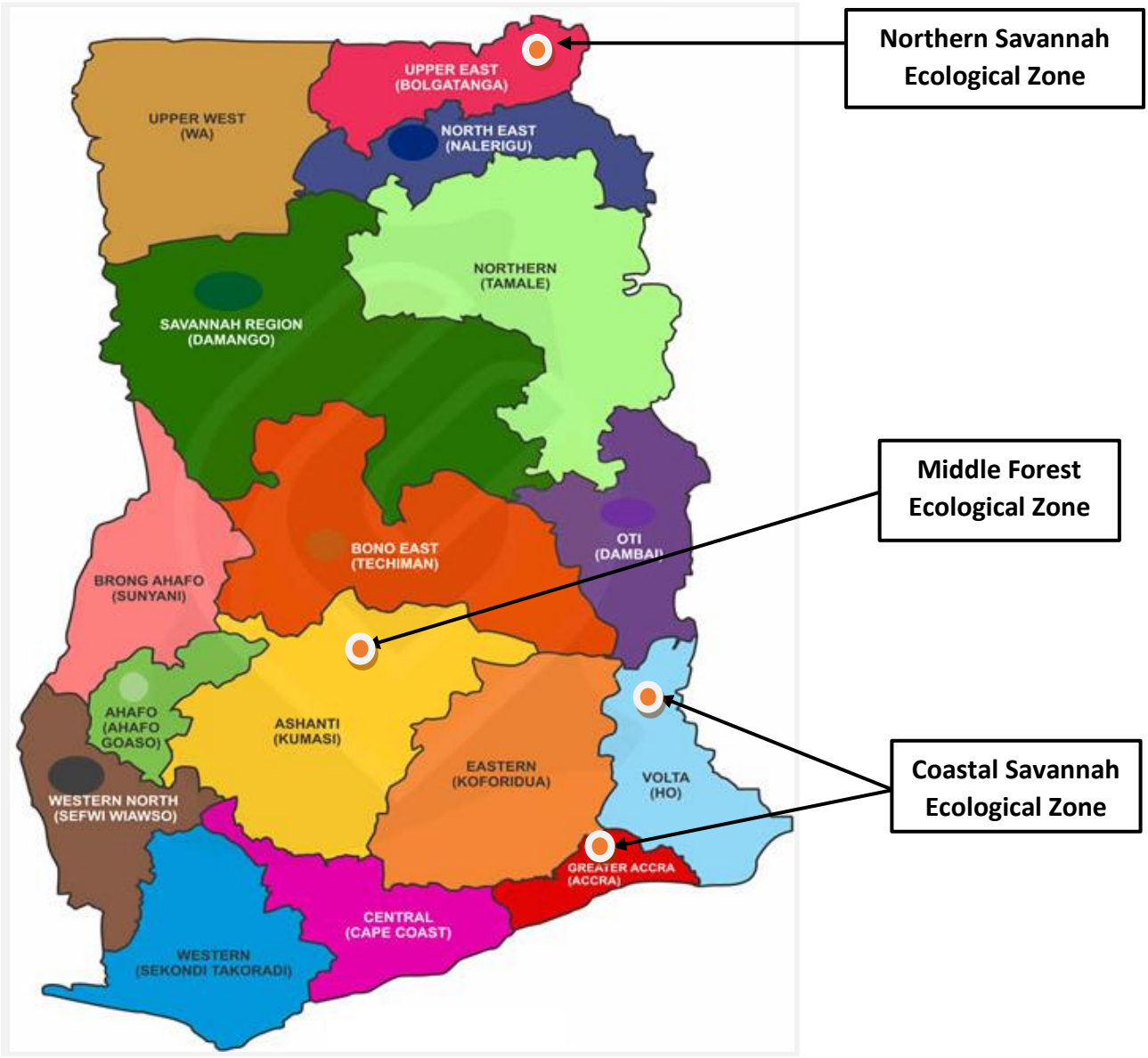


Fig 3.1: A map showing the Ecological Zones of the various study sites

Geographical location of the study sites are pointed out by filled red circles ( ) within the four participating regions in Ghana.

3.2.4 Ethics, Study population and Sample Collection

Ethical and study protocols were reviewed and approved by the Scientific and Technical Committee and Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Accra-Ghana (Appendix 3.1). Children aged 0-59 months who sought medical care and were hospitalized with the diagnosis of AGE and met the study inclusion criteria were enrolled in the study after informed consent had been taken from their parents or guardians. Non-participation in the study did not affect the medical treatment provided. Participation in the study was voluntary and the participants could exit from the study voluntarily without any consequences. A standard questionnaire was used to collect clinical data which included: episodes of diarrhea, vomiting, fever, clinical signs, treatment given, and case outcome from the parents and hospital folders of children enrolled in the study. AGE or diarrhoea was defined as the passage of three (3) or more 'looser than normal' (or) watery stools within 24 hours and of less than seven (7) days duration. Stool samples were collected into labeled screw-top containers no later than 7 days from the onset of the illness and within 48 hours of hospitalization. The samples were kept at −20 °C until transported to the Noguchi Memorial Institute for Medical Research in Accra, where they were stored at −20 °C until ready to be tested. To preserve the integrity of viral RNA, glycerol was added to samples before storage.

3.2.5 Laboratory Analysis

3.2.5.1 Preparation of 20% stool suspension

Twenty percent (20%; w/v) fecal suspensions were made by adding approximately 0.2 g of the stool samples to 1.0 mL of phosphate-buffered saline (PBS) of pH 7.2 in 1.5 mL Eppendorf tubes and vortexed to mix. Stool suspensions were stored at 4 °C until ready to be used.

3.2.5.2 Extraction and Purification of Viral RNA

Viral RNA was extracted from all the stool samples that were collected. Single-stranded RNA (ssRNA) was extracted from the 20% fecal suspension using the QIAamp Viral RNA Mini kit (QIAGEN, Germantown, MD, USA). This kit combines the selective binding properties of a silica-based membrane with the speed of micro-spin to generate high-quality purified RNA. The QIAamp Viral RNA isolation was done manually following the manufacturer's instructions. Briefly, the prepared 20% (w/v) stool suspensions were vortexed and centrifuged at 5,000 rpm [using Eppendorf 5418, a bench-top centrifuge (Eppendorf International, USA)] for 3 minutes to obtain a clarified supernatant. Five-sixty microliters (560 µl) of lysis buffer (Buffer AVL) containing carrier RNA was added to 140 µl of sample supernatant (from previously prepared 20% stool suspension) in sterile 1.5 Eppendorf tubes, vortexed and incubated at room temperature (15 °C -25 °C) for 10 minutes. An equal volume of absolute ethanol (560 µl) was added to the samples and mixed by pulse-vortexing for 15 secs after which the contents were briefly centrifuged to remove drops from inside the lid of the Eppendorf tubes. To purify the lysed viral RNA, 630 µl of the lysate solution was carefully applied onto the QIAamp mini spin column and centrifuged at 8,000 rpm for 1 min. The spin columns were placed in clean 2 ml collection tubes, and the tubes containing the filtrate were discarded. This step was repeated until all the lysate had been loaded onto the spin columns. To wash away contaminants and inhibitors, 500 µl of wash buffer AW1

was added to each spin column, centrifuged at 8,000 rpm for 1 min and the filtrate discarded. The spin columns were once again placed in clean 2 ml collection tubes, 500 µl of wash buffer AW2 was added, centrifuged at 8,000 rpm for 3 mins, and the tube containing the filtrate discarded. To eliminate any chance of possible residual wash buffer AW2 carryover into elute which may cause problems in downstream applications, the empty spin columns were placed in clean 2 ml collection tubes and centrifuged at full speed (14,000 rpm) for 3 mins. The spin columns were placed in clean 1.5 ml microcentrifuge tubes and 40 µl of elution buffer AVE added, incubated at room temperature for 1 min, and centrifuged at 8000 rpm for 1 min. To increase the yield of purified viral RNA in elute, double elution (2 x 40 µl of Buffer AVE) was performed. Extracted viral RNA was immediately stored at −20 °C before norovirus detection.

3.2.5.3 Norovirus Detection and Genogrouping

The study employed two different protocols for the detection and characterization of noroviruses. Study protocol one used genogroup specific primers (GISKF or GIFFN/GISKR; GIISKF or GIIFBN/GIISKR) as previously described (Kojima et al., 2002) and targeted the 5' end of the capsid gene (designated region C) of the norovirus genome (Fig 2.8) for the analysis of samples collected between 2008 and 2012. Since recombination events frequently take place in noroviruses, Kroneman and colleagues in 2013 (Kroneman et al., 2013) recommended a dual strain typing system by the amplification of partial regions of the capsid (VP2) (C-typing/genotype) and polymerase (VP1) (P-typing/genotype) genes of the genome. Study protocol two was therefore a P-C typing assay that used modified genogroup-specific primers (Mon432/GISKR; Mon431/GIISKR) previously described (Anderson et al., 2001; Kojima et al., 2002). The second assay was used for the analysis of the samples that were collected from 2013 to 2017. Both

protocols involved a two-step reaction of reverse transcription of ssRNA gene and nested PCR amplification of the target gene using genogroup-specific primers, and offered a qualitative detection and differentiation of norovirus genogroups I and II. Table 3.1 describes fully the primers used as well as their targeted regions and expected band sizes.

3.2.5.3.1 Synthesis of Complementary DNA (cDNA)

Viral RNA extracts were reversed transcribed as previously described by Gallimore et al., (Gallimore et al., 2005). One microliter (1.0 µl) of 20 µM Pd(N)6™ hexanucleotide random primers was added to 9.0 µl of ssRNA template. Reverse transcription master mix (10.0 µl) consisting of 4 µl of 5X AMV reaction buffer (Promega); 5U of Avian Myeloblastosis Reverse Transcriptase (Promega); 1.0 µl each of 10 mM dNTP's (dATP, dCTP, dGTP, dTTP) and 1.5 µ1 of RNase free water was prepared and added to each reaction. Double distilled water was substituted as the ssRNA template in a negative control reaction. The reverse transcription assay was carried out in an Eppendorf Master® thermal cycler (Eppendorf AG, Hamburg) at 42 °C for 4 hours to produce cDNA. The cDNAs were stored at -20 °C for future use.

PCR amplification and Genogrouping

Generated cDNAs were subjected to nucleic acid amplification for norovirus detection. For the amplification of the various genogroups, both forward and reverse genogroup-specific primers (described in Table 3.1) were used. Nested PCR in a 45.0 µl reaction mixture containing 0.25 µl each of 10 mM dNTP's; 10.0 µl of 5X Green GoTaq® reaction Buffer (Promega); 0.25 µl GoTaq® DNA Polymerase (Promega); 30.75 µl of RNase free water and 5.0 µl of cDNA template. For amplification of the genogroup one (GI) norovirus genome, 1.0 µl of 20 µM GISKF/GIFFN and 2.0 µl of 20 pmol GISKR primers were added to the master mix solution while for amplification

of the genogroup two (GII) genome, 1.0 µl of 20 µM GIISKF/GIIFBN and 2.0 µl of 20 µM GIISKR primers were used. Forty (40) cycles of amplification consisting of 10 mins of initial denaturation at 95 °C, 30 secs of annealing at 48 °C, 5 mins of extension at 72 °C were performed using the Eppendorf Master® Thermocycler (Eppendorf AG, Hamburg). To complete the PCR, a final extension cycle was carried out for 5mins at 72 °C.

To determine the success of the PCR amplification, all the amplicons were resolved by electrophoresis on a 1.5% agarose gel (Hi-Res Standard Agarose, AGTC Bioproducts) stained with 5µg/ml ethidium bromide (Invitrogen). The amplicons were electrophoresed in 1X Tris-Acetic acid-EDTA (TAE) buffer (pH 7.9), alongside a 100 bp molecular weight marker (Promega) at 120V for one (1) hour, viewed under ultraviolet (UV) illumination and documented using UVP BioDoc-IT^{TM} imaging system (UVP, Upland CA, USA). The various genogroups were determined based on the molecular weights of the amplicons (Table 3.1).

3.2.5.3.2 One-Step RT-PCR Assay

Study protocol two was a One-Step RT-PCR assay that typed the overlap junction of the regions B-C of the norovirus genome in a single reaction. Briefly, extracted RNA was reverse transcribed and amplified using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer's protocol with slight modifications. A total reaction mixture volume of 25 µl containing: 5.0 µl of 5× QIAGEN OneStep RT-PCR buffer; 1.0 µl of 10 mM dNTP mix; 1.0 µl of enzyme mix (containing both reverse transcriptase and Taq polymerase); 0.5 µl each of 50 µM forward and reverse primers; 0.5µl of 20 U/ µl RNase-Inhibitor, 11.0 µl RNase-free water and 5 µl RNA template. Cycling conditions were performed as follows: reverse transcription for 30 min at 42 °C, followed by initial PCR activation at 95 °C for 15 mins, and then 45 cycles of denaturation

at 95 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 mins. All PCR amplicons were electrophoresed on a 2.0% agarose gel (Hi-Res Standard Agarose, AGTC Bioproducts) containing ethidium bromide (Invitrogen) alongside a 100 bp molecular weight marker (Promega). The various genogroups were determined based on the molecular weights of the amplicons also described in Table 3.1. All gels were also documented as described previously above.

3.2.5.3 Norovirus Gene Sequencing

Clean PCR products, after gel electrophoresis were directly purified with a master of ExoSAP-IT (Applied Biosystems, Foster City, CA) or QIAquick® PCR Purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Positive bands were excised from gels with non-specific products and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Sequencing in both the forward and reverse directions was based on the dideoxy- chain terminator method, using the BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the same norovirus genogroup specific primers as used in both RT-PCR procedures (Table 3.1). Sequencing assays were carried out in a 10 µl reaction mix under the cycling conditions of initial denaturation at 95 °C for 2 mins followed by 25 cycles of 94 °C for 30 secs, 50 °C for 15 secs and 60 °C for 4 mins in a GeneAmp 9700 ABI thermocycler (Applied Biosystems, Santa Clara, CA, USA). To confirm the success of the cycle sequencing amplification, a control reaction consisting of 1.0µL BigDye® Terminator mix, 1.0 µM of control M13 forward primer, 2µL of pGEM 3Z (+) vector DNA was also set up to run alongside the samples. Post-sequencing purification was done using an in-house ethanol/sodium acetate precipitation method (Appendix 3.2). Purified and dried sequenced DNA products were resuspended in 10.0 µl of Hi-

Di™ Formamide (Applied Biosystems, USA), and samples were resolved on POP–7™ polymer (Applied Biosystems, USA) in an ABI 3130 automated genetic sequence analyzer platform (Applied Biosystems, Hitachi).

Table 3.1: Oligonucleotide primers used for norovirus genogroup detection in this study

Primer Sets	Primer Name	Polarity	Sequence (5'- 3')	Target Region (Position)	Amplicon Weight	Genogroup	Reference
Primer Set I	GI-SKF	+	CTGCCCGAATTYGTAAATGA	ORF2 (5342–5361)[a]	330 bp	GI	(Kojima et al., 2002)
	GI-SKR	-	CCAACCCARCCATTRTACA	5671–5653			
	GII-SKF	+	CNTGGGAGGGCGATCGCAA	ORF-2 (5046–5064)[b]	345 bp	GII	
	GII-SKR	-	CCRCCNGCATRHCCRTTRTACAT	5389 - 5367			
Primer Set II	GI-FFN	+	GGAGATCGCAATCTTCCTGCC	ORF1/ORF2 (5313 - 5330)[a]	310 bp	GI	(Armah et al., 2006a; Gallimore et al., 2005)
	GI-SKR	-	CCAACCCARCCATTRTACA	5671–5653			
	GII-FBN	+	TGGGAGGGCGATCGCAATTCT	ORF1/ORF2 (5048 - 5067)[b]	300 bp	GII	
	GII-SKR	-	CCRCCNGCATRHCCRTTRTACAT	5367- 5389			
Primer Set III	MON 432	+	TGG ACI CGY GGI CCY AAY CA	ORF1/ORF2 (5093- 5113)[a]	579 bp	GI	(Anderson et al., 2001; Cannon et al., 2017a; Kojima et al., 2002)
	GI-SKR	-	CCAACCCARCCATTRTACA	5671–5653			
	MON 431	+	TGG ACI AGR GGI CCY AAY CA	ORF1/ORF2 (5093 - 5113)[b]	570 bp	GII	
	GII-SKR	-	CCRCCNGCATRHCCRTTRTACAT	(5367- 5389)			

H: A, T or C; **R:** A or G; **N:** A, T, C or G **Y:** C, or T **I:** C > T > G **+:** Forward Primer **—:** Reverse Primer

The positions of the primers are relative to the entire genome of: [a] the GI Norwalk/68 virus (GenBank ID: M87661; NoV GI.1) and [b]GII Lordsdale virus (GenBank ID: X86557; NoV GII.4)

3.2.6 Data Analysis

3.2.6.1 Sequence analysis and Norovirus genotyping

Analysis and editing of the retrieved chromatograms were performed by BioEdit Sequence Alignment Editor v7.2.1 program (Seah et al., 1999) and Molecular Evolutionary Genetic Analysis (MEGA) v7.0.26 software package (Tamura et al., 2007). Sequences from this study were compared and aligned with sequences held in the National Center for Biotechnology (NCBI) public GenBank database using the Basic Local Alignment Search Tool (BLAST) server. Genotyping results were confirmed by using an automated online genotyping tool (v.2.0) which is available on https://www.rivm.nl/mpf/typingtool/norovirus/. The sequences characterized in this study were submitted to the GenBank database (NCBI, USA-[www.ncbi.nlm.nih.gov]).

3.2.6.2 Statistical Analysis

Norovirus prevalence was calculated using data from all the 10-year-period of surveillance (2008-2017). Rotavirus pre-vaccination years were defined as January 2008 to April 2012 and post-vaccination years as May 2012 to December 2017. Norovirus negative cases were defined as cases where neither norovirus GI nor GII strains were detected whilst mixed infections were also defined as the detection of both norovirus GI and GII strains in a single sample simultaneously. All statistical analyses were performed by using STATA version 12.1 (StataCorp LP, College Station, USA). Missing values were excluded from analyses, thus the denominators for some comparisons differ. Chi-square test was used to test for the association among variables and p-values <0.05 were considered statistically significant. Categorical variables were reported as frequencies and percentages, and continuous variables were reported as means ± standard deviations (SDs) or as medians with interquartile ranges (IQRs). The associations between demographic data, clinical

characteristics, and norovirus infections were determined by calculating odds ratios (OR) and 95% confidence intervals (CI) in logistic regression models. Age was categorized into groups: 0 - <6 months, 6 – 11 months, 12 – 18 months, 19 – 24 months, 25 – 36 months and ≥37 months; or <6 months, 6 – 24 months, and >24 months to analyze age-specific infection dynamics. Norovirus seasonal variability was determined by evaluation of monthly prevalence and calendar seasons (rainy versus dry seasons).

3.2.6.3 Vesikari Clinical Scoring System for Severity of Norovirus Infection

The severity of norovirus-associated acute gastroenteritis in infected children was evaluated by applying the Vesikari scoring system according to clinical manifestations (Ruuska & Vesikari, 1990). This system was originally developed for rotavirus-associated diarrhoea but has since been used to score the severity of norovirus-associated diarrhoea in recent studies (Wang et al., 2016; Wikswo et al., 2013). This scoring system considers the general symptoms of acute gastroenteritis including the presence of diarrhoea and its duration; vomiting episodes and duration, dehydration, fever, and hospitalizations. The seven parameters and the corresponding scores provided for each categorical level of severity are outlined in Appendix 3.3. Severity scores above 10 points (i.e., ≥11 points) were considered severe, scores between 7 and 10 moderate, and scores less than 7 considered mild.

3.3 Results

3.3.1 Demographic and Clinical Characteristics of Study Subjects

A total of 1337 diarrhoea stool samples from children <5 years were collected from the sentinel study sites from January 2008 to December 2017 (Table 3.2) and tested for noroviruses. The samples were unevenly distributed with the majority of samples (57.8%; 773/1337) coming from the Northern belt, 37.8% (506/1337) from the southern belt, and 4.3% (58/1337) from the middle belt. Slightly more males (663/1197; 55.4%) were recruited compared to females (534/1194; 44.6%). Enrolled study children were aged 2 weeks to 58 months with a median age of 12 months (IQR: 0.5-50 months). The proportion of children with diarrhoea decreased gradually with age. Children within the 6-11 months old age group recorded the most diarrhoea cases (31.9%), followed by the 12-18 months old (31.0%), and then those <6 months old (17.5%). The 0-24-month-old age group alone accounted for 90.0% of all diarrhoea cases recorded. The age distribution of diarrhoea cases is summarized in Fig. 3.2. All cases used in the study were acute gastroenteritis cases for which non-bloody stool samples were collected. The major clinical symptoms related to gastroenteritis in this study were vomiting, dehydration, and fever. Vomiting was present in 71.5% (817/1139) of the cases studied whilst 57.9% (659/1139) of the children were found with mild to severe dehydration. Vomiting (39.5%; 323/817) and severe dehydration (43.2%; 64/148) were most frequently present in children between 6 and 11 months old. The mean duration of diarrhoea and vomiting in study participants was 3.2 (SD: ±1.8) and 2.0 (SD: ±1.7) days respectively. Most children (66.9%; 759/1140) reported fever with temperatures ranging between 37.1 °C and 38.4 °C.

Table 3.2: Shows the annual distribution of study samples

Year	Frequency	Percent	Cum.
2008	231	17.28	17.28
2009	142	10.62	27.90
2010	123	9.20	37.10
2011	108	8.08	45.18
2012	48	3.59	48.77
2013	154	11.52	60.28
2014	223	16.68	76.96
2015	186	13.91	90.88
2016	66	4.94	95.81
2017	56	4.19	100
Total	**1,337**	**100**	

*In the year 2012, active surveillance slowed down following vaccine introduction

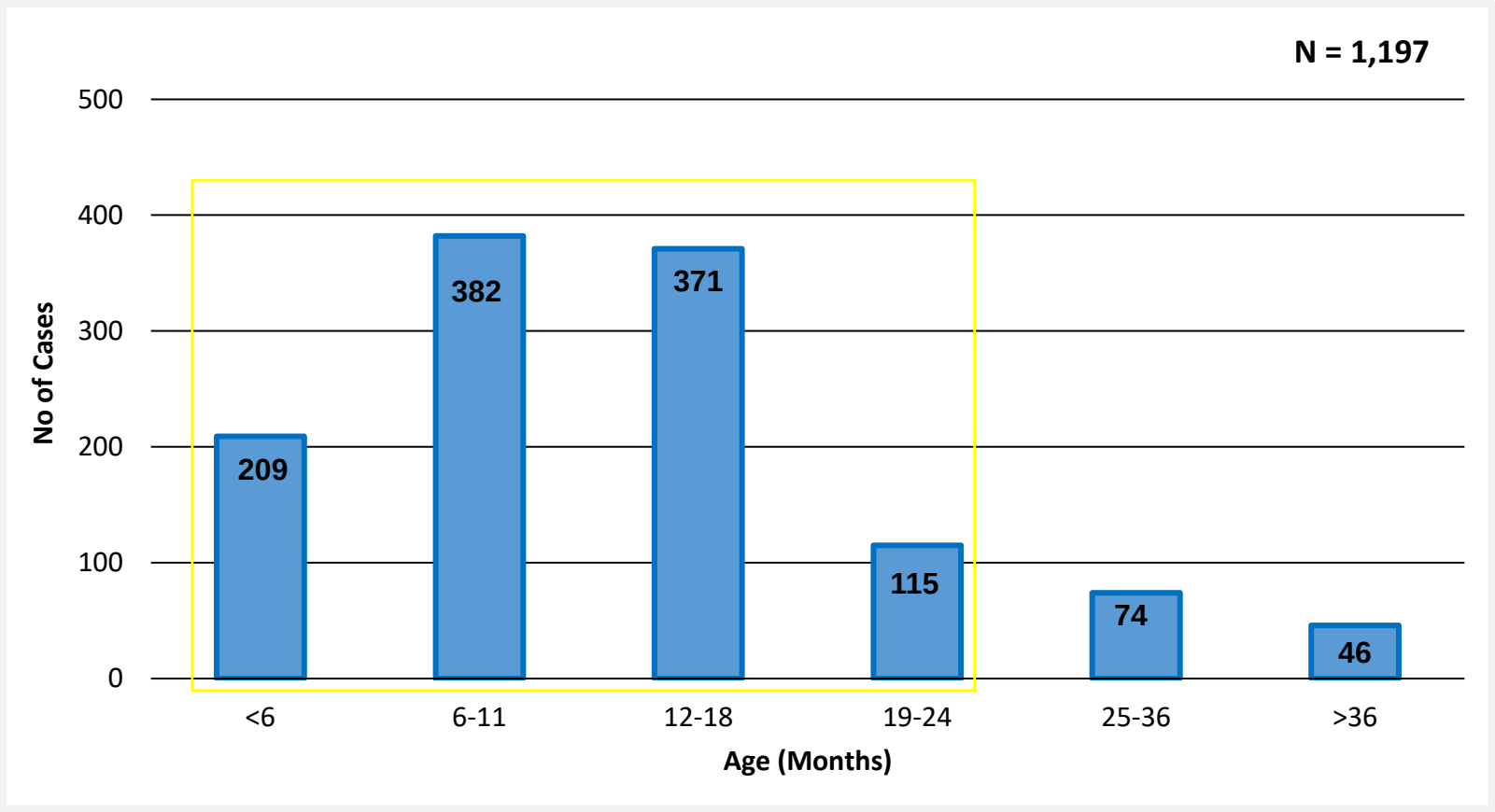
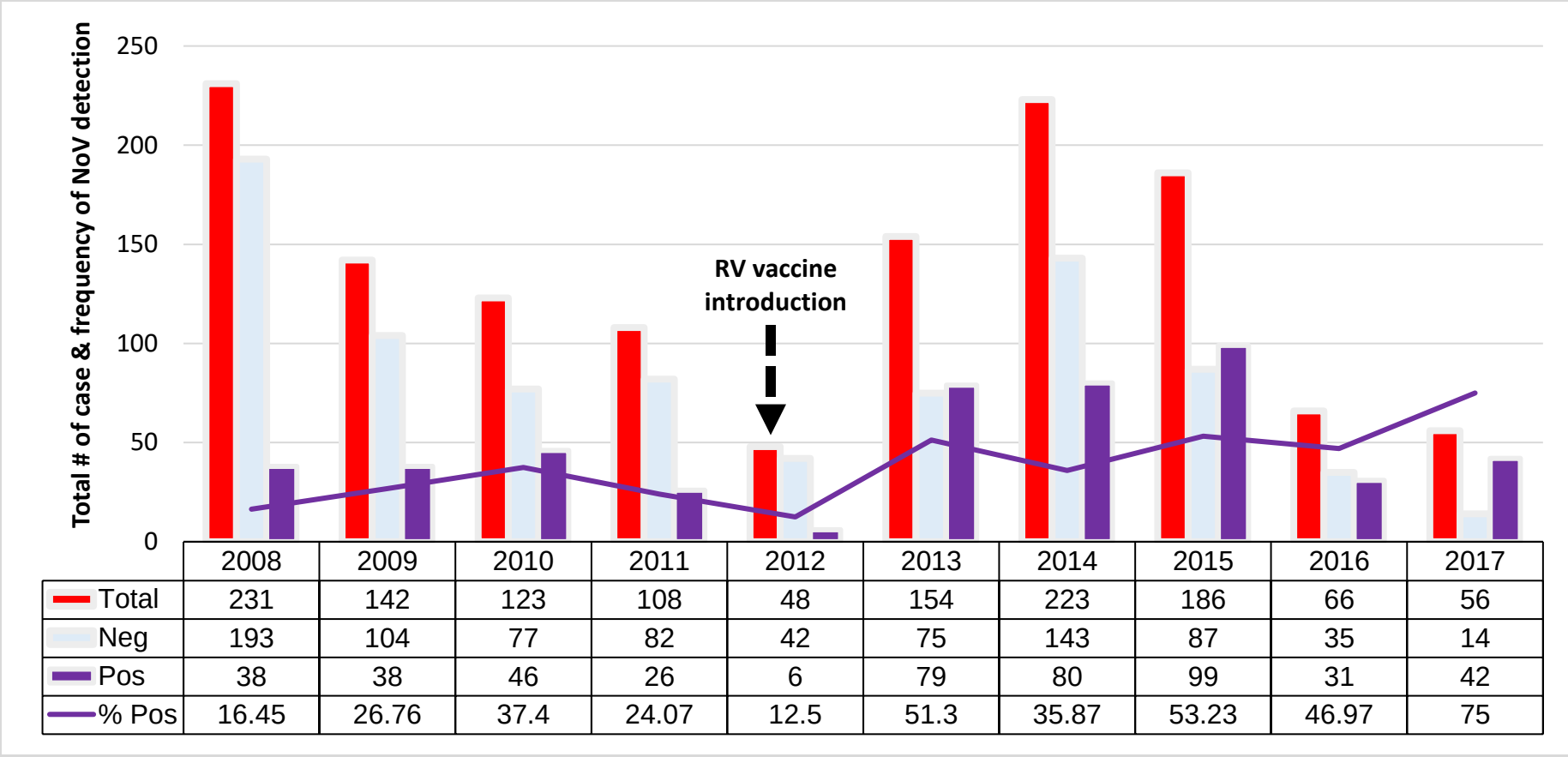


Fig. 3.2: Age distribution of diarrhoea cases recruited from 9 sites across Ghana

The yellow box highlights the age groups that are generally considered to be the most vulnerable, and who recorded the most diarrhoeal cases

3.3.2 Norovirus Detection and Epidemiology

Between 2008 and 2017, norovirus was detected in 36.2% (485/1337) of the samples tested. Norovirus positivity rate varied significantly over the years of study (ranging from 12.5% - 75.0%; P=0.000) with the year 2017 recording the highest positivity rate (Fig 3.3). The proportion of norovirus-positive cases in the pediatric population was also observed to increase significantly (Table 3.3) from 23.0% in the pre-rotavirus vaccination years to 48.3% in the post-rotavirus vaccination years. Children had a 3.0- fold higher likelihood to be positive for norovirus infection in the post-rotavirus vaccine years when compared to the pre-vaccine years (OR 3.0; 95% CI 2.391 – 3.824; P=0.000). The norovirus incidence in the Southern-Coastal plain was 2-fold higher (45.8% (232/506); OR 2.04; 95% CI 1.130 - 3.690; P=0.018) compared to the northern (30.5% (236/773); OR 1.06; 95% CI 0.590 – 1.903; P=0.846) and middle belts (29.3%; 17/58) respectively. Infection was not gender-dependent, however, the incidence of infection was found to be higher in males compared to females (37.9% vs. 36.7%, P=0.682). Noroviruses were detected across all the age groups with a median age of infection of 13.4 months (IQR: 0.5 – 52 months) but common in children under two (2) years of age (Table 3.3). Though not statistically significant, the peak of infection was in the 19-24 months age group (42.6%; OR 1.27; 95% CI 0.627 – 2.559; P=0.510).



	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Total	231	142	123	108	48	154	223	186	66	56
Neg	193	104	77	82	42	75	143	87	35	14
Pos	38	38	46	26	6	79	80	99	31	42
% Pos	16.45	26.76	37.4	24.07	12.5	51.3	35.87	53.23	46.97	75

Fig. 3.3: Yearly distribution of diarrhoea cases and norovirus infection in the Ghanaian paediatric population between January 2008 and December 2017

Detection rates significantly varied over the years (P=0.000)

Table 3.3: Analysis of demographic features associated with norovirus detection in the Ghanaian pediatric population

Parameters	Total Tested	NoV Pos (%)	NoV Neg (%)	OR (95% CI)	P-Value
All study Samples	1337	485 (36.3)	852 (63.7)	-	-
Study Period					
Pre-vaccine Years	652	154 (23.6)	498 (76.4)	Ref	
Post-Vaccine Years	685	331 (48.3)	354 (51.7)	3.02 (2.391 - 3.824)	0.000
Study Site					
Middle Belt	58	17 (29.3)	41 (70.7)	Ref	
Southern Belt	506	232 (45.8)	274 (54.2)	2.04 (1.130 - 3.690)	0.018
Northern Belt	773	236 (30.5)	537 (69.5)	1.06 (0.590 - 1.903)	0.846
Gender					
Male	663	251 (37.9)	412 (62.1)	Ref	
Female	534	196 (36.7)	338 (63.3)	0.95 (0.752 - 1.205)	0.682
*Unknown	140	38 (27.1)	102 (72.9)	-	-
Age (Months)					
>36	46	17 (37.0)	29 (63.0)	Ref	
25 - 36	74	29 (39.2)	45 (60.8)	1.10 (0.514 - 2.349)	0.807
19 - 24	115	49 (42.6)	66 (57.4)	1.27 (0.627 - 2.559)	0.510
12 - 18	371	136 (36.7)	235 (63.3)	0.99 (0.523 - 1.863)	0.968
6 - 11	382	135 (35.3)	247 (64.7)	0.93 (0.494 - 1.758)	0.829
<6	209	81 (38.8)	128 (61.2)	1.08 (0.558 - 2.089)	0.820
*Unknown	140	38 (27.1)	102 (72.9)	-	-
General Condition of Patient					
Normal	392	115 (29.3)	277 (70.7)	Ref	
Irritable	461	164 (35.6)	297 (64.4)	1.33 (0.996 – 1.776)	0.053
Drowsy and Lethargic	51	27 (52.9)	24 (47.1)	2.71 (1.500 – 4.895)	0.001
Unconscious	1	0	1 (100)	-	-

***missing data excluded from all statistical analysis; CI== Confidence Interval; OR== Odds Ratio; NoV== Norovirus

3.3.3 Clinical characteristics of norovirus infection within the study population

The clinical characteristics of the children with and without norovirus infections are summarized in Table 3.4. While the mean diarrhoeal frequency (episodes/24hr period) of norovirus infected children did not differ significantly from those without infection (5.2 ± 2.5 vs 5.5 ±2.8 respectively, P=0.05), there was a significant difference in mean diarrhoeal duration between norovirus infected and uninfected children (2.9±1.6 vs 3.3±1.7 respectively, P=0.019). Vomiting was more frequently identified in norovirus positive compared with negative children (OR 1.56; 95% CI 1.189-2.056; *P* = 0·001). Compared with uninfected children, norovirus positive children were more likely to report 2–4 vomiting episodes per day (OR 1·62; 95% CI 1·192–2·212; *P* = 0·002) and vomiting duration of up to a day (OR 1·91; 95% CI 1·282–2·847; *P* = 0·002). Norovirus infection was not significantly associated with severe dehydration (OR 1.18; 95% CI 0.798 - 1.735; P=1.176). Norovirus-infected children were however 2.7 times more likely to report with temperatures ≥ 39.0 ^{o}C (P=0.002) and 4.1 times more likely to be hospitalized (P=0.000). Children reporting norovirus-associated gastroenteritis were generally more likely to be drowsy and lethargic (OR 2.71; 95% CI 1.500 – 4.895; P=0.001) than irritable (OR 1.33; 95% CI 0.996 – 1.776; P=0.053 [Table 3.3].

Evaluation of the severity of norovirus-associated diarrhoea using the 20-point numerical score (Vesikari score) showed scores that ranged between 5-20 points. There was a significant difference between the mean severity score of norovirus infected and uninfected children (13.3±3.0 vs 12.5±3.2 respectively; P=0.005). Norovirus infection was also significantly associated with the presentation of severe gastroenteritis (V-score ≥11) in all the age groups of children assessed in the study (OR 1.88; 95% CI 1.393 - 2.529; P=0.000). There was however no significant association in the clinical severity of infection in children presenting with either norovirus genogroup I (GI)

or GII infection (GI: OR 0.90; 95% CI 0.324 - 2.506; P=0.840 vs GII: OR 0.94; 95% CI 0.401 - 2.237; P=0.902 respectively) [(Appendix 3.4)].

Table 3.4: Clinical Severity of AGE according to the norovirus infection status of study participants

	Total Tested	NoV Pos (%)	NoV Neg (%)	OR (95% CI)	P-Value
Subject Number	**1, 142**	**435**	**707**	-	-
Clinical Profile					
Diarrhoea Duration/day					
1 - 4	967	381 (39.4)	586 (60.6)	Ref	
5	73	23 (31.5)	50 (68.5)	0.71 (0.425 - 1.179)	0.184
≥6	102	31 (30.4)	71 (69.6)	0.67 (0.432 - 1.044)	0.077
Max No. of diarrhoeal stools/day					
≥6	496	166 (33.5)	330 (66.5)	Ref	
4 - 5	378	169 (44.7)	209 (55.3)	1.61 (1.220 - 2.117)	0.287
1 - 3	268	100 (37.3)	168 (62.7)	1.18 (0.868 - 1.613)	0.001
Vomiting					
Absent	325	100 (30.8)	225 (69.2)	Ref	
Present	817	335 (41.0)	482 (59.0)	1.56 (1.189 - 2.056)	0.001
vomit Duration/day					
0	321	99 (30.8)	222 (69.2)	Ref	
1	150	69 (46.0)	81 (54.0)	1.91 (1.282 - 2.847)	0.001
2	234	91 (38.9)	143 (61.1)	1.43 (1.002 - 2.033)	0.049
≥3	437	176 (40.3)	261 (59.7)	1.51 (1.115 - 2.050)	0.008
Max No. of Vomiting episodes/day					
0	324	103 (31.8)	221 (68.2)	Ref	
1	49	16 (32.7)	33 (67.3)	1.04 (0.548 - 1.975)	0.904
2 - 4	383	165 (43.1)	218 (56.9)	1.62 (1.192 - 2.212)	0.002
≥5	386	151 (39.1)	235 (60.9)	1.38 (1.010 - 1.880)	0.043
Temperature (Fever)					
37.1-38.4	759	254 (33.5)	505 (66.5)	Ref	
38.5-38.9	333	154 (46.3)	179 (53.8)	1.71 (1.315 - 2.225)	0.000
≥39.0	48	27 (56.3)	21 (43.8)	2.56 (1.417 - 4.610)	0.002
Dehydration (as assessed by the clinician)					
None	480	151 (31.5)	329 (68.54)	Ref	
Mild	238	81 (34.0)	157 (66.0)	1.11 (0.801 - 1.549)	0.523
Moderate	273	150 (55.0)	123 (45.0)	2.65 (1.950 - 3.599)	0.000
Severe	148	52 (35.1)	96 (64.9)	1.18 (0.798 - 1.735)	1.176
Treatment					
Rehydration	216	50 (23.2)	166 (76.8)	Ref	
Hospitalization	155	86 (55.5)	69 (44.5)	4.14 (2.645 - 6.474)	0.000
Rehydration/Hospitalization	770	299 (38.8)	471 (61.2)	2.11 (1.488 - 2.984)	0.000
Vesikari Score					
Non-Severe (<11)	273	75 (27.5)	198 (72.5)	Ref	
Severe (>=11)	864	359 (41.6)	505 (58.4)	1.88 (1.393 - 2.529)	0.000

*Only children with complete clinical data were included in the analysis; AGE== acute gastroenteritis

3.3.4 Norovirus Seasonality

The monthly distribution plot showed that norovirus was detected throughout the years of study (Fig 3.4) with the detection rates varying between months and years. The seasonal pattern and peaks of norovirus detection became more pronounced in the post-rotavirus vaccine years (2013-2017) compared to the pre-rotavirus vaccine years (2008-2011). Two different peaks were observed for the seasonal onset of norovirus cases; a major peak spanning from the cool dry months of October to January the following year and a minor peak beginning from the wet months of May to July of each year (Fig 3.4). In 2015, increased norovirus activity was observed to have started from August before coinciding with the major peak season in October. Prolonged norovirus activity was also observed in 2017 with the norovirus season starting from October as usual but this time ending in April the following year (2018). Apart from the increased numbers of norovirus positive cases seen in the post-rotavirus vaccine years, the seasonal patterns in the pre-and post-vaccine years were comparable.

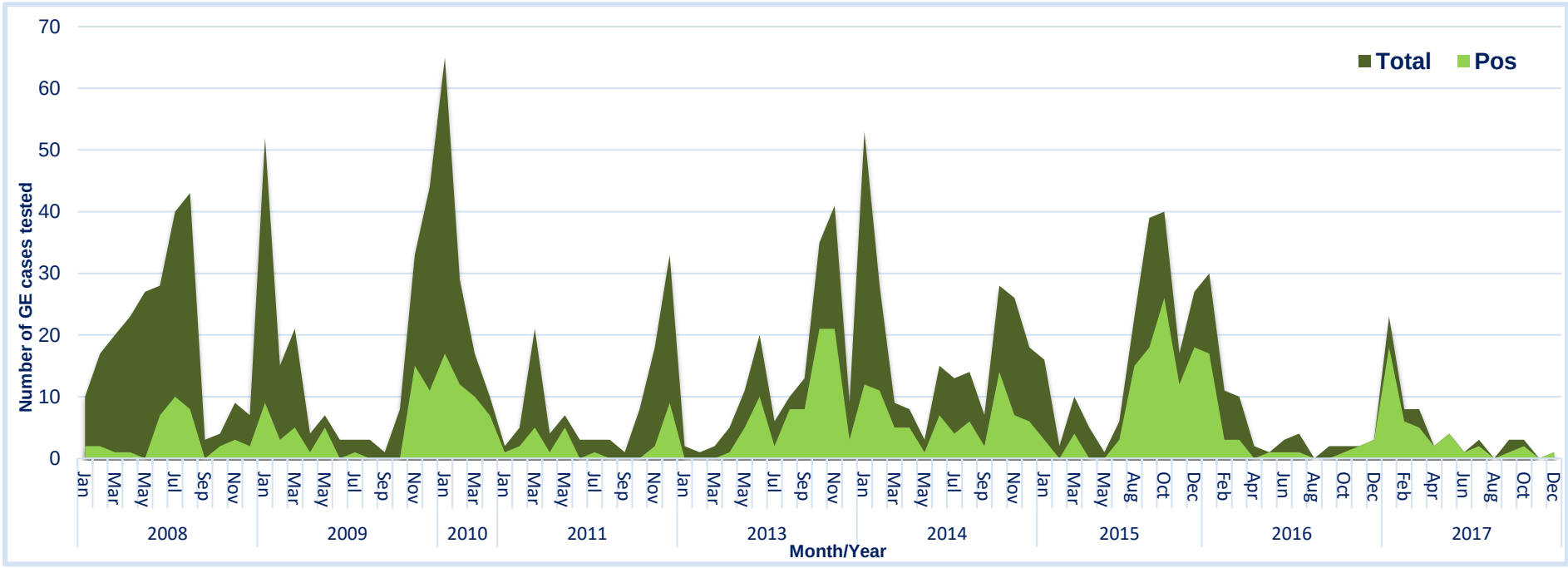


Fig 3.4: The stacked area chart represents, the total number of gastroenteritis (GE) cases tested and the number of GE cases testing norovirus-positive cases per month /year

The year 2010 shows only four months of data collection (January to April). *The graph excludes the year 2012 that had only 2 months of data collected. Filled deep green area==total number of GE cases; Filled light green area==number of norovirus positive cases

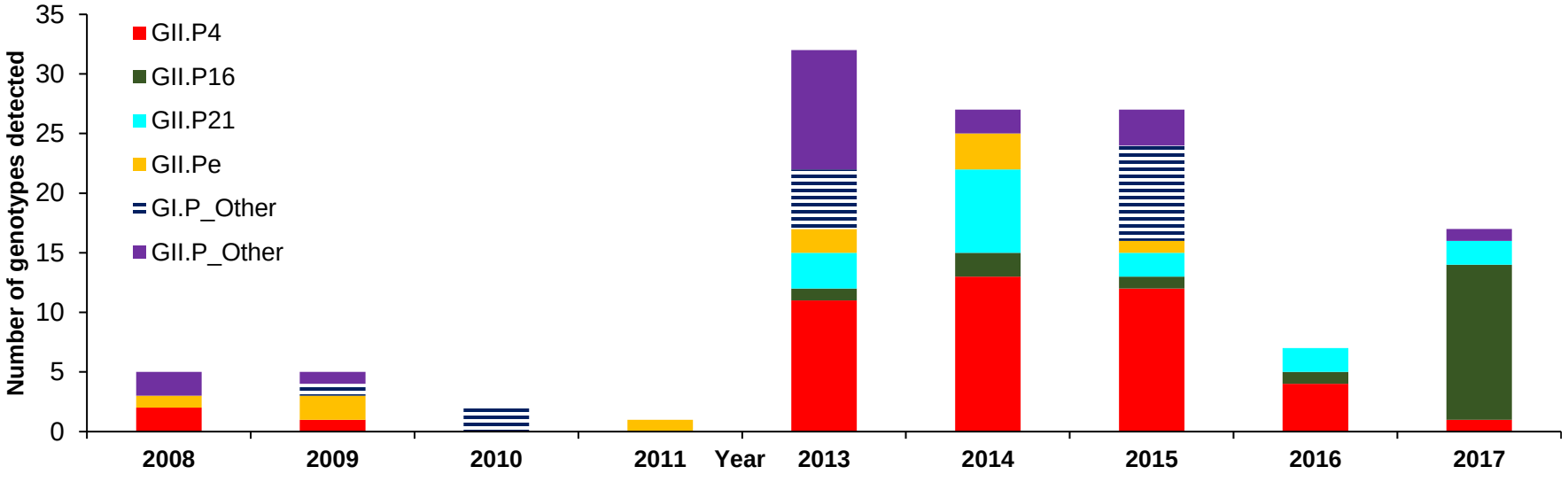
3.3.5 Distribution of polymerase and capsid genotypes of circulating norovirus strains

Out of the 485 norovirus positive isolates, 372 (76.7%) belonged to norovirus genogroup II (GII), 70 (14.4%) to genogroup I (GI) whilst 43 (8.9%) tested positive for both GI and GII. Sequence reads of the capsid and/or polymerase regions were successfully obtained for 35.9% (174/485) and 25.4% (123/485) respectively of positive samples sequenced.

Sequence analysis showed the presence of a diverse variety of both polymerase and capsid norovirus genotypes. There was a high prevalence of GII genotypes (n =25 (14 capsid and 11 polymerase genotypes) compared to the GI genotypes (n=10 (4 capsid and 6 polymerase genotypes) throughout the study period. Figures 3.5 and 3.6 show the temporal trends of polymerase and capsid genotypes for both norovirus genogroups I and II. Of the genogroup II's, GII.P4 was the most predominant (35.7%) polymerase genotype detected, followed by GII.P16 (14.6%), GII.P21 (13.0%), and GII.Pe (8.1%). Likewise, GII.4 (54.1%) was the most predominant capsid genotype detected, followed by GII.3 (7.7%), GII.6 (5.3%), and GII.17 (4.7%).

In addition, GI.P4 (37.5%) and GI.7 (31.3%) constituted the major detected polymerase and capsid genotypes of the GI genogroup respectively. Norovirus GII.P4 and GII.4 were the most consistent and commonly circulating polymerase and capsid genotypes and were detected all through the study years. The remaining proportions were a diverse mixture of 16 (64.2%) polymerase and 17 (47.1%) capsid genotypes, with some genotypes only sporadically detected in low frequency whereas others were detected more often in some years (Figures 3.5 and 3.6).

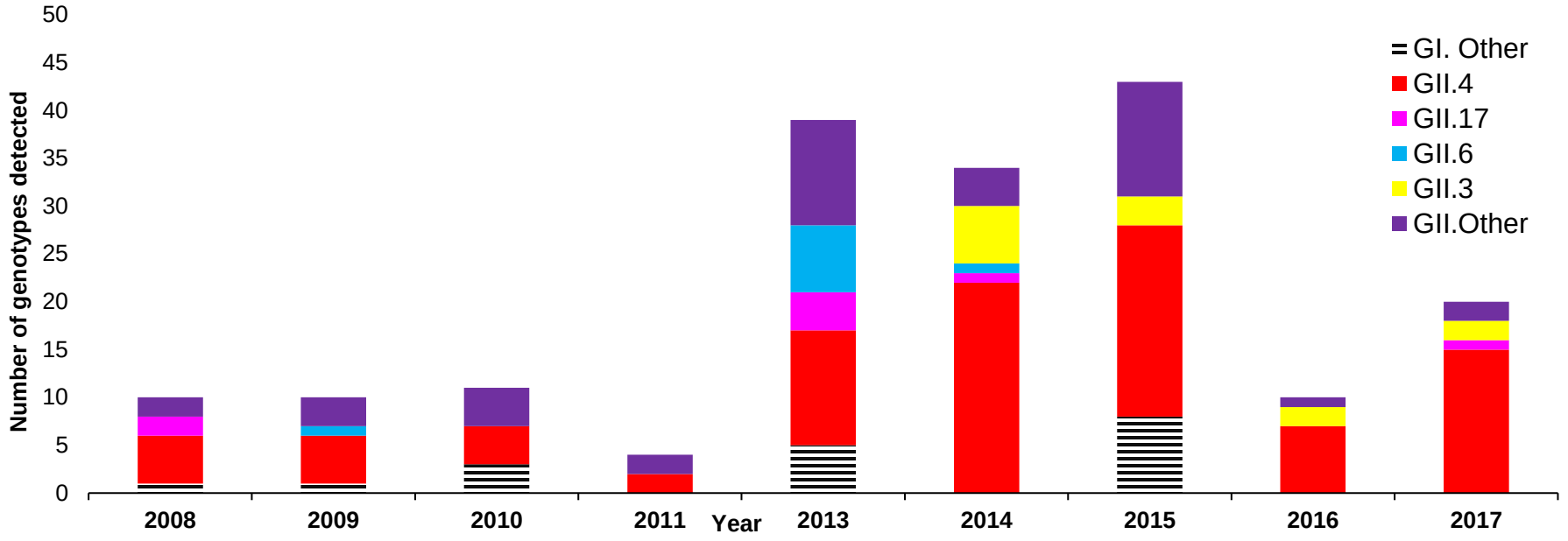
In 2017, there was a rapid increase in the number of detected GII.P16 polymerase genotype, and these co-circulated with the ever dominating GII.P4 polymerase genotype (figure 3.5). A broad spectrum of both capsid (n=14) and polymerase (n=16) GI and GII genotypes were observed in the post-rotavirus vaccine era compared to the pre-rotavirus vaccine era (capsid: n=9; polymerase: n=6) [Fig 3.6].



	2008	2009	2010	2011	2013	2014	2015	2016	2017
GII.P4*	2 (40.0)	1 (20.0)	0	0	11 (34.4)	13 (48.1)	12 (44.4)	4 (57.1)	1 (5.9)
GII.P16*	0	0	0	0	1 (3.1)	2 (7.4)	1 (3.7)	1 (14.3)	13 (76.5)
GII.P21*	0	0	0	0	3 (9.4)	7 (25.9)	2 (6.3)	2 (26.6)	2 (11.8)
GII.Pe*	1 (20.0)	2 (40.0)	0	1 (100)	2 (6.3)	3 (11.1)	1 (3.7)	0	0
GI.P1	0	0	0	0	0	0	1 (3.7)	0	0
GI.P3	0	0	0	0	1 (3.1)	0	1 (3.7)	0	0
GI.P4	0	0	0	0	0	0	6 (22.2)	0	0
GI.P7	0	1 (20.0)	2 (100)	0	2 (6.3)	0	0	0	0
GI.Pd	0	0	0	0	1 (3.1)	0	0	0	0
GI.Pf	0	0	0	0	1 (3.1)	0	0	0	0
GII.P13	0	0	0	0	1 (3.1)	0	0	0	0
GII.P17	1 (20.0)	0	0	0	1 (3.1)	1 (3.7)	0	0	0
GII.P3	0	0	0	0	1 (3.1)	0	0	0	0
GII.P7	0	1 (20.0)	0	0	5 (15.6)	1 (3.7)	1 (3.7)	0	1 (5.9)
GII.P8	0	0	0	0	2 (6.3)	0	0	0	0
GII.Pc	0	0	0	0	0	0	2 (6.3)	0	0
GII.Pg	1 (20.0)	0	0	0	0	0	0	0	0
Total	**5 (100)**	**5 (100)**	**2 (100)**	**1 (100)**	**32 (100)**	**27 (100)**	**27 (100)**	**7 (100)**	**17 (100)**

Fig 3.5: Yearly distribution of the circulating Polymerase genotypes in Ghanaian children between 2008 and 2017

The attached table have been stratified to show the four most predominant Polymerase genotypes, GI genotypes (shown in dark blue fonts) and other GII genotypes (shown in Purple fonts)



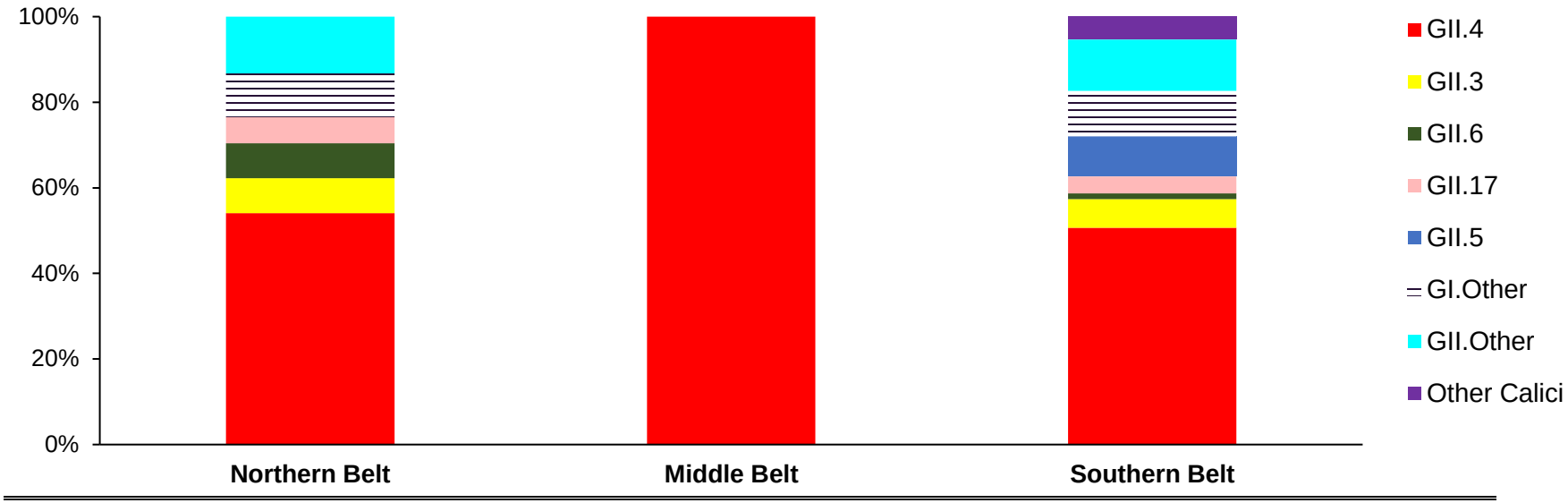
GII.4*	5 (50.0)	5 (55.6)	4 (40.0)	2 (50.0)	12 (34.2)	22 (66.7)	20 (46.5)	7 (70.0)	15 (75.0)
GII.3*	0	0	0	0	0	6 (26.1)	3 (7.0)	2 (20.0)	2 (10.0)
GII.6*	0	1 (11.1)	0	0	7 (20.0)	1 (3.0)	0	0	0
GII.17*	2 (20.0)	0	0	0	4 (11.4)	1 (3.0)	0	0	1 (5.0)
GI.1	0	0	0	0	0	0	1 (2.3)	0	0
GI.3	0	0	0	0	3 (8.6)	0	1 (2.3)	0	0
GI.4	0	0	0	0	0	0	6 (14.0)	0	0
GI.7	1 (10.0)	1 (11.1)	3 (30.0)	0	2 (5.7)	0	0	0	0
GII.1	1 (10.0)	0	0	0	0	0	0	0	0
GII.2	0	0	1 (10.0)	0	2 (5.7)	0	1 (1.3)	0	1 (5.0)
GII.5	0	0	0	0	0	0	7 (16.3)	0	0
GII.7	0	1 (11.1)	0	0	0	0	0	0	0
GII.8	0	0	0	0	2 (5.7)	0	0	0	0
GII.9	0	0	0	0	0	0	1 (2.3)	0	1 (5.0)
GII.10	0	0	0	0	0	0	1 (2.3)	0	0
GII.12	1 (10.0)	1 (11.1)	2 (20.0)	0	0	0	0	0	0
GII.13	0	0	0	2 (50.0)	0	0	0	0	0
GII.21	0	0	0	0	3 (8.6)	2 (6.1)	0	0	0
Total	**10 (100)**	**9 (100)**	**10 (100)**	**4 (100)**	**35 (100)**	**33 (100)**	**43 (100)**	**10 (100)**	**20 (100)**

Fig 3.6: Yearly distribution of the circulating Capsid genotypes in Ghanaian children between 2008 and 2017

The attached table have been stratified to show the four most predominant Capsid genotypes, GI genotypes (shown in dark blue fonts) and other GII genotypes (shown in Purple fonts)

Comparable proportions of genotype distributions were observed in the northern (72.2%; n=13/18 capsid genotypes) and southern (72.2%; n=13/18 capsid genotypes) belts with only one (7.7%; n=1/13) capsid genotype being detected in the middle belt (Fig 3.8). GI norovirus strains were only detected in the northern (GI.3, GI.7) and southern (GI.1, GI.3, GI.4) belts. Across the 3 belts, GII.4 norovirus strains were the most predominant and common cause of infection whilst GII.3 strains were the second most predominant source of infection in the southern and northern belts. Norovirus GII.5 strain was only detected in the southern belt. Figure 3.8 summarises the diversity in norovirus capsid genotypes detected across the three different belts of the country. Other presumptive calicivirus types including Nacovirus, Valovirus, Bat Sapovirus, and Chicken Calicivirus (n=1 each) were detected only in the southern belt.

Results on the characterization of the norovirus genotypes detected from each age groupings are shown in Table 3.5. More genotype diversity was observed in children aged 6-24 months (n=17/18 capsid genotypes) compared to infected children <6 months (n=10/18 capsid genotypes) and >24 months (n=4/18 capsid genotypes) of age. GII.4 and GI.4 were the only norovirus capsid genotypes detected across all the different age groupings. Norovirus GII.4 capsid genotype also represented the most predominant genotype detected across all of the age groupings, with prevalences higher than 50% in each age group. On the other hand, norovirus GII.3, GII.6 and GII.17 capsid genotypes were the second, third, and fourth highest genotypes commonly detected in norovirus positive children aged between 6 and 24 months (92.3% (12/13); 88.9% (8/9); 87.5% (7/8)) respectively. All other uncommon GI and GII genotypes were sporadically detected albeit at low prevalence in all age groups (Table 3.5).



	Northern Belt	Middle Belt	Southern Belt	
No. NoV (+)	**236 (48.7)**	**17 (3.5)**	**232 (47.8)**	**485 (100%)**
No. Seq Anal	98 (41.5)	1 (5.9)	75 (32.3)	**174 (35.9)**
GII.4	53 (57.6)	1 (1.1)	38 (41.3)	**92 (52.9)**
GII.3	8 (61.5)	0	5 (38.5)	**13 (7.5)**
GII.6	8 (88.9)	0	1 (11.1)	**9 (5.2)**
GII.17	6 (66.7)	0	3 (33.3)	**9 (5.2)**
GII.5	0	0	7 (100.0)	**7 (4.0)**
GI.Other	10 (55.6)	0	8 (44.4)	**18 (10.3)**
GII.Other	13 (59.1)	0	9 (40.9)	**22 (12.6)**
Other Calici*	0	0	4 (100.0)	**4 (2.3)**

Fig 3.7: Distribution of circulating Capsid genotypes across the three belts of the Country

The attached table shows the five most predominant circulating capsid genotypes, other detected GI and GII genotypes and other calicivirus types. **No. NoV (+)** ==number of norovirus positive samples; **No. Seq Anal**==number of genotype sequence analysed; Other Calici*==other presumptive calicivirus types identified

Table 3.5: Age-stratified norovirus genotypes detected in children with AGE in Ghana

	Age Group (Months)				
	< 6	**6-24**	**>24**	***Unknown age**	**Total**
No. Samples Pos	**81 (16.7)**	**320 (66.0)**	**46 (9.5)**	**38 (7.8)**	**485 (100)**
No. GI Pos	14 (20.6)	43 (63.2)	11 (16.2)	2 (2.9)	
No. GII Pos	60 (17.8)	248 (73.6)	29 (8.6)	35 (9.4)	372 (76.7)
No. GI/GII Pos	7 (16.7)	29 (69.1)	6 (14.3)	1 (2.3)	43 (8.9)
No. of Seq Analysed	**34 (40.5)**	**126 (39.4)**	**7 (15.2)**	**7 (18.4)**	**174 (35.9)**
GI Genotypes					**18 (10.3)**
GI.1	1 (2.9)	0	0	0	1 (0.6)
GI.3	0	4 (3.2)	0	0	4 (2.3)
GI.4	3 (8.8)	2 (1.6)	1 (14.3)	0	6 (3.4)
GI.7	2 (5.9)	4 (3.2)	0	1 (14.3)	7 (4.0)
GII Genotypes					**152 (87.4)**
GII.1	0	1 (0.8)	0	0	1 (0.6)
GII.2	1 (2.9)	4 (3.2)	0	0	5 (2.9)
GII.3	0	12 (9.5)	1 (14.3)	0	13 (7.5)
GII.4	18 (52.9)	65 (51.6)	4 (57.1)	5 (71.4)	92 (52.9)
GII.5	4 (11.8)	3 (2.4)	0	0	7 (4.0)
GII.6	1 (2.9)	8 (6.3)	0	0	9 (5.2)
GII.7	0	1 (0.8)	0	0	1 (0.6)
GII.8	0	2 (1.6)	0	0	2 (1.1)
GII.9	0	1 (0.8)	1 (14.3)	0	2 (1.1)
GII.10	0	1 (0.8)	0	0	1 (0.6)
GII.12	1 (2.9)	2 (1.6)	0	1 (14.3)	4 (2.3)
GII.13	1 (2.9)	1 (0.8)	0	0	2 (1.1)
GII.17	1 (2.9)	7 (5.6)	0	0	8 (4.6)
GII.21	0	5 (4.0)	0	0	5 (2.9)
Other Caliciviruses					**4 (2.3)**
Nacovirus*	0	1 (0.8)	0	0	1 (0.6)
Valovirus*	0	1 (0.8)	0	0	1 (0.6)
Bat Sapovirus*	0	1 (0.8)	0	0	1 (0.6)
Chicken Calicivirus*	1 (2.9)	0	0	0	1 (0.6)

No. Samples Pos==number of positive samples; **No. of Seq Analysed**==number of sequences analysed; * other presumptive calicivirus types detected; *Unknown age- not included in statistical analysis

3.3.6 Norovirus Genotype and Associated Disease Severity

Compared to the other norovirus genotypes, increased frequency of diarrhoea (≥6 stool episodes per day) and vomiting (≥5 vomiting episodes per 24 hours) was observed in more than half of the children infected with norovirus GII.3 and GII.4 strains. Compared to the other genotypes, approximately 50% of children infected with norovirus GII.4 strain showed prolonged vomiting duration of more than three (3) days. More children infected with GII.3 norovirus genotype (~50%) experienced severe dehydration compared to children infected with GII.4 and GII.17 strains who experienced mild to moderate dehydration. Patients infected with strains from the GI genogroup generally presented with less severe clinical symptoms.

Evaluation of the association of the clinical outcome in infected children with circulating predominant norovirus genotypes showed all patients infected with either norovirus GII.3, GII.17, or GII.21 strains presented with severe gastroenteritis with Vesikari scores ≥11. Moderate to severe disease (7 ≤ score ≥11) was observed in children infected with either GII.4, GII.6, GII.5 strains (Fig 3.8). Among the GI norovirus strains, all children infected with GI.3 exhibited severe diarrhoeal disease with Vesikari scores ≥ 11 whilst patients infected with GI.4 and GI.7 strains experienced moderate to severe diarrhoea with scores (7 ≤ score ≥11) [Fig 3.8; Appendix 3.4].

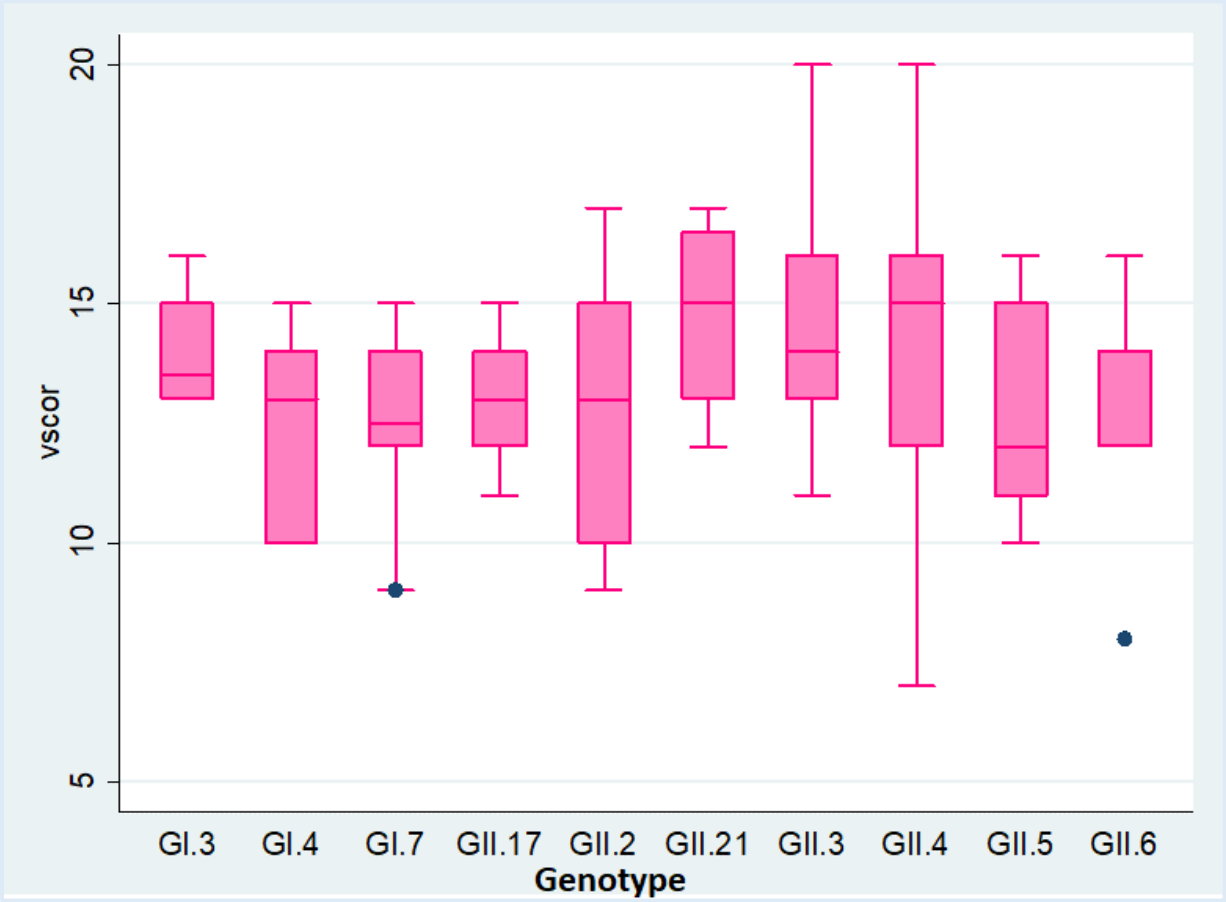


Fig 3.8: Shows the association between genotypes and clinical outcomes in norovirus positive children as measured the Vesikari severity scoring system

Vesikari score is represented by the Y-axis labelled (**VScor**); Scores: <7==mild; 7 ≤ score < 11==moderate; score ≥11==severe

3.4 Discussion

Noroviruses are increasingly being recognized as one of the more important viral agents of childhood diarrhoeal disease worldwide (Lopman et al., 2016b; Mans, 2019). To account for the still high numbers of diarrhoeal cases seen within our region even after the rotavirus vaccine introduction, this current study investigated the role played by noroviruses in the high burden of diarrhoea disease in hospitalized Ghanaian children. This study spanned a period of ten years from 2008 to 2017 and included eight (8) sentinel sites from four different geographic locations within the country.

The findings from this study showed the importance of norovirus in children under 5 years hospitalized with acute gastroenteritis post- rotavirus vaccine era and identified vomiting, fever, and diarrhoea as important clinical features associated with norovirus infection. Overall, severity scores were significantly higher for norovirus positive compared to norovirus negative gastroenteritis cases. Norovirus genotypes GII.4, GII.3, GII.17, GII.6, GII.5, and GI.7 were identified as the predominant circulating strains and were usually associated with moderate to severe acute gastroenteritis in Ghanaian children. Children ≤2 years were identified to be the most vulnerable to a host of norovirus strains.

Earlier studies in Ghana detected noroviruses in 10 -16.5% of hospitalized children less than five years (Armah et al., 2006a; Chen JC et al., 2013; Reither et al., 2007). Previous studies from few countries within sub-Saharan Africa also identified noroviruses at prevalence rates ranging from 4.6% to 32.4% (Munjita, 2015) with an average rate of 13.5% (Mans et al., 2016a). Similarly, a global systematic review and meta-analysis studies on the global prevalence of norovirus gastroenteritis conducted between 2008 and 2014 estimated norovirus to be responsible for 18% (95% CI 17–20) of all acute gastroenteritis cases in children <5 years (Ahmed et al., 2014). The

detection rate of 36.2% of noroviruses in the children in this study was higher than those previously reported. This discrepancy might be due to the varying study periods, varying study durations as well as the geographical distribution of the studied population. Also, a very rapid uptake of the rotavirus vaccines mainly due to the government policy on routine vaccine administration, leading to a decline in rotavirus-associated AGE and a subsequent increase in norovirus-associated gastroenteritis as observed (Fig 3.3).

Though norovirus-associated diarrhoeal cases were seen across all the different age groups in this study, it was observed more frequently in the children between the ages of 6-24 months (66%) when compared to those below 6 months of age (16%) and those above 24 months of age (9.5%). This finding was not surprising as children within this age group have usually lost maternal antibodies and maybe are being introduced to semisolid foods which may have been contaminated because of improper handling. Also, children within this age group have started moving about by crawling or walking, thus likely to pick anything they see into their mouth. These findings are consistent with studies from reports by Shioda (Shioda et al., 2015) and from other parts of Africa including Libya, Nigeria, and Madagascar that have also reported the high detection of noroviruses in stools of children between the ages of 6 – 24 months with gastroenteritis (Abugalia et al., 2011; Ayolabi et al., 2010; Papaventsis et al., 2007). This finding also goes to support and emphasize the suggestion that global norovirus immunization schedules (when they come in place) should be completed before 6 months of age as that would have the potential of saving about 85% of the pediatric population from severe infection.

Unlike the temperate northern hemisphere were where the seasonality of norovirus infection is well-defined peaking during the winter period, the seasonality of infection on the African continent

is less obvious (Ahmed et al., 2013; Mans et al., 2016a; Patel et al., 2009). Interestingly, in this current study except for the years 2015 and 2017 when prolonged norovirus activity was observed (Fig 3.5), norovirus seasonality in Ghana was fairly consistent and was detected in two seasonal peaks; a major peak occurring during the cool dry months of October to January and a minor peak in the wet months of May to July. Ghana has two diarrhoea seasons, the first occurring during the rainy season of June to August and the second during the dry cool months of October to February with the latter being associated with rotavirus infection (Armah et al., 2003). The peaks of norovirus activities coincided with these two diarrhoeal seasonal peaks. The observations of this study were also comparable with a study by Armah and colleagues (2006) that reported the peak of norovirus detection to coincide with the cool dry months. In some West African countries including Burkina Faso and Nigeria norovirus infections were reported to have peaked in the dry season as well (Nordgren et al., 2013; Oluwatoyin Japhet et al., 2012).

Of the two most prevalent human norovirus genogroups (G), GII was more commonly detected accounting for ~ 80% of all positive cases tested compared to the GI's which accounted for ~14% of cases. A significant difference was also observed in circulating genogroups across the different geographical study sites. Norovirus GII was seen to be predominantly in circulation across all the 3 geographical (southern, northern, and middle) belts whilst GI and mixed infections of GI/GII occurred only in the southern and northern belts of the country. The differences observed could have been due to the sampling time and sample size difference. Only a small number of samples were collected from the middle belt (n=58) compared to the northern (n=773) and southern (n=506) belts.

Though the relationship between pathogenicity, virulence, and symptoms of infection by either GI or GII norovirus strains remains speculative, our study showed both genogroups were able to cause severe infections within the pediatric population as they each caused over 80% of the severe diarrheic cases (Appendix 3.4). It has been suggested elsewhere that, of the two genogroups, the GI noroviruses which are more common in environmental samples (Gentry et al., 2009; Lee & Kim, 2008; Lysen et al., 2009) tend to cause asymptomatic infections or infections with mild clinical symptoms not requiring hospitalization (Kitajima et al., 2012). Since this study was from hospital-based surveillance that enrolled children presenting with severe diarrhoea, a lot of the GI cases would have been missed and hence the small GI infection rate was observed.

The noroviruses, similar to other RNA viruses, are naturally diverse even within genogroups. A plethora of genotypes was identified in this study; a total of 31 genotypes were detected, 17 by the polymerase gene and 14 by the capsid gene method (Fig 3.6 and 3.7). During the study period, 2008 to 2017, there was a high prevalence of GII genotypes (n=25) compared to the GI genotypes (n=10). This diversity in genotypes is a known feature of norovirus epidemiology not only in other sub-Saharan African countries but globally (Mans et al., 2016a; Parra et al., 2017b). Diversity in circulating genotypes was also observed across the different ecological regions of Ghana with the Coastal and Northern savannah ecological zones showing the highest genotype diversity. Differences in hygiene and sanitation, as well as differences in socioeconomic status across the different belts (Immurana & Urmi, 2017), could be a factor driving the norovirus genotype diversity as seen.

Overall, GII.4 (54.1%) was the most predominant genotype detected in hospitalized Ghanaian children throughout the study period. Our results are comparable with global reports in which most cases of norovirus-associated gastroenteritis were attributable to GII.4, co-circulating with other

genotypes. Additionally, norovirus genotypes GII.3 (7.7%) and GII.6 (5.6%) were the second and third most predominant genotype of gastroenteritis among our study participants. Similar findings have been reported in recent systemic reviews that have tried to account for the genotypic distribution of norovirus among the pediatric population both globally (Hoa Tran et al., 2013) and within the sub-Saharan African region (Mans et al., 2016; Mans, 2019). In the Coastal savannah ecological zone, norovirus GII.5 capsid genotype was an important source of infection and was responsible for approximately 4.0 % of infections post-rotavirus vaccine era. Among the GI's, GI.7 (38.9%), GI.4 (33.3%), and GI.3 (22.2%) were the predominantly detected capsid genotypes, and more commonly detected in the Northern savannah ecological zone. The GI capsid genotypes reported in this study are a common source of food and waterborne norovirus outbreaks as well as their isolation from environmental specimens in several countries (Inoue et al., 2016; Nenonen et al., 2012; Verhoef et al., 2015). Their presence in clinical specimens from hospitalized children could be an indication of food or waterborne norovirus transmission considering that majority of these GI genotypes were detected from the northern belt, a rural community where good hygiene and sanitation practices are relatively poor. Environmental norovirus studies are however necessary to establish the environmental transmission route of noroviruses in this community. The globally emerging norovirus GII.17 which has been a predominant genotype detected in recent times particularly in the Asian countries (de Graaf et al., 2015a; Fu et al., 2015; Jung et al., 2017; Lu et al., 2015) ranked as the fourth most common genotype causing approximately 5% of infections in our study. Other capsid genotypes that showed relatively low prevalence included GII.1 GII.2, GII.8, GII.13, GII.21, and GI.1 (Fig 3.6). The low frequency of these genotypes did not allow any analysis to study their trends within our study population.

In an attempt to evaluate the clinical implications and importance of circulating norovirus genotypes, we investigated the association between circulating norovirus genotypes and their clinical outcomes using the Vesikari severity scoring system. Infections with GII.3 norovirus genotype were common in children <24 months and were usually associated with severe dehydration whilst infections with GII.4 strains associated with prolonged vomiting duration but mild to moderate dehydration. Moreover, we observed that children infected with non-GII.4 norovirus strains (Appendix 3.3) recorded equally severe clinical illness comparable to that caused by GII.4 norovirus strains. A probable explanation for this observation might be the naivety and minimal immune state of children to these virus strains and hence their experiencing more severe symptoms upon infection for the first time in life. Only a few studies have tried to correlate norovirus genotypes to clinical features presented by infected children (Desai et al., 2012; Huhti et al., 2011; Mathew et al., 2019). Contrary to our observations, these studies suggested norovirus GII.4 genotype had greater virulence compared to other non-GII.4 genotypes. Shilu et al. however, reported on the ability of norovirus GII.3 genotype to cause severe infections in children less than a year old.

A limitation of this study was our inability to genotype all positive samples because of the cost limitations. This might cause a probable shift in the predominant circulating genotypes after all positive samples have been sequenced for genotyping.

3.5 Conclusion

This report describes the epidemiology and diversity of norovirus infecting genotypes in hospitalized children under five years of age with gastroenteritis in Ghana. The study confirmed the significant role played by noroviruses as a major cause of acute watery diarrhoea. A detection rate of 36.2 % of norovirus infection was recorded in Ghanaian children. During the 10-year surveillance period, GII.4 and GII.3 were the two most predominant norovirus strains circulating in the Ghanaian pediatric suggesting developed future vaccines would need to be multivalent and effective to protect against these two capsid genotypes and the many genotypes in circulation. Since the epidemiology of norovirus changes rapidly, the establishment of systematic surveillance within sentinel sites across the country would enhance the monitoring of circulating norovirus strains and allow us to have a continuous understanding of the current state of norovirus infection within our settings. This would then put us in a better position to support appropriate future public health intervention strategies against childhood norovirus-associated gastroenteritis. Studies enhancing our understanding of norovirus evolution and adaption to immunological pressures would also be critical for future vaccine effectiveness studies.

CHAPTER FOUR

Molecular Epidemiology of Noroviruses in Ghana

Molecular epidemiology, temporal dynamics, and evolution of norovirus associated acute gastroenteritis in the pediatric population of Ghana

Abstract

Noroviruses are a highly diverse group of diarrheagenic RNA viruses that commonly display variations in the antigenic epitopes of their capsid gene, which presents a challenge for the development of broadly protective norovirus vaccines. Monitoring the genetic diversity exhibited by the noroviruses is very important for vaccine development, understanding norovirus evolution, characterizing and documenting the changes that occur in the neutralizing epitopes of the different norovirus strains over time.

Between January 2008 and December 2017, noroviruses were found to be associated with 36.2% (485/1337) of all acute gastroenteritis cases in Ghanaian children. Further analysis of the genetic diversity of identified strains, their temporal dynamics, and evolution was conducted. Genotyping of isolated strains was performed by amplifying and sequencing segments of the viral VP1 and RNA-dependent RNA polymerase (RdRp) regions. Phylodynamic studies and evolutionary relationships were performed on the norovirus capsid genes using MEGA7.0 and BEAST software respectively.

A high genotype diversity was observed to be characterized by the circulation of both GII.4, and non-GII.4 strains. The evolution of these study norovirus strains was usually a result of intra- and intergenic recombination between the capsid and RdRp genes. A total of 25 capsid/RdRP combinations were detected with GII.4[P4] (47/174; 27.0%); GII.4[P16] (16/174; 9.2%);

GII.3[P21] (11/174; 6.3%); GII.4[P31] (8/174; 4.6%) and GII.6P[7] (8/174; 4.6%) being the most common strains. Data from this research work also suggest that most of the study norovirus strains circulated at low prevalence within the study population before their recognition as epidemic and pandemic strains associated with increased norovirus outbreaks globally

4.1 Introduction

Noroviruses are considered as agents of public health importance globally as they are associated with approximately 18% of all non-bacterial acute gastroenteritis cases worldwide (Ahmed et al., 2014). Noroviruses belong to the family *Caliciviridae* family and the genus *Norovirus.* They are non-enveloped, non-segmented, single-stranded RNA viruses. Their genome comprises ~ 7.5 Kb and contains three open reading frames (ORF1, ORF2, and ORF3) which respectively code for a large polyprotein that is proteolytically cleaved into six non-structural proteins including an RNA-dependent RNA polymerase (RdRp); a major structural (VP1) protein; and a minor structural (VP2) protein (Thorne & Goodfellow, 2014a). The major structural protein which forms the capsid protein of the virus contains important determinants of antigenicity and has been the target of several vaccine strategies (de Graaf et al., 2016b).

The mechanisms and drivers for the emergence and predominance of human norovirus strains in the population are not entirely understood. However, compared to other RNA viruses, noroviruses evolve rapidly with estimated mutation rates between 1.21×10^{-2} to 1.44×10^{-2} nucleotide substitutions/site/year (Victoria et al., 2009). In addition to this, their genome undergoes frequent recombination events especially in the ORF1 - ORF2 overlap regions, further driving viral diversity with the emergence of new variants and novel strains (Chan et al., 2017c; de Graaf et al., 2016b). A plausible hypothesis extrapolated from this observed phenomenon is that this viral diversity provides host immune escape for the noroviruses. It also increases the infectivity and efficient transmission of the viruses during human norovirus-associated acute gastroenteritis (Parra et al., 2017b).

Divergence observed in sequences of either the RdRp and/or VP1 genes has been used to classify noroviruses into genogroup, genotypes, and variants (Koopmans, 2008). Phylogenetic analysis of

noroviruses based on the viral capsid gene has shown that the noroviruses genetically cluster into seven (GI – GVII) distinct genogroups (Vinje, 2015). Strains belonging to genogroups I, II, and IV primarily infect humans, with the genogroup II strains identified as being the most predominant circulating strains globally (de Graaf et al., 2016b; van Beek et al., 2018a). Norovirus genogroups III, V, VI, and the recently proposed genogroup VII infect animals (Mesquita et al., 2010; Vinje, 2015). Norovirus genogroups I and II strains have further been classified into 9 and 22 genotypes respectively. During the past two decades, norovirus GII.P4/GII.4 strain has been of high public health importance as it has been found in a lot of studies to be associated with 70-80% of all human gastroenteritis events both in the adult and children (Bok et al., 2009; Green, 2013; Hoa Tran et al., 2013). New GII.4 variants have been found to emerge every 2-4 years replacing previous circulating GII.4 variants with an increased ability to induce gastroenteritis disease with more severe clinical symptoms (de Graaf et al., 2015a; de Graaf et al., 2015b; Debbink et al., 2013; Gustavsson et al., 2017; Lindesmith et al., 2013). Currently the GII.4 variant, the GII.4/Sydney_2012 identified in 2012–2013 norovirus season is now a globally circulating strain. In recent years, however, some previously rare genotypes have also emerged as the major cause of norovirus-associated disease in some parts of the world. During the winter season of 2014–2015 in Asia and Europe, a new GII.P17/GII.17 (GII.17 Kawasaki 2014) norovirus strain replaced the predominant GII.4, emerging as a major cause of acute gastroenteritis outbreaks (de Graaf et al., 2015a; Fu et al., 2015; Jung et al., 2017; Lindesmith et al., 2017; Lu et al., 2015). The winter seasons of 2016-2017 also saw the emergence and widespread circulation of a potential epidemic recombinant new strain GII.P16/GII.2 (Lu et al., 2017; Niendorf et al., 2017; Tohma et al., 2017).

Studies from Ghana (Armah et al., 2006a; Chen JC et al., 2013) and paper one of this thesis show a high prevalence of norovirus-associated disease in the Ghanaian pediatric population. Currently,

no licensed vaccines are available, but some vaccine candidates have advanced into clinical phase trials. The design of these vaccines has been complicated with the frequent antigenic variations that occur within the norovirus genus and present vaccines only target the most common and predominant GII.4 genotype (Mallory et al., 2019, Lucero et al., 2018). The epidemiology and prevalence of distinct genotypes are bound to differ from country to country. Therefore, studies that aim to define the genotypic variability of noroviruses are important as they help to provide more complete molecular-epidemiological data for countries allowing for a better understanding of the infection dynamics associated with these infecting strains. In this study, archived norovirus positive samples obtained from previous studies were further characterized and their molecular epidemiology is reported. The study also reports the identification of GII.4 variants and recombinants and the evolutionary dynamics of GII.4 and non-GII.4 norovirus strains.

4.2 Methods

4.2.1 Study samples and Norovirus molecular characterisation

Two hundred (200) of the four hundred and eight-five (485) norovirus positive samples previously identified from infants admitted with AGE were selected for analysis in this study. These study samples originally were collected as part of an ongoing national rotavirus surveillance program conducted between January 2008 and December 2017. Samples were selected based on the quantity and quality of the available, and to span the whole time frame of sample collection. To investigate genotype diversity, OneStep RT-PCR was performed targeting the overlap region B-C of the norovirus gene using the primers MON432/GISKR and MON431/GIISKR specific for norovirus genogroup I and II genomes respectively (Cannon et al., 2017b). Nucleotide sequences were determined directly from purified DNA amplicons using the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, CA) with the same PCR primers as the sequencing primers. The sequenced amplicons were then resolved on an ABI 3130 automated DNA sequencer (Applied Biosystems).

4.2.2 Sequence and Phylogenetic analyses

4.2.2.1 Genotyping and sequence submission

The study sequences obtained were subjected to genotyping using the online Norovirus genotyping tool v.2.0 available on http://www.rivm.nl/norovirus/typingtool (Kroneman et al., 2011). To obtain accession numbers, the sequences of the successfully genotyped strains were submitted to the GenBank database (National Center for Biotechnology Information, USA-[www.ncbi.nlm.nih.gov]).

4.2.2.2 Phylogenetic Analysis

To build a suitable multiple sequence alignment file for phylogenetic analysis, the sequences of the successfully genotyped strains were subjected to a search in the GenBank using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Similar sequences were downloaded for each sequence query and included in the dataset for the phylogenetic analysis. Multiple sequence alignment files were generated using the online version of Multiple Alignment using Fast Fourier Transform (MAFFT version 7; https://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2017; Katoh & Standley, 2013). For each genotype dataset, the best fit nucleotide substitution model was selected based on the lowest Bayesian Information Criterion value upon model testing in the MEGA v.6.06 software package. Maximum likelihood phylogenetic trees were constructed using the best fit models with 1000 bootstrap replicate trials. The region of sequences included for the phylogenetic analysis was the partial sequences of the overlap region of the ORF1-ORF2 of the norovirus genome.

4.2.2.3 Bayesian Evolutionary Analysis by Sampling Trees (BEAST)

Bayesian evolutionary analysis by sampling trees was performed on the datasets generated for each genotype i.e. GII.3, GII.4, GII.6, and GII.17 to determine the time of the most recent common ancestor of the Ghanaian strains as well as to determine the evolutionary rate of these sequences. The same dataset used in the maximum likelihood phylogenetic trees were maintained in this analysis. These datasets comprised of a global collection of the respective GII genotypes i.e. Forty GII.3 taxa collected between 2000 and 2017; seventy-seven GII.4 taxa collected from 1974-2017; thirty-seven GII.6 collected from 1971-2018 and seventeen GII.17 taxa collected from 1978 to

2015. The Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST v1.8.1 (Drummond et al., 2012a) was employed. An uncorrelated lognormal relaxed-clock model (Drummond et al., 2006), a coalescent constant size tree (Drummond et al., 2002) together with the best-fit nucleotide substitution models (based on Bayesian Information Criterion) were used. The MCMC calculations were carried out with a 10% burn-in and the effective sampling sizes (ESS) were monitored for each continuous parameter using the Tracer v.1.6 software ((http://tree.bio.ed.ac.uk/software/tracer/) until convergence was reached. It was observed that convergence was reached (ESS ≥200) for the datasets at MCMC chains ranging from 15 million (genotype GII.17) generations to 50 million generations (genotype GII.4). The BEAGLE library was used to enhance the computational speed of the analysis (Suchard & Rambaut, 2009). Maximum clade credibility trees were annotated with the TreeAnnotator software v1.8.1 and edited in FigTree.

4.3 Results

4.3.1 Temporal distribution of circulating norovirus strains (2008 – 2017)

Of the 200 samples tested, 170 (85.0%) were successfully assigned to human norovirus capsid and/or the polymerase genotypes and four samples to other non-human calicivirus types - namely Bat sapovirus, chicken calicivirus, Nacovirus, and Valovirus as indicated in Table 4.1. A total of 88.0% (152/174) samples characterized were as GII noroviruses while 10.9% (18/174) were GI noroviruses. Approximately 71% (123/174) of the sequenced samples genotyped for both the capsid and polymerase genes whilst 27.0% (47/174) genotyped for only the capsid region. Overall 20 different norovirus GII strains (genotype combinations) were obtained, of which GII.4[P4] constituted 27.0%, GII.4[P16] (9.2%), GII.3[P21] (6.3), GII.4[P31] (4.6%) and GII.6[P7] (4.6%) (Table 4.1). The norovirus strains detected were GI.4[P4] (26.3%), GI.7[P7] (21.1%), GI.3[P3] (10.5%). No co-infection of different norovirus genotypes was observed in any of these samples.

GII.4 Strains

The temporal strain distribution revealed that three different GII.4 norovirus genotypes namely: GII.4[P4], GII.4[P16], and GII.4[P31] co-circulated during the study period (2008-2017) in Ghana albeit at varying rates of 27.0%, 9.2%, and 4.6%, respectively (Table 4.2). Except for 2010 and 2011 where no GII.4[P4] strains were detected, the proportions of the predominant GII.4[P4] norovirus strain varied from as high as 42.4 % to as low as 5.0 % over the study period. In the year 2017 however, the recombinant GII.4[P16] became the predominant circulating strain accounting for 65.0% of norovirus-associated gastroenteritis while the prevalence of GII.4[P4] declined from 40.0% to 5.0% (Table 4.2).

Table 4.1 Genotypes of noroviruses isolated from 174 positive stool samples obtained from Ghanaian Children with AGE between 2008 and 2017

GI genotypes:		GII genotypes:		*Other*		
Cap/Pol	**No of cases (%)**	**Cap/Pol**	**No of cases (%)**	***Caliciviruses***	**No of cases (%)**	**Total**
GI.4[P4]	5 (2.9)	GII.4[P4]	45 (25.9)	Bat Sapovirus	1 (0.6)	
GI.7[P7]	4 (2.3)	GII.4[P16]	16 (9.2)	Chicken Calicivirus	1 (0.6)	
GI.3[P3]	2 (1.1)	GII.3[P21]	11 (6.3)	Nacovirus	1 (0.6)	
GI.1[P1]	1 (0.6)	GII.4[P31]	8 (4.6)	Valovirus	1 (0.6)	
GI.3[P13]	1 (0.6)	GII.6[P7]	7 (4.0)			
GI.3[P14]	1 (0.6)	GII.21[P21]	5 (2.9)			
		GII.17[P17]	3 (1.7)			
		GII.8[P8]	2 (1.1)			
		GII.9[P7]	2 (1.1)			
		GII.1[P33]	1 (0.6)			
		GII.2[P30]	1 (0.6)			
		GII.2[P31]	1 (0.6)			
		GII.3[P16]	1 (0.6)			
		GII.3[P30]	1 (0.6)			
		GII.4[P7]	1 (0.6)			
		GII.5[P16]	1 (0.6)			
		GII.17[P3]	1 (0.6)			
		GII.17[P13]	1 (0.6)			
		GII.17[P31]	1 (0.6)			
	14 (77.8)		**109 (71.7)**			**123 (70.7)**
Cap/Pol[nd]		**Cap/Pol[nd]**				
GI.7[Pnd]	3 (1.7)	GII.4[Pnd]	22 (12.6)			
GI.4[Pnd]	1 (0.6)	GII.5[Pnd]	6 (3.4)			
		GII.12[Pnd]	4 (2.3)			
		GII.2[Pnd]	3 (1.7)			
		GII.13[Pnd]	2 (1.1)			
		GII.17[Pnd]	2 (1.1)			
		GII.6[Pnd]	1 (0.6)			
		GII.7[Pnd]	1 (0.6)			
		GII.10[Pnd]	1 (0.6)			
		GII.16[Pnd]	1 (0.6)			
	4 (22.2)		**43 (28.3)**			**47 (27.0)**
Total	**18 (10.3)**		**152 (87.4)**		**4 (2.3)**	**174 (100)**

Cap: Capsid gene genotype; **Pol:** Polymerase gene genotype; **[P_ND]:** Polymerase genotyped not determined; **Other Caliciviruses:** other presumptive calicivirus types identified

GII.3, GII.6, and GII.5 strains

The third most prevalent strain GII.3[P21] appeared in 2014 and remained relatively stable over the study period at a rate of 6.3%. The fourth most common strain GII.6 [P7] was first detected in 2009 (11.1%), then in 2103 (17.1%) and 2014 (3.0%) whereas the fifth most common strain GII.5 [PNA] emerged in 2015 with a prevalence of 14.0% (Table 4.2).

Recombinant norovirus strains

The study invested inter-genotype recombination among circulating strains by sequencing and genotyping of the partial polymerase (ORF1) and capsid (ORF2) overlap regions, a known hotspot for recombination (Kroneman et al., 2013).

Overall 33.5% (57/170) of the sequenced samples were recombinant strains, of which GII.4[P16] accounting for 28.1% (n=16/57), were the most predominant inter-genotypic recombinant strains. In addition, GII.3[P21] were the second most prevalent (19.3%; n=11) followed by GII.4[P31] (14.0%; n = 8) and GII.6[P7] (12.3%; n =7).

Other recombinant viruses identified included: GII.9[P7] (2 cases); GI.3[P13], GI.3[P14], GII.1[P33], GII.2[P30], GII.2P[31], GII.3[P16], GII.3[P30], GII.4[P7], GII.5[P16], GII.17[P3], GII.17[P13] and GII.17[P31] (1 case each) [Table 4.1]. The remaining 118 (67.8%) norovirus strains were identified as wild-type viruses with identical genotypes for both polymerase and capsid sequences. Furthermore, 17 (68.0%) of the 25 strains (genotype combinations; Table 4.1) identified in this study were inter-genotypic recombinant strains possessing discordant polymerase and capsid genotypes.

Table 4.2 Trends in circulating norovirus strains (2008 to 2017)

	Number (%)									
Genotype	**2008**	**2009**	**2010**	**2011**	**2013**	**2014**	**2015**	**2016**	**2017**	**Total**
GII.3[P21]	0	0	0	0	0	5 (15.1)	2 (4.7)	2 (20.0)	2 (10.0)	**11 (6.3)**
GII.4[P4]	2 (20.0)	1 (11.1)	0	0	11 (31.4)	14 (42.4)	14 (35.6)	4 (40.0)	1 (5.0)	**47 (27.0)**
GII.4[P16]	0	0	0	0	1 (2.9)	1 (3.0)	0	1 (10.0)	13 (65.0)	**16 (9.2)**
GII.4[P31]	1 (10.0)	2 (22.2)	0	1 (25.0)	0	3 (9.1)	1 (2.3)	0	0	**8 (4.6)**
GII.4[P_ND]	2 (20.0)	2 (22.2)	3 (30.0)	1 (25.0)	0	4 (12.1)	6 (14.0)	2 (20.0)	1 (5.0)	**21 (12.1)**
GII.5[P_ND]	0	0	0	0	0	0	6 (14.0)	0	0	**6 (3.4)**
GII.6[P7]	0	1 (11.1)	0	0	6 (17.1)	1 (3.0)	0	0	0	**8 (4.6)**
GII.17[P17]	1 (10.0)	0	0	0	4 (11.4)	1(3.0)	0	0	0	**6 (3.4)**
GII.21[P21]	0	0	0	0	3 (8.6)	2 (6.1)	0	0	0	**5 (2.9)**
Others	4 (40.0)	3 (33.3)	7 (70.0)	2 (50.0)	16 (45.7)	2 (6.1)	14 (32.6)	3 (30.0)	3 (15.0)	**54 (31.0)**
Total	**10 (5.8)**	**9 (5.2)**	**10 (5.7)**	**4 (2.3)**	**35 (20.1)**	**33 (19.0)**	**43 (24.7)**	**10 (5.8)**	**20 (11.5)**	**174 (100.0)**

[P_ND]: Polymerase gene genotype not determined; **Others:** other detected GI and GII genotypes

4.3.2 Genetic analysis of the major circulating Ghanaian norovirus strains

4.3.2.1 Circulating GII.4 Variants

Based on capsid genotyping, seven (7) GII.4 variants were identified during the study period (Table 4.3). At least two of these GII.4 variants co-circulated each year albeit at varying frequencies. The capsid GII.4_Sydney 2012 variant circulated all through the surveillance period and remained the most predominant GII.4 variant detected. It is however worth noting that no single GII.4 variant strain circulated nor remained predominant through the study period when variants were analyzed. Figure 4.1 provides detailed results of both the capsid and polymerase genotypes of circulating GII.4 variant strains. GII.4_Sydney 2012[P31] variant was the predominant variant detected from 2008-2011 and 2013-2015. This variant co-circulated with Yerseke_2006b, Cairo_2007, and Den Haag_2006b GII.4 variants between 2008 and 2011 and also with GII.4_Sydney 2012[P4_New Orleans 2009] variant from 2013-2015. In 2017 however, these variants were replaced and predominated by the recently emerging recombinant GII.4_Sydney 2012[P16] variant (Fig 4.1).

Table 4.3: Yearly distribution of detected norovirus GII.4 variants (2008 -2017)

	Year of GII.4 variant detection								
Capsid Genotype	**2008**	**2009**	**2011**	**2013**	**2014**	**2015**	**2016**	**2017**	**Total**
GII.4_Asia 2003	0	0	0	1 (8.3)	0	0	0	0	**1 (1.1)**
GII.4_Cairo 2007	2 (33.3)	0	0	0	1 (4.2)	1 (5.3)	0	0	**4 (4.6)**
GII.4_Den Haag 2006b	0	1 (20.0)	0	0	2 (8.3)	0	1 (16.7)	0	**4 (4.6)**
GII.4_Farmington Hills 2002	0	0	0	1 (8.3)	2 (8.3)	0	0	0	**3 (3.4)**
GII.4_New Orleans 2009	0	0	0	0	1 (4.2)	0	0	0	**1 (1.1)**
GII.4_Sydney 2012	3 (50.0)	2 (40.0)	1 (100)	10 (83.3)	17 (70.8)	16 (84.2)	5 (83.3)	9 (64.3)	**63 (72.4)**
GII.4_Yerseke 2006b	1 (16.7)	2 (40.0)	0	0	0	0	0	0	**3 (3.4)**
GII.4_na	0	0	0	0	1 (4.2)	2 (10.5)	0	5 (35.7)	**8 (9.2)**
Total	**6 (6.8)**	**5 (5.7)**	**1 (1.1)**	**12 (13.8)**	**24 (27.6)**	**19 (21.8)**	**6 (6.8)**	**14(16.1)**	**87 (100)**

GII.4_na==capsid genotype not assigned a variant type

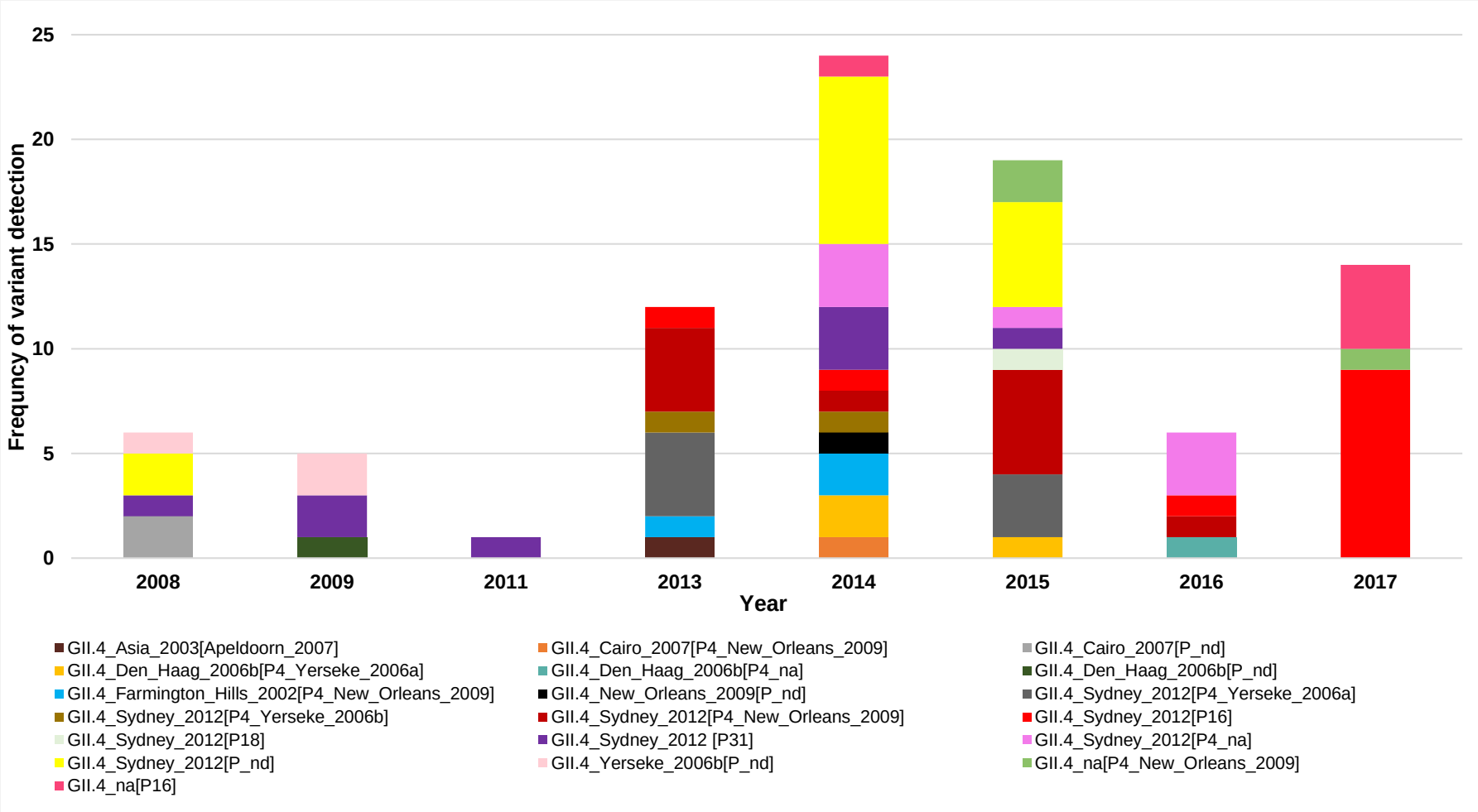


Fig 4.1: Temporal dynamics in the distribution of norovirus GII.4 variants in the Ghanaian pediatric population from 2008 to 2017

Circulating GII.4 variants are represented in this study by different colors as shown in the legend

P_nd==Polymerase gene not determined/genotyped; **GII.4_na** and/or **P4_na**==capsid and/or polymerase genotype not assigned a variant type

4.3.2.2 Sequence and Phylogenetic Analysis of GII.4

Sequence Comparison

Study strains detected in 2017 and belonging to the GII.4_Sydney 2012[P16] cluster were highly identical with nucleotide sequence identity ranging from 99.0%-100%. Contrastingly, GII.4_Sydney 2012[P16] study strains detected in different years (2013 – 2014; 2017) shared nucleotide identities ranging from as low as 87.6% - 100%. Similarly, GII.4 study strains detected between 2014 and 2015 and belonging to the GII.4_Sydney 2012[P4_New Orleans 2009] cluster were distantly related to each other with sequence identities ranging from 83.8% to 100%.

Maximum Likelihood Phylogenetic Analysis

The evolutionary history of the Ghanaian GII.4_Sydney 2012 variants was inferred using Maximum Likelihood phylogeny based on the partial VP1 genes. The Ghanaian GII.4 strains segregated into two clusters designated cluster 1 and cluster 2 (Suchard et al., 2018). Cluster 1 contained the GII.4_Sydney 2012[P16] strains detected between 2013 and 2017 together with the Japanese strain OH16002 detected in Osaka city in 2016. Cluster 2 on the other hand contained Ghanaian GII.4_Sydney 2012[P4_New Orleans 2009] strains detected in 2013 – 2016 together with the GII.4_Sydney 2012[P4_New Orleans 2009]/Ellsworth 7118 and Pima0257 strains detected in the USA in 2014 and earlier GII.4_Sydney 2012 variants detected from Africa (Cameroon) and the USA circulating between 2011 and 2015 (Fig 4.2a).

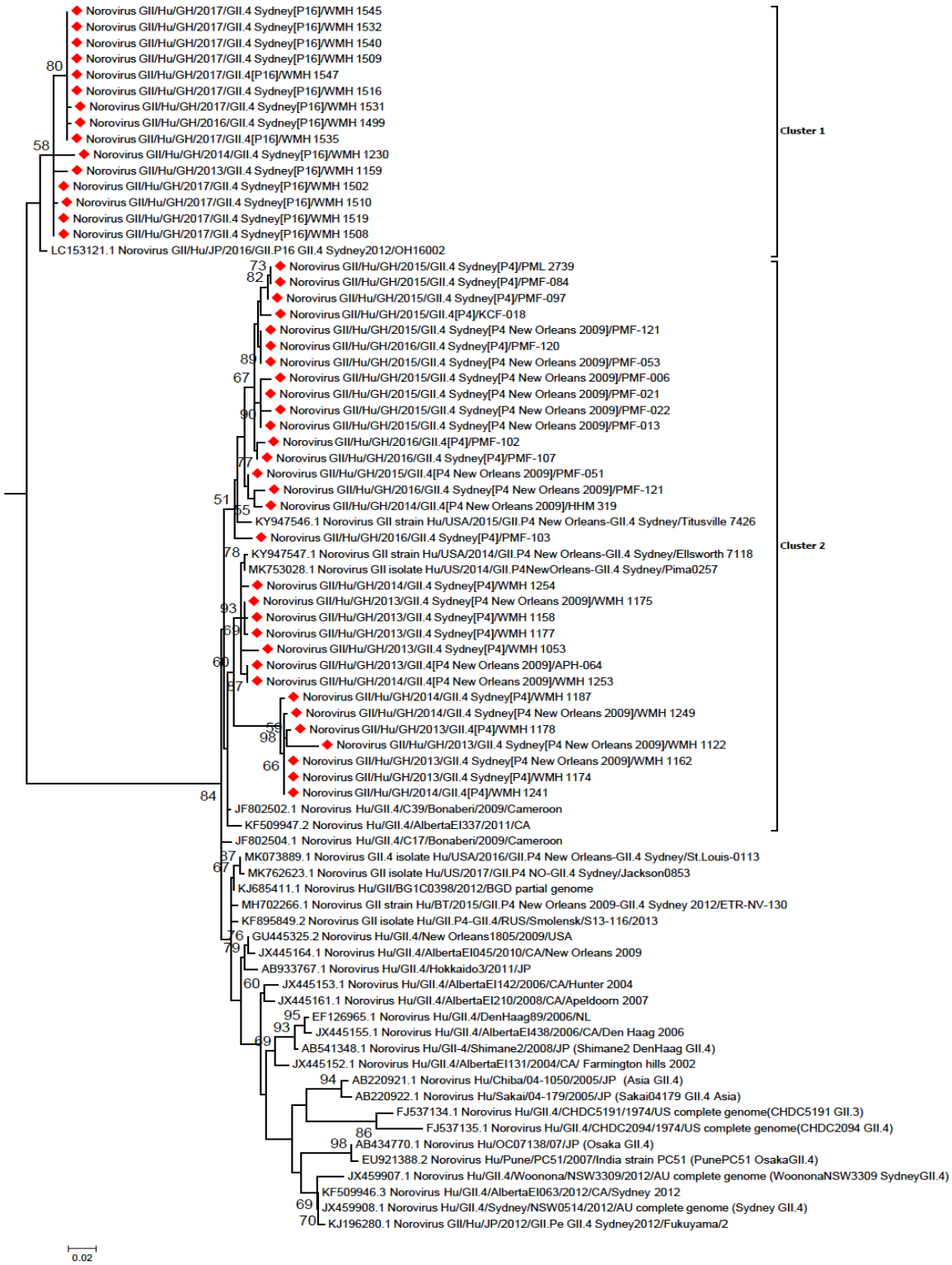


Fig 4.2a: Phylogenetic tree of the partial VP1 gene sequences of GII.4 norovirus strains, showing the genetic relationship between Ghanaian study GII.4 strains and other globally circulating GII.4 strains

Strains characterized in this study are indicated with red squares or diamonds. Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model with a discrete gamma distribution in the mega 6 software package. Significant bootstrap values (1000 replicates) of ≥ 70 % are indicated at each node. The outgroup sequences were excluded from the trees. Bar indicates genetic distance expressed as number of nucleotide substitutions per site.

Determination of the time of the most recent common ancestor of Ghanaian GII.4 strains

The time of the most recent common ancestor of the GII.4 variants (Fig 4.2b) was determined using the BEAST program (Drummond et al., 2012b; Suchard et al., 2018). It was noted that the tMRCA of the Ghanaian GII.4 variants in cluster 1 was 2012 (95% Highest Posterior Density interval: 2011- 2013) whereas that of stains in cluster 2 was 2009 (95% Highest Posterior Density interval: 2007- 2012). Within cluster 2 however, there were sub-clusters of Ghanaian GII.4 variants that had tMRCAs ranging from 2011 to 2013 (Fig. 4.2b). The mean evolutionary rate of the GII.4 dataset was determined to be 5.3×10^{-3} substitutions/site/year (95% Highest Posterior Density interval: $3.7 \times 10^{-3} – 7.2 \times 10^{-3}$). These varying tMRCAs determined for the two major clusters together with the evolutionary rate lend support to a hypothesis that there is a continuous diversification of the GII.4 variants with varied GII.4 strains emerging at different time points during the study period.

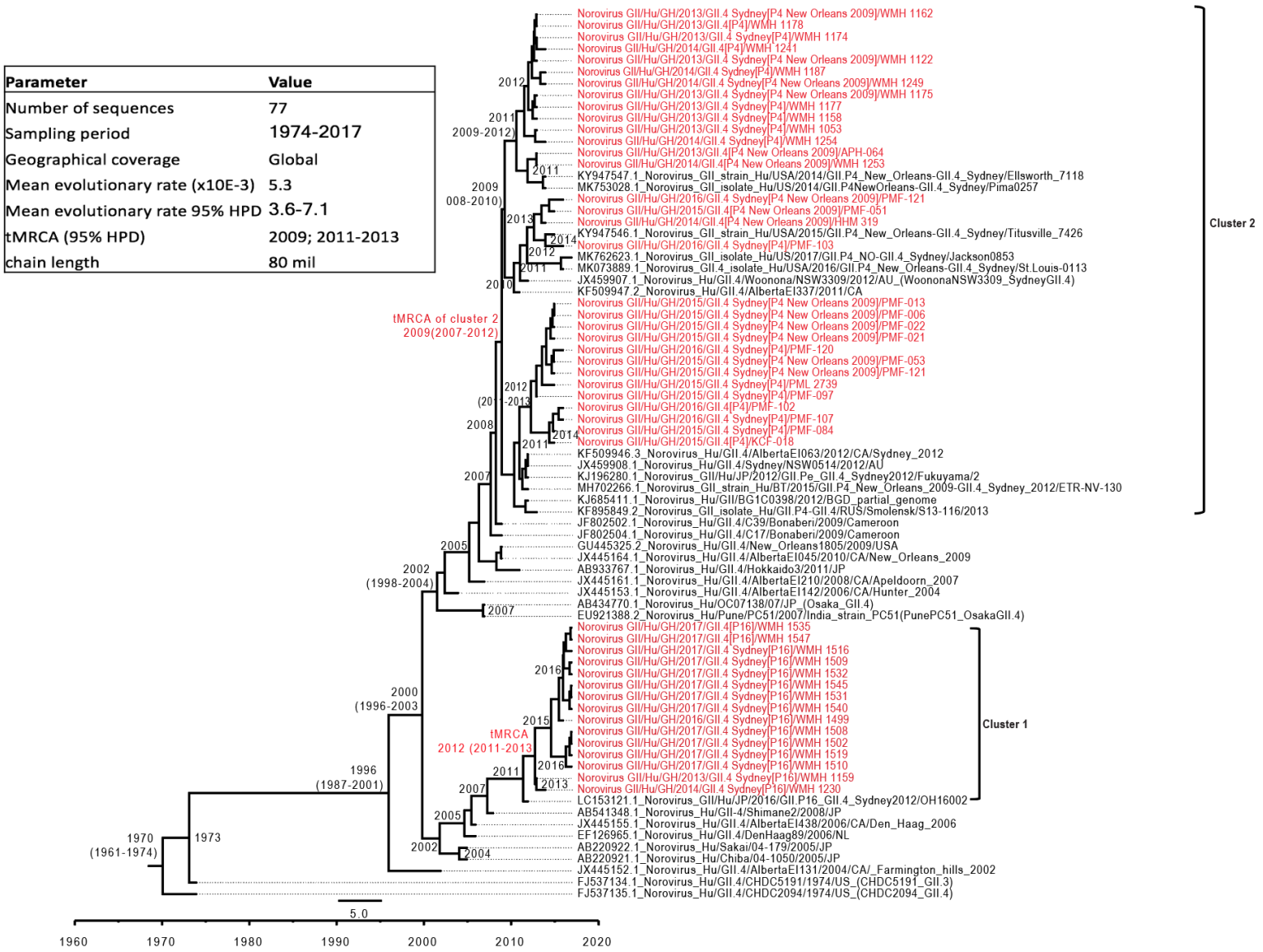


Fig 4.2b: Evolutionary analysis of Ghanaian GII.4 noroviruses

Maximum clade credibility (MCC) tree of 77 dated GII.4 partial VP1 nucleotide sequences reconstructed in the Bayesian MCMC framework. Bar indicates genetic distance expressed as number of nucleotide substitutions per site. The time of the most common recent ancestor (tMRCA) is indicated in red faced fonts for each cluster. Study strains are also indicated in red faced fonts while variants are identified by names. The MCC tree was constructed using the BEAST package and visualized in FigTree v1.4.3.

4.3.3 Sequence and Phylogenetic Analysis of GII.3 Strains

GII.3 study strains

The GII.3 study strains were all detected two years into the post-rotavirus vaccine years (2014-2017). Interestingly, all the Ghanaian GII.3 strains detected were recombinants possessing the discordant [P21] polymerase genotype and accounted for 6.3% (n=11) of gastroenteritis cases in the pediatric wards at the time of the study (2008-2017).

Sequence Comparison

The partial capsid gene sequences of the Ghanaian GII.3 study strains isolated during the different study years were distantly related with sequence identities ranging from 78.0% to 99.6%. Study strains detected within the same year were however closely related (94.3% to 100%) except for GII.3 strains detected in 2014 that differed by 14.3% in sequence homology.

Maximum Likelihood Phylogenetic Analysis

Phylogenetically, the study GII.3 strains formed a monophyletic cluster with the Indian strain (Nov/Hu/V1714) detected in 2007 (Fig 4.3a) but shared low sequence identities ranging from 74.3% to 90.4%.

Determination of the time of the most recent common ancestor of Ghanaian GII.3 strains

Using BEAST, the time of the most recent common ancestor of the GII.3 strains was determined. The time tMRCA of the Ghanaian GII.3 strains was estimated to be 2010 (95% HPD interval: 2007-2012) (Fig. 4.4b). The Ghanaian GII.3 cluster shared a common ancestor with an Indian strain Hu/V1714/07/IND in 2006 (95% HPD interval: 2004-2007). The mean evolutionary rate of the GII.3 dataset was determined to be 6.3×10^{-3} substitutions/site/year (95% HPD interval: $3.6 \times 10^{-3} – 9.2 \times 10^{-3}$).

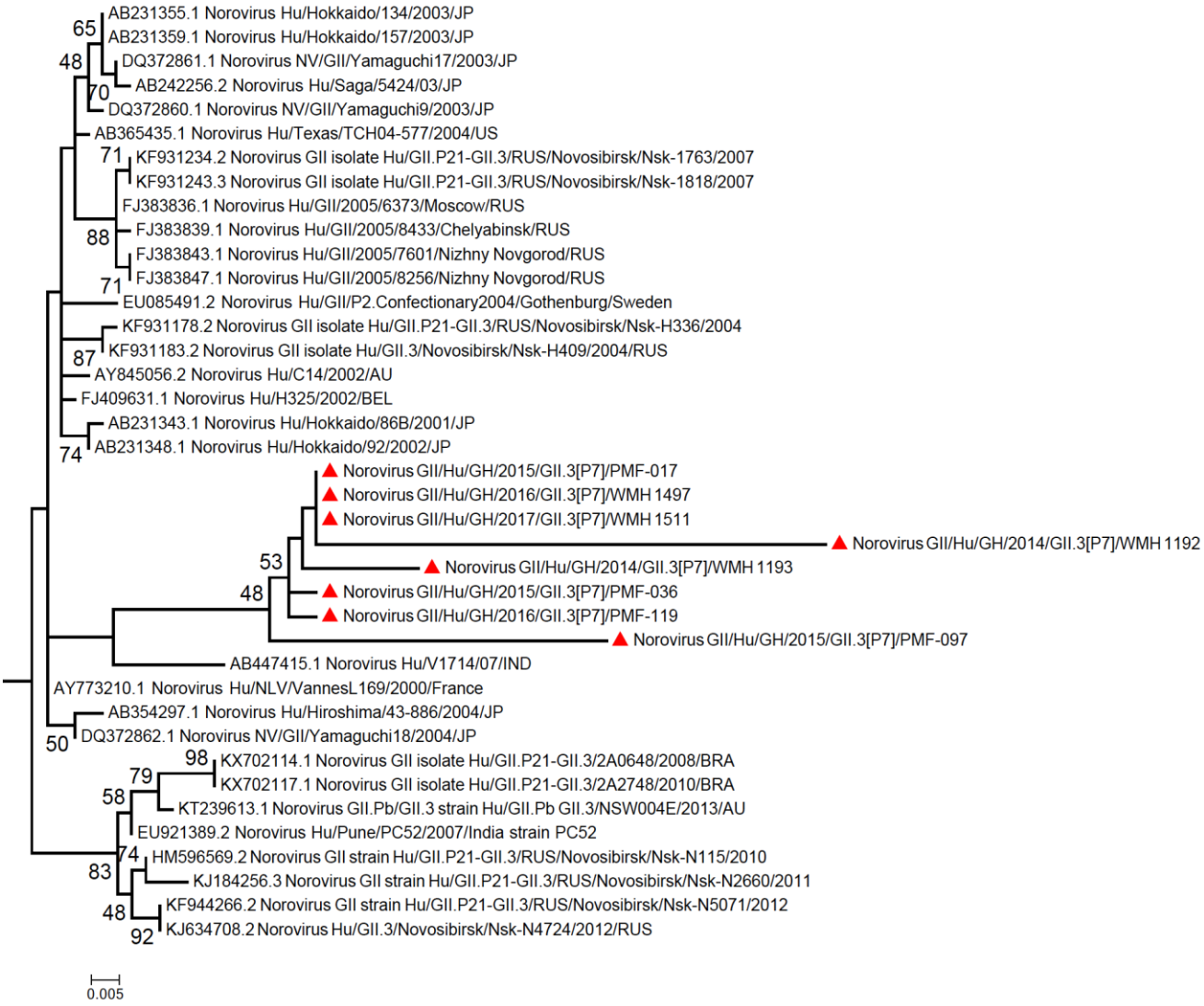


Fig 4.3a: Phylogenetic tree of the partial VP1 gene sequences of GII.3 norovirus strains, showing the genetic relationship between Ghanaian study GII.3 strains and other globally circulating GII.3 strains.

Strains characterized in this study are indicated with red triangles. Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model with a discrete gamma distribution in the mega 6 software package. Significant bootstrap values (1000 replicates) of ≥ 70 % are indicated at each node. The outgroup sequences were excluded from the trees. Bar indicates genetic distance expressed as the number of nucleotide substitutions per site.

Parameter	Value
Number of sequences	40
Sampling period	2000-2017
Geographical coverage	Global
Mean evolutionary rate (x10E-3)	6.3
Mean evolutionary rate 95% HPD	3.6-9.2
tMRCA (95% HPD)	2010 (2007-2012)
chain length	50 mil

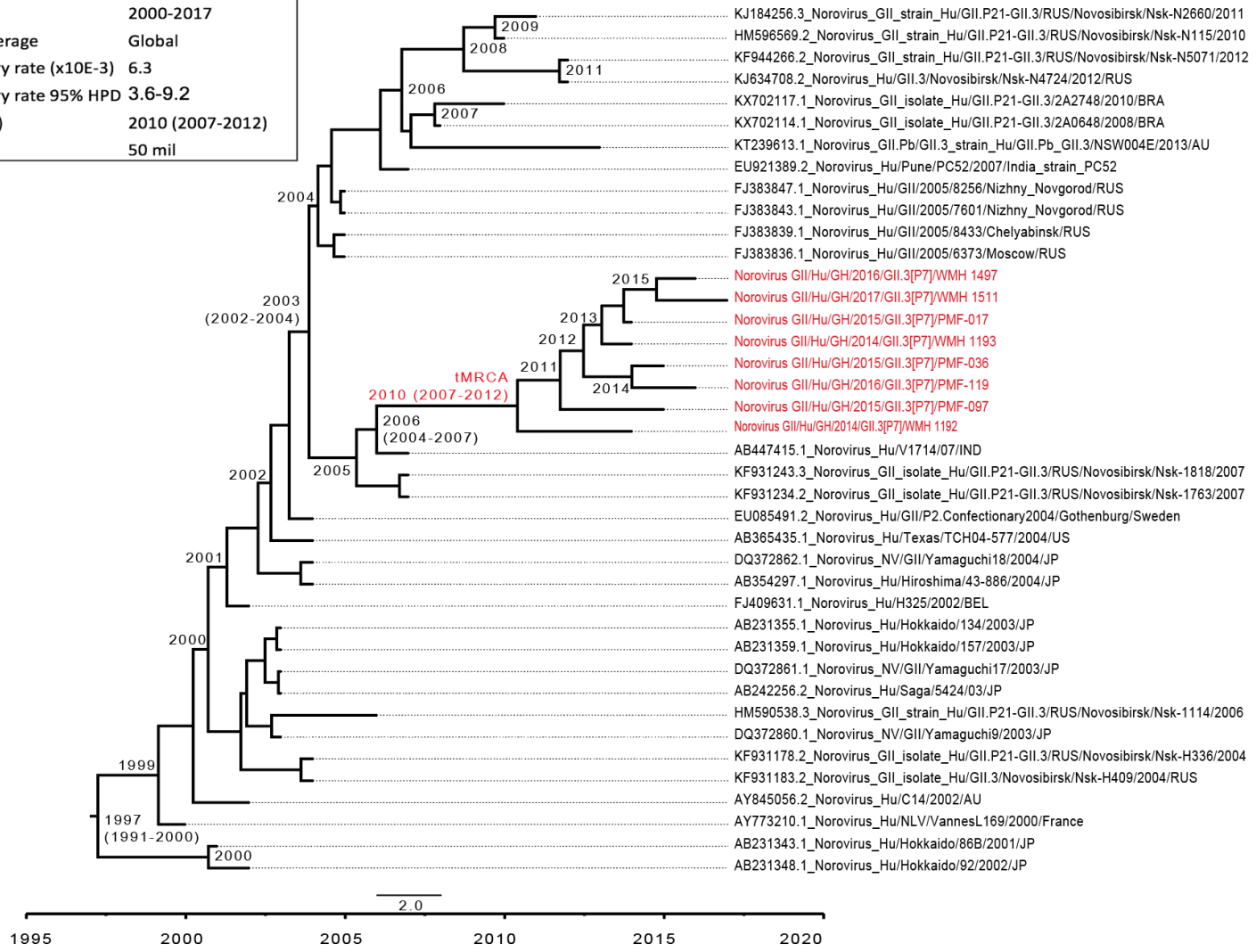


Fig 4.3b: Evolutionary analysis of Ghanaian GII.3 noroviruses

Maximum clade credibility (MCC) tree of 40 dated GII.3 partial VP1 nucleotide sequences reconstructed in the Bayesian MCMC framework. Bar indicates genetic distance expressed as number of nucleotide substitutions per site. The time of the most common recent ancestor (tMRCA) is indicated in red faced fonts for each cluster. Study strains are also indicated in red faced fonts while variants are identified by names. The MCC tree was constructed using the BEAST package and visualized in FigTree v1.4.3

4.3.4 Sequence and Phylogenetic Analysis of GII.6 Strains

GII.6 Study Strains

The GII.6 strains constituted one of the five most prominent genotypes associated with diarrhoea in the Ghanaian pediatric population. The Ghanaian GII.6 study strains first appeared in 2009 but were more frequently (17.1%) detected in the year 2013. All the study GII.6 strains were inter-genotype recombinants with the [P7] polymerase genotype (Table 4.2).

Sequence Comparison

Comparison of the partial VP1 gene sequences of the study strains revealed low genetic identity between strains detected within different months of the same year with nucleotide sequence identity ranging from as low as 86.3% - 99.0%.

Maximum Likelihood Phylogenetic Analysis

The sequence diversity among the capsid genes of the Ghanaian GII.6 strains segregated them into two different clusters according to their month of detection; strains (WMH 1080, WMH 1089, and WMH 1087) all detected in May 2013 belonged to Cluster I whereas those detected in August (WMH 1139) and September (WMH 1151) of the same year belonged to Cluster II (Fig 4.4a). Study strains found in Cluster I were related to earlier reference strains from Japan and the USA detected between 1971 and 1993 whilst study strains belonging to Cluster II were related to more recent (2002-2014) published strains from Africa, Asia, South America, and Europe. The Ghanaian GII.6 strains in Cluster II were also found to be closely related to Burkinabe T209 GII.6 isolate detected in 2011 and shared over 98.0% nucleotide sequence identity.

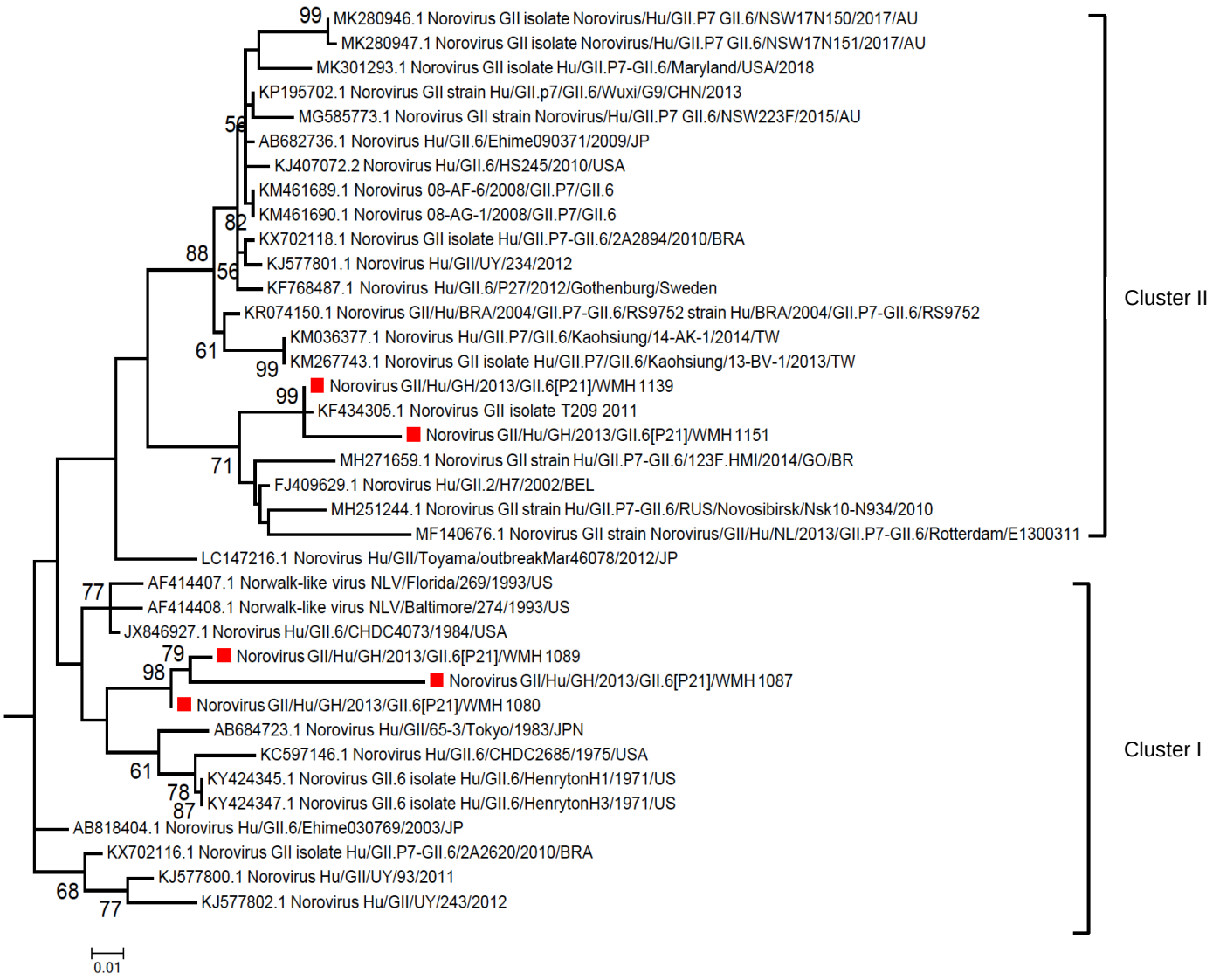


Fig 4.4a: Phylogenetic tree of the partial VP1 gene sequences of GII.6 norovirus strains, showing the genetic relationship between Ghanaian study GII.6 strains and other globally circulating GII.6 strains

Strains characterized in this study are indicated with red squares. Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model with a discrete gamma distribution in the mega 6 software package. Significant bootstrap values (1000 replicates) of ≥ 70 % are indicated at each node. The outgroup sequences were excluded from the trees. Bar indicates genetic distance expressed as the number of nucleotide substitutions per site.

Determination of the time of the most recent common ancestor of Ghanaian GII.6 strains

The time of the most recent common ancestor of the GII.6 strains was determined using BEAST (v1.8.1) software. As noted in the maximum likelihood tree, the GII.6 strains were located in two different clusters. Two strains detected in 2013 had their tMRCA estimated to be 2012 (95% HPD interval: 2009-2013) whereas the remaining three GII.6 strains also detected in 2013 had their tMRCA to be 2011 (95% HPD interval: 2007-2013(Fig. 4.4b). This indicates that around the same time frame, a diverse pool of GII.6 strains were co-circulating in the study area. The mean evolutionary rate of the GII.6 dataset was determined to be 2.4×10^{-3} substitutions/site/year (95% HPD interval: $1.6 \times 10^{-3} – 3.3 \times 10^{-3}$).

Parameter	Value
Number of sequences	37
Sampling period	1971-2018
Geographical coverage	Global
Mean evolutionary rate (x10E-3)	2.4
Mean evolutionary rate 95% HPD	1.6-3.3
tMRCA (95% HPD)	2011 (2007-2013); 2012 (2009-2013)
chain length	50 mil

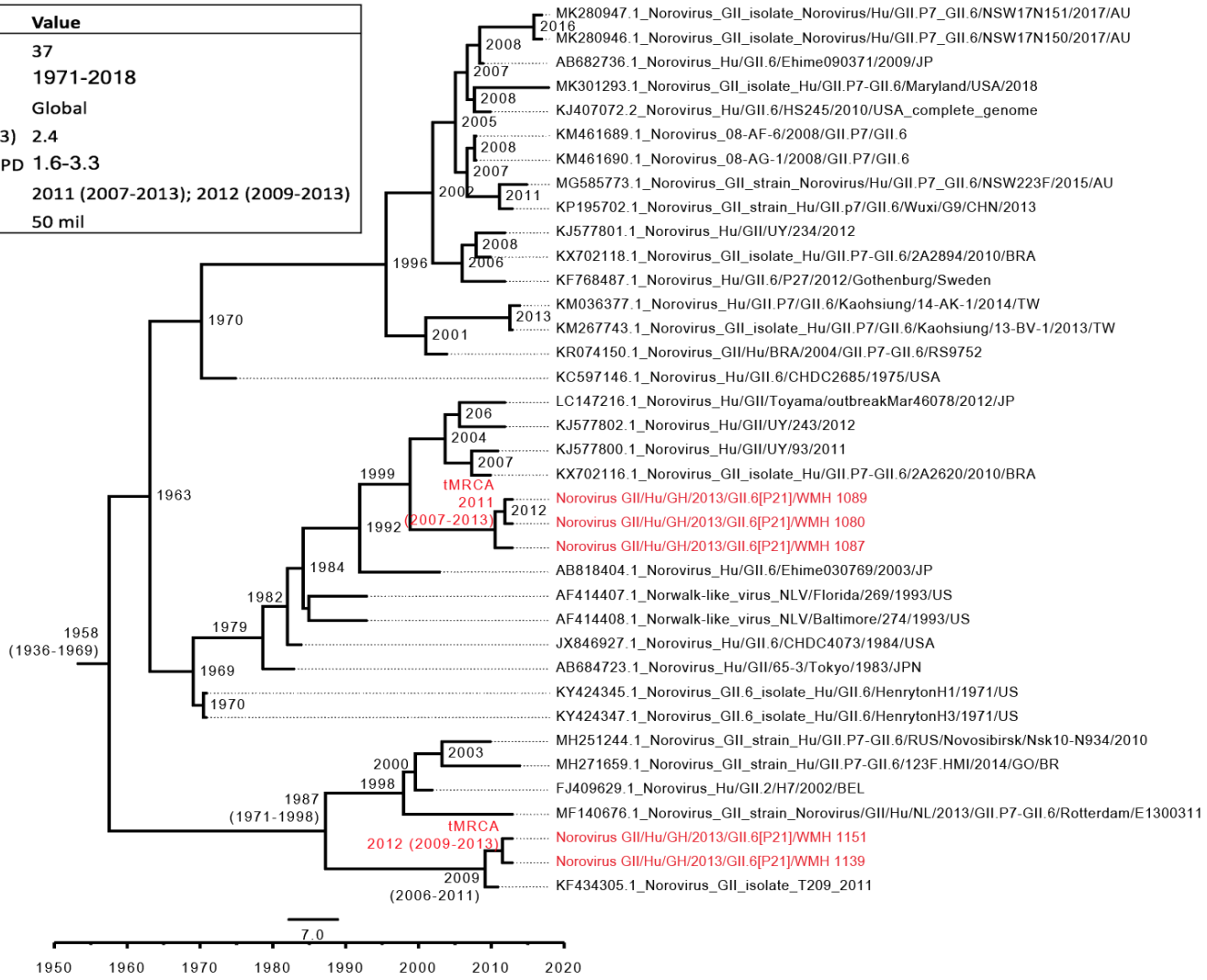


Fig 4.4b: Evolutionary analysis of Ghanaian GII.6 noroviruses

Maximum clade credibility (MCC) tree of 37 dated GII.6 partial VP1 nucleotide sequences reconstructed in the Bayesian MCMC framework. Bar indicates genetic distance expressed as number of nucleotide substitutions per site. The time of the most common recent ancestor (tMRCA) is indicated in red faced fonts for each cluster. Study strains are also indicated in red faced fonts while variants are identified by names. The MCC tree was constructed using the BEAST package and visualized in FigTree v1.4.3

4.3.5 Sequence and Phylogenetic Analysis of GII.17 Strains

GII.17 Study Strains

The GII.17 norovirus strains accounted for 3.4% of AGE hospitalized cases within the study period. Of the six detected GII.17 strains, three (3) possessed the concordant polymerase genotype [P17] whilst the other three were recombinants possessing discordant polymerase genotypes GII.Pe (n=1), GII.P13 (n=1) and GII.P3 (n=1) [Table 4.1].

Sequence Comparison

Sequence analysis of the Ghanaian GII.17 strains detected from 2008-2013 showed they were highly diverse both at the nucleotide and amino acid sequence level. The nucleotide sequence identities of their partial capsid gene ranged from 81.4%-95.9%. Even among strains detected within the same norovirus season (2013), nucleotide sequence diversity was as high as 8.7% to 17.6%.

Maximum Likelihood Phylogenetic Analysis

At the phylogenetic level, the Ghanaian GII.17 strains were observed to belong to multiple lineages. Also, strains detected in 2013 from within the same hospital clustered into different lineages (Fig 4.5a). While the Ghanaian norovirus WMH-1164 GII.17 strain shared ancestral history with the prototype GII.17 strain C142 detected in 1978, the others including WMH-1079 and WMH-1111 GII.17 Ghanaian strains clustered together with contemporary GII.17 strains circulating in Cameroon, South Africa, and many Asian countries. The earliest Ghanaian GII.17 strain (WMH-048) detected in 2008 was however distinct from any other lineage in the phylogenetic tree.

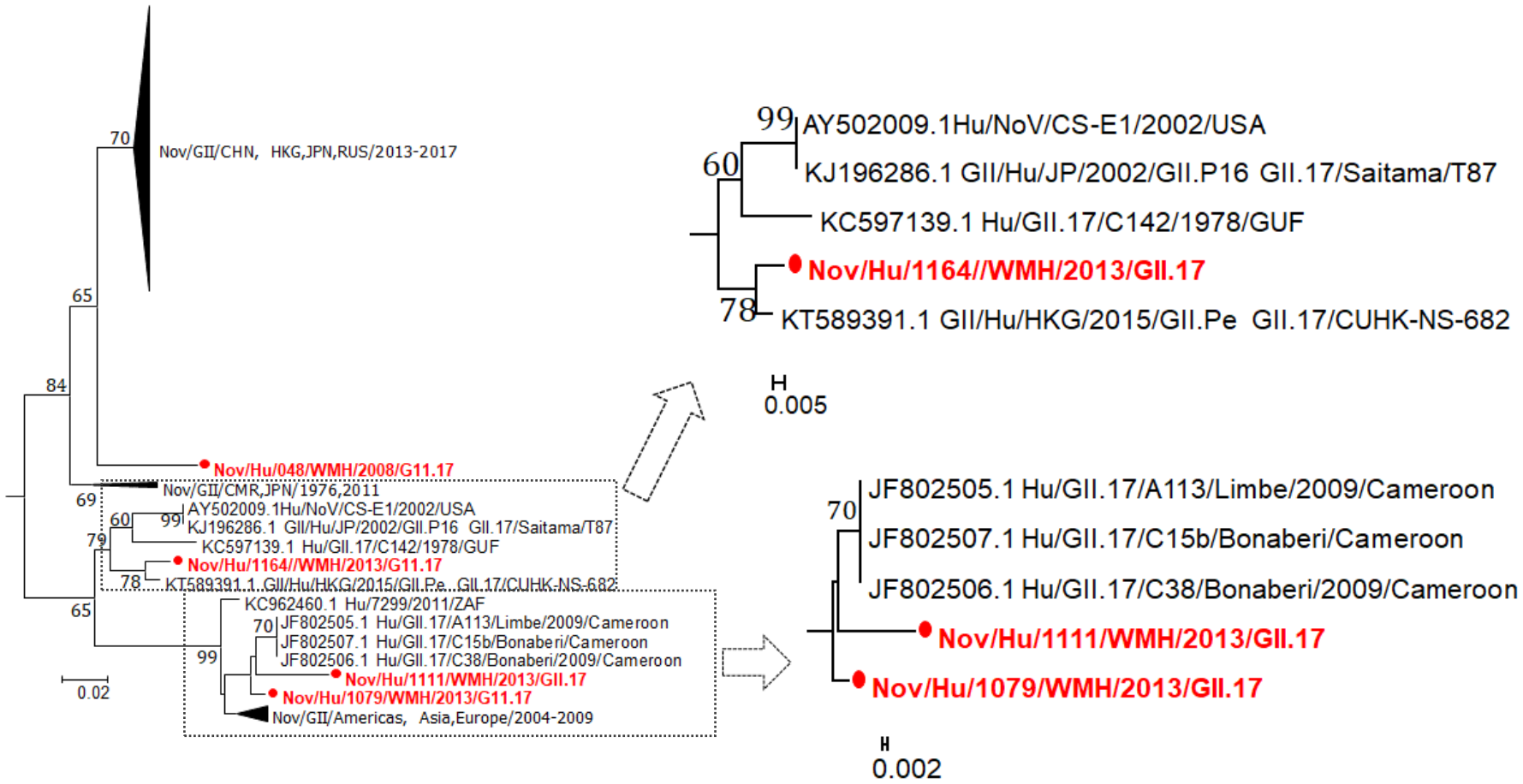
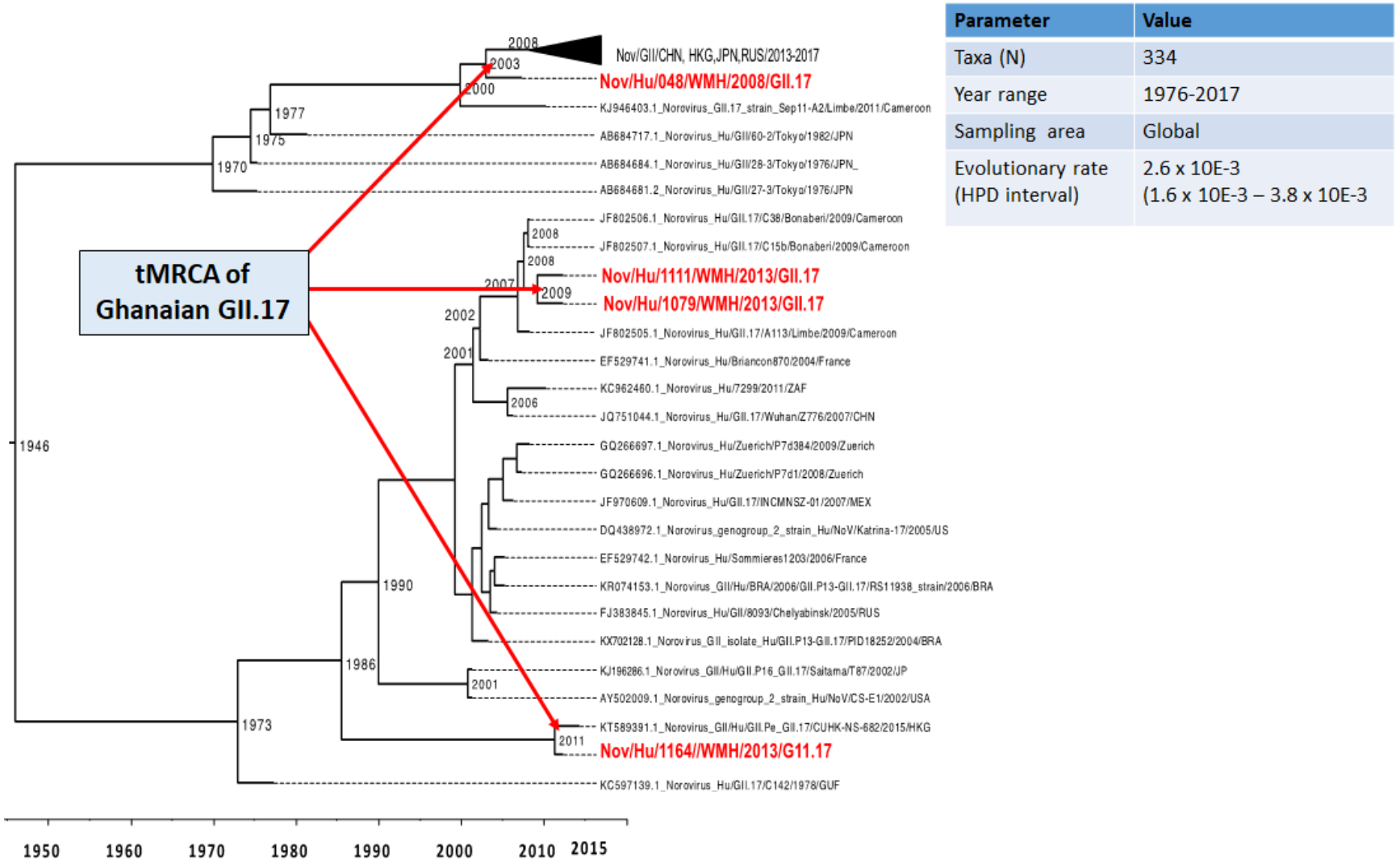


Fig 4.5a: Phylogenetic tree of the partial VP1 gene sequences of GII.17 norovirus strains, showing the genetic relationship between Ghanaian study GII.17 strains and other globally circulating GII.17 strains

Strains characterized in this study are indicated with red-faced fonts and filled red circles. Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model with invariant sites in the mega 6 software package. Significant bootstrap values (1000 replicates) of ≥ 70 % are indicated at each node. The outgroup sequences were excluded from the trees. Bar indicates genetic distance expressed as the number of nucleotide substitutions per site.

Determination of the time of the most recent common ancestor of Ghanaian GII.17 strains

Evolutionary analysis revealed the Ghanaian GII.7 strain and its variants were introduced into our population at different time points (Fig 4.5b) with the earliest Ghanaian GII.17 ancestor dating as far back as 2002.9 (2003). The evolutionary rate of the detected GII.17 study strains was estimated to be approximately 2.6 x 10E-3 substitutions/site/year (HPD interval: 1.6 x 10E-3 - 3.7 x 10E-3).



Parameter	Value
Taxa (N)	334
Year range	1976-2017
Sampling area	Global
Evolutionary rate (HPD interval)	2.6 x 10E-3 (1.6 x 10E-3 – 3.8 x 10E-3

Fig 4.5b: Evolutionary analysis of Ghanaian GII.17 noroviruses

Maximum clade credibility (MCC) tree of 4 dated GII.17 partial VP1 nucleotide sequences reconstructed in the Bayesian MCMC framework. Bar indicates genetic distance expressed as number of nucleotide substitutions per site. The time of the most common recent ancestor (tMRCA) is by red faced arrows. Study strains are also indicated in red faced fonts. The MCC tree was constructed using the BEAST package and visualized in FigTree v1.4.3.

4.3 Discussion

In Ghana norovirus infection is not a notifiable disease, consequently not much is known of the virus infection dynamics within our population. This current study comprehensively examined and reports the molecular epidemiology of noroviruses associated with childhood diarrhoea along with the temporal circulation of GII.4 variants and norovirus recombinant strains. The study also reports the evolutionary dynamics of GII.4 and non-GII.4 norovirus strains with our findings suggesting that the different norovirus genotypes exhibit different evolutionary patterns on their capsid genes.

While many genotypes were detected at varying rates (Table 4.1), this study confirmed the dominance of the GII.4 capsid genotype in frequent circulation with [P4], [P16], and [P31] polymerase genotype similar to earlier studies reported across the globe (van Beek et al., 2018a). The mechanism for the persistence and establishment of the GII.4 genotypes over the other genotypes is not fully understood. It is however hypothesized that, like the influenza viruses, the GII.4 viruses undergo epochal evolution with the accumulation of mutations on their capsid gene. This results in the chronological production of new GII.4 viruses (variants) that can escape immunity developed against GII.4 variants previously in circulation (Lindesmith et al., 2008a; White, 2014b). This new variant then persists for a couple of years within the population without any phenotypic change until it is replaced by the next variant (Tohma et al., 2019). Our study detected the circulation of 7 different GII.4 variants (table 4.3), five of which are well-established pandemic strains (Asia_2003, FarmingtonHills_2002, Den-Haag 2006b, New Orleans_2009, and Sydney_2012) while the other two (Yerseke_2006b and Cario_2007) are described as epidemic strains with limited geographic circulation and no pandemic characteristics (Siebenga et al., 2009; White, 2014b). Interestingly, in this current study at least 2 or more variants co-circulated at each time point (Fig 4.1). The GII.4 variants identified in this study appeared for a maximum of 36

months and was replaced by a new variant, a distinct feature of GII.4 viruses which has also been reported elsewhere (Rahman et al., 2013). No single variant was detected or found in circulation continuously throughout the study period.

The norovirus capsid contains antigenic epitopes that are targets for protective antibody responses. Understanding the diversity that exists within the capsid gene of circulating noroviruses is important for vaccine formulations as well as the evaluation of candidate vaccines (Lindesmith et al., 2013). Our epidemiological investigations also revealed equally important capsid genotypes including GII.3 (6.3%), GII.6 (4.6%), GII.5 (3.4%), and GII.17 (3.4%). These non-GII.4 capsid genotypes have been associated with multiple polymerase types during their evolutionary history. All the study GII.3's and GII.6's capsid genotypes possessed the [P21] and [P7] polymerase genotypes respectively. Circulating GII.17 study strains possessed the [P17], [P3], [P13] polymerase genotype while the polymerase type for the GII.5 could not be determined for all samples. Bayesian estimation revealed the evolutionary rates of the GII.4 (3.7–7.2 x 10^{-3} nt substitutions/site/year) and GII.3 (3.6–9.2 x 10^{-3} nt substitutions/site/year) genotypes to be higher than that of the GII.6 (1.6–3.3 x 10^{-3} nt substitutions/site/year), and GII.17 genotypes (1.6–3.7 x 10^{-3} nt substitutions/site/year). The high evolutionary rate of the GII.4 and GII.3 strains supports the predominance of these two norovirus strains associated with AGE infections.

The observed varying tMRCAs of each of the predominant capsid genotypes (Figs 4.2b-4.5b) also implies there was the introduction of these genotypes into the Ghanaian population at different times. In combination with the different lineages the strains belonged to, we provide evidence that there is the need for critical monitoring of the antigenic variations that might occur in these strains with time and the implications for the vaccine development strategies.

Approximately 35.0% of norovirus isolates sequenced were found to be recombinants possessing discordant capsid and polymerase genotypes (Table 4.1), as was detected in the European and Asian, and Oceania regions (van Beek et al., 2018b). We also observed a high prevalence (55.4%) of these recombinant strains among the non-GII.4 study samples analysed compared to the GII.4's (44.6%). The GII.4_ Sydney 2012[P16] and GII.3[P21] were the most frequent recombinants and unlike the other detected recombinant strains, they were in circulation for a long period (2013 – 2017). The norovirus GII.4_Sydney 2012[P16] strain was first described during an outbreak in the Oceania Region in the mid-2015 (Lun et al., 2018a) and by 2016 had become an epidemic strain globally responsible for 14-42% of all norovirus outbreak cases (Cannon et al., 2017b; Choi et al., 2017; Lun et al., 2018b). This strain is believed to have acquired the [P16] polymerase gene (Ruis et al., 2017) whilst maintaining the previously circulating GII.4 Sydney_2012 capsid, this modification to its non-structural regions might have contributed to its ability for immune escape and virological fitness and hence it's increased prevalence (Lindesmith et al., 2018). The recombinant GII.3[P21] was the second most frequently found in this study. This recombinant type has been described as one of the prevalent norovirus strains associated with sporadic AGE in children globally (Boon et al., 2011b; Puustinen et al., 2012). Recombinant strains including GII.2[P30], GII.3[P16], GII.4[P7], GII.5[P16], GII.9[P7], and GII.17[P13] were uncommon recombinants types that were infrequently detected. This finding suggests that these strains could be less virulent compared to the GII.4[P16] and GII.3[P21] recombinants and hence are less associated with hospitalizations. The use of only symptomatic clinical samples might therefore have resulted in an underestimation of their prevalence. Nevertheless, put together these infrequent strains were responsible for approximately 23% of AGE cases in the Ghanaian pediatric population hence their presence cannot be ignored.

Although more analysis of the junction of the Pol and VP1 genes is needed to confirm the recombination breakpoints of the study samples, our results clearly show the importance of recombination in driving viral diversity as well as the evolution of novel strains. It also emphasizes the need for the continuous monitoring of circulating norovirus strains of both the capsid and polymerase regions for the early detection of new strains with the ability to cause global epidemics or pandemics.

4.3 Conclusion

One major question in the field of norovirus research has been whether the emergence of new variants or novel strains with pandemic characteristics could be predicted. Over the years, however, molecular epidemiological studies and surveillance of noroviruses have proved to be an essential tool for identifying new circulating recombinants as well as emerging strains both at the population and global level. In our study, although the GII.4 lineage continued to be the cause of the majority of the norovirus-associated AGE, the non-GII.4 strains were observed to have contributed to the generation of the wide genetic diversity of circulating strains. Of these, at least 18 different recombinant types were identified with the majority belonging to the GII viruses. The high genetic diversity and frequent detection of recombinants circulating within our population demonstrate the importance of ongoing surveillance as well as the importance of dual ORF1/ORF2 genotyping to better understand the role these recombinants play in norovirus infection dynamics in addition to their evolutionary pathways.

CHAPTER FIVE

Association between Norovirus Infection and Histo-Blood Group Antigen (HBGA)

Genetic Susceptibility of Ghanaian Children to Symptomatic Norovirus Infection

Abstract

Histo-blood group antigens (HBGAs) have been identified as key receptors for virion attachment and host entry. The HBGAs expressed on the epithelium of the intestines are largely controlled by the fucosyltransferase (*FUT2* (secretor) and *FUT3* (Lewis)) genes which encode the FUT2 and FUT3 enzymes respectively. Polymorphisms that occur in these fucosyltransferase genes have been associated with an individual's susceptibility to various infectious diseases. In norovirus infections, different norovirus genotypes infect different HBGA phenotypes. This has raised questions of whether the prevalence and circulating norovirus strains would also vary as well as whether the percentage of secretors within a population would influence the prevalence of specific norovirus genotypes as a function of their ability to infect secretors compared to non-secretors or vice versa. This study aimed to evaluate the HBGA profile in norovirus-infected Ghanaian children and to investigate potential associations if any between norovirus genotypes and HBGA patterns in these patients.

Paired diarrhoeic stool and saliva samples were collected between August 2015 and March 2016 from selected sentinel sites participating in the national rotavirus diarrhoea surveillance in Ghana**.** Norovirus genotyping was performed by amplifying and sequencing parts of the viral VP1 and RNA-dependent RNA polymerase (RdRp) genes. The entire coding region of the *FUT2* gene was

amplified by conventional PCR and genotyped using Restriction Fragment Length Polymorphism (RFLP).

Eighty-seven percent of the participants tested (126/145) were found to be secretors whilst 13% (13/145) were classified as non-secretors. Within the secretor group, 38 (26.2%) were sub-classified as homozygous secretors and 88 (60.7%) heterozygous secretors. More than 50% (79/145) of the children tested were found to be shedding noroviruses in their stools. Of those who tested positive for noroviruses, 69 (87.3%) were secretors whilst 8 (10.1%) were non-secretors.

The four most common capsid genotypes were GII.4 (50.0%; 22/44), GI.4 (13.6%; 6/44), GII.3 (9.1%; 4/44) and GII.5 (9.1%; 4/44), whereas GII.4P[4] (50.0%; 14/28) and GI.4P[4] (21.4%; 6/28) were the most prevalent genotype combinations. Norovirus genotype GII.4 infected more secretor-positive children who possessed heterozygous allele of the *FUT2* gene compared to the non-GII.4 strains (60.0% vs 40.0%, $p=0.035$) which had a preference for secretor-positive children with homozygous allele of the gene (62.5% vs 37.5%, $p<0.05$).

From the study, neither secretor status nor genotype difference affects susceptibility to norovirus infection in Ghana.

5.1 Introduction

Histo-blood group antigens (HBGAs) are complex carbohydrates found on the mucosal surfaces of host cell membranes, body fluids, and secretions (Heggelund et al., 2017). These carbohydrate complexes under the control of α1,2-fucosyltransferase (FUT2) and α1-3,4 fucosyltransferase (FUT3) enzymes mostly expressed on the mucosal epithelial surfaces, genetically determines an individual's ABO (H), secretor, and Lewis status. Persons carrying at least one functional FUT2 allele expressing the α1,2 fucosyltransferase 2 enzyme are termed "secretors" and can express the A and B blood group antigens as well as H-type 1 antigen. Individuals with a recessive allele of the gene have a missense or nonsense mutation in their *FUT2* gene and are unable to produce a functional FUT2 enzyme and hence are termed non-secretors (Ferrer-Admetlla et al., 2009). The FUT3 enzyme on the other hand mediates the expression of the Lewis antigens, either Lewis a (for non-secretors) or Lewis b (for secretors) with Lewis-negative individuals (inactive FUT3 enzyme) unable to express either of the Lewis a/b antigens. Changes in the expression of these HBGAs on the mucosal surface are thought to be a major determinant of an individual's disease susceptibility, as HBGAs can serve as receptors and attachment sites for some microorganisms and as such is important for the establishment of infection (Ramani et al., 2016).

Serving either as ligands or restriction factors, both FUT2 secretor or non-secretor status have been reported to be associated with either protection against or susceptibility to different gastrointestinal enteric organisms (Karst, 2010; Nordgren & Svensson, 2019). The genetic status has long been associated with an individual's susceptibility to norovirus infection and this has been shown in several challenge studies (Hutson et al., 2002; Lindesmith et al., 2003; Marionneau et al., 2002; Parrino et al., 1977). In these studies, secretors exhibited strong affinity to the most commonly circulating GII.4 norovirus strain whiles non-secretors remained highly resistant to infection with

this virus strain although they were susceptible to infection caused by other strains of the norovirus (Frenck et al., 2012a; Lindesmith et al., 2003; Parrino et al., 1977). A study among the Burkinabe pediatric diarrhoeal population also showed the GI noroviruses exclusively infecting Lewis-positive children, while the GII noroviruses infected both Lewis-negative and Lewis-positive children (Nordgren et al., 2013). In 2005, studies by Tan and colleagues suggested the different strains of the noroviruses exhibited different properties about their ability to bind to different HBGAs (Tan & Jiang, 2005c). Currently, two major binding profiles have been described for the noroviruses: those that bind A or B and H antigens and those that bind Lewis or H antigens (Harrington et al., 2002; Huang et al., 2005b).

Several mutations are known to occur within the *FUT2* and *FUT3* genes with some of these mutations exhibiting high indigenous specificity (described in chapter 2 of this thesis). This genetic diversity found existing within human HBGAs implies the proportions of secretor, ABO (H) and Lewis HBGA types differ and vary across geographical populations (Ferrer-Admetlla et al., 2009). Approximately 80% of the African and European populations are secretors while the Mesoamericans have as high as 95% of their population as secretors (Nordgren et al., 2016a). Additionally, in Africa, the Lewis-negative phenotype population is significantly higher (20–33%) (Lindesmith et al., 2003) compared to the European and Asian populations (6–11%) (King et al., 2018; Nordgren et al., 2013).

Both challenge studies (Frenck et al., 2012b; Lindesmith et al., 2003) and prospective surveillance studies conducted in countries including Ecuador, the USA, China and Burkina Faso on the association between norovirus and host genetic susceptibility confirmed the near resistance of non-secretors to infection with the predominate GII.4 norovirus genotype (Currier et al., 2015; Liu et al., 2014; Lopman et al., 2015; Nordgren et al., 2013). A study by Van Trang and colleagues on

host susceptibility to norovirus infection among Vietnamese children also showed that norovirus GII.3 genotype predominantly infected secretors compared to non-secretors (Van Trang et al., 2014a). Nevertheless, these studies also showed the ability of a host of other norovirus genotypes including GII.7, GII.2, GII.1, GI.6, GI.3, and GI.7 to equally infect secretors as well as non-secretors.

Since different norovirus genotypes infect different HBGA phenotypes, it raises questions of whether the prevalence and circulating norovirus strains would also vary as well as whether the percentage of secretors within a population would influence the prevalence of specific norovirus genotypes as a function of their ability to infect secretors compared to non-secretors or vice versa. Answers to these questions would be important as this would inform on what other norovirus strain types would be important to include in norovirus vaccine formulations as well as help explain immunologic responses to vaccine components.

The aim of this study was to evaluate the HBGA profile (secretor, and Lewis status) in norovirus-infected children in Ghana and to investigate potential associations if any between norovirus genotypes and HBGA patterns in these patients.

5.2 Methodology

5.2.1 Sample collection

This study was conducted with approval from the Institutional Review Board of Noguchi Memorial Institute, University of Ghana. It was a cross-sectional hospital-based study carried out between August 2015 and February 2016 from selected sentinel sites including the Child Health Department of Korle-Bu Teaching Hospital, and the Princess Marie Louise Children's Hospital participating in the national rotavirus diarrhoea surveillance in Ghana. All children under five years old with non-bloody diarrhoea, defined as the passage of more than three watery stools in 24 hours, were enrolled. Paired diarrhoeic stool and saliva samples were then collected from each consenting participant for analysis.

5.2.2 Nucleic Acids Extraction

Viral RNA was extracted from clarified 20% w/v stool suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN, CA, USA) following the manufacturer's instructions with slight modifications. To increase the yield of extracted RNAs, a double elution (2 x 40 µl) using buffer AVE was performed. Genomic DNA from swabs of buccal epithelial cells of study subjects was also extracted with the Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All extracted products were stored at -20^{o}C until ready to be used.

5.2.3 Determination of Single Nucleotide Polymorphism in the FUT2 Gene

5.2.3.1 FUT2 Gene Amplification

For all samples of subjects, a 1,125 bp fragment containing the entire coding region (exon 2, 1032 bp) of the *FUT2* gene was amplified using FUT2_F and FUT2_R published primers (Ferrer-Admetlla et al., 2009) [Table 5.2]. Conventional PCR was carried out in a 50 µl reaction mix containing 20mM dNTP mix, 1X green PCR buffer (Promega), 1.25 U GoTaq DNA Polymerase (Promega), 20 µM each of forward and reverse primers and 5 µl genomic DNA extract. PCR cycle conditions were as follows: 2 mins of initial denaturation at 94 °C; followed by 35 cycles of 30 secs of denaturation at 94 °C, 30 secs of annealing at 48 °C, 5 mins of extension at 72 °C; and a final extension cycle at 72 °C for 10 mins. All amplicons were electrophoresed on a 2.0% ethidium bromide-stained agarose gel alongside a 100 bp molecular weight marker and bands visualized under UV to confirm the success of PCR.

5.2.3.2 Restriction Fragment Length Polymorphism (RFLP) of Amplified FUT2 genes

An in-house NaOAc Ethanol purification method was used to clean the PCR amplicons and the purified DNA eluted in double-distilled water (ddH_2O). Restriction Fragment Length Polymorphism (RFLP) was carried out in a 25 µL reaction mix (5U AvaII restriction enzyme (NEB) and 1X RE buffer) at 37 °C for 120 minutes in a heat block. The RFLP setup also included a digest control (pGEM 3Zf(+) circular plasmid DNA giving 2 expected fragments of 2975 bp and 222 bp) and an uncut FUT2 amplified gene (no AvaII RE added, therefore single 1125 bp fragment expected) to confirm restriction enzyme activity. Digested products were electrophoresed on 2.0% ethidium bromide-stained agarose gels alongside a 100 bp molecular marker and the bands

visualized under UV light. FUT2 G428A SNP genotypes were determined based on the published RFLP patterns described in table 5.1 below:

Table 5.1: Interpretation to RFLP Patterns of the *FUT2* gene

Digest Product	Fragment Sizes	Interpretation
3 fragments	202 bp - 425 bp - 498 bp	**Genotype AA** Non-secretor
4 fragments	202 bp - 295 bp - 130 bp - 498 bp	**Genotype GG** Homozygous secretor
5 fragments	202 bp - 295 bp - 130 bp - 425 bp - 498 bp	**Genotype GA** Heterozygous secretor

5.2.4 Determination of Single Nucleotide Polymorphism in the *FUT3* Gene

5.2.4.1 *FUT3* Gene Amplification

To amplify the entire coding sequence region of the *FUT3* gene, touchdown-PCR (TD-PCR) targeting the coding sequence region of exon3 was carried out as previously described (Elmgren et al., 1996; Nordgren et al., 2014) with some modifications. The gene was amplified in four (4) fragments using different primer pairs (Table 5.2).

Gene amplification using Ex3.1_F/ Ex3.1_R and Ex3.2_F/ Ex3.2_R primer pairs: TD-PCR (65 ^{o}C – 55 °C, 20+20 cycles) was carried out in 50 µL reactions containing 1X green PCR buffer (Promega); 10nM dNTPmix; 10 µM primer each (forward and reverse), 1.25 U GoTaq DNA Polymerase (Promega) and 2 µL genomic DNA extract.

Gene amplification using Ex3.3_F/ Ex3.3_R and Ex3.4_F/ Ex3.4_R primer pair: TD-PCR (70^{o}C - 60°C, 20+20 cycles) was also carried out in 50 µL reactions consisting of, 1X green PCR buffer (Promega), 10 µM primer each (forward and reverse), 6 µL and 2 µL genomic DNA extract respectively. All PCR amplicons were then electrophoresed on a 2.0% ethidium bromide-stained agarose gel alongside a 100 bp molecular weight marker and bands visualized under UV to confirm the success of PCR.

5.2.4.2 FUT3 Gene Sequencing

Amplicons for Ex3.1_F/ Ex3.1_R and Ex3.2_F/ Ex3.2_R were purified directly using the QIAquick PCR purification kit (Qiagen) while the expected band size-specific for Ex3.3_F/ Ex3.3_R and Ex3.4_F/ Ex3.4_R primers were excised from gels, and DNA extracted from agarose gels by dissolving inappropriate volume of 6 M guanidinium isothiocyanate (GITC) and later purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's

instructions with some modifications. All purified amplicons were subjected to dideoxy chain-terminating sequence PCR using BigDye® Terminator v 3.1 Cycle Sequence kit (Life Technologies). Sequenced-PCR products were cleaned using an in-house NaOAc-ethanol purification method. Purified amplicons were then resuspended in Hi-Di Formamide, and loaded onto an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

5.2.5 Norovirus gene amplification and sequencing

Two sets of primers (GISKR/MON432) and (GIISKR/MON431) were used for norovirus genogroup one (I) and genogroup two (II) detection to generate 579 or 570 amplicons respectively. The reaction was performed in a 25 µl mixture of 5 µl RNA, 5U QIAGEN OneStep RT-PCR enzyme (QIAGEN, USA) containing the HotStarTaq DNA polymerase and 50 µM each (forward and reverse primers) (Table 3.1). Reverse transcription was first performed at 42 ^{o}C for 30 mins followed by 45 cycles of PCR amplification. PCR products were visualized on agarose gels.

For genotyping, the 543 and 557 bp genogroup-specific band sizes for GI and GII were excised from the gel and DNA extracted from weighted agarose gels by dissolving in an appropriate volume of 6 M GITC and subsequently purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. Bidirectional DNA sequencing of the purified PCR products was then performed using both forward and reverse primers Nucleotide sequencing reactions were performed by fluorescent di-deoxynucleotide chain termination chemistry using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit, version 3.1 (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Nucleotide sequences were determined using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Table 5.2: Primer sequences for the amplification of FUT2 and FUT3 genes in this study

Primer Name	Polarity	Sequence (5'- 3')	Target Region (Position)	Expected Band Size	Reference
FUT2_F	+	CCA GCT AAC GTG TCC CGT TTT CC		1125 bp	(Ferrer-Admetlla et al., 2009)
FUT2_R	-	GGC ACT CAT CTT GAG GGA GGC A			
FUT3_EX3.1F	+	GGA GCT TTG GTA AGC AGG AG	nt 6562 - nt 6581	484 bp	(Nordgren et al., 2014)
FUT3_EX3.1R	-	AAG TTG AAC CAG ATC CAG CG	nt 7026 - nt 7045		
FUT3_EX3.2F	+	AGT GGG TCC TCC CGA CAG GAC ACC ACT CCC	nt 6780 - nt 6809	470 bp	(Elmgren et al., 1996)
FUT3_EX3.2R	-	GCT GAG TCC GGC TTC CAG TT	nt 7230 - nt 7249		
FUT3_EX3.3F	+	TCC GAC ATC TTC ACG CC	nt 7122 - nt 7138	387 bp	(Nordgren et al., 2014)
FUT3_EX3.3R	-	GAT GAA GGC GTC GGG TG	nt 7492 - nt 7508		
FUT3_EX3.4F	+	ATC ACC GAG AAG CTG TGG A	nt 7401 - nt 7419	389 bp	(Nordgren et al., 2014)
FUT3_EX3.4R	-	AAA GGA CTC CAG CAG GTG AG	nt 7770 - nt 7789		

5.2.6 Data Analysis

5.2.6.1 Sequence Analysis

All nucleotide sequences were edited using the BioEdit software. Multiple sequence alignment with cognate gene sequences was carried out using the CLUSTALW algorithm within the BioEdit and Mega software. For allele identification, *FUT3* gene sequences were aligned to *FUT3* reference genomes to identify and establish SNP loci (G13A, T59G, T202C, C314T, G484A, G508A, G808A, T1067A, G179A, C445T, C548T, G667A, A858G, and G1022A) reported to producing null genotypes by either inactivating or decreasing Lewis (FUT3) enzyme activity. Phylogenetic analysis was performed using the MEGA v7.0.26 package (Tamura et al., 2007). Genotyping results of norovirus sequences were further confirmed using the automated online genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool).

5.2.6.1 Statistical Analysis

The data were analyzed using STATA 12.1 for Windows (StataCorp LP). Descriptive analysis was performed for all explanatory variables. Chi-square test was used to test the association among variables and P values <0.05 were considered statistically significant. Diarrhoeal disease severity was assessed as previously described (Chapter 3 of this thesis)

5.3 Results

5.3.1 Clinical Features of Study Population

Between August 2015 and March 2016, a total of 145 paired diarrhoeic stool and saliva samples collected from the pediatric wards of Korle-Bu Teaching and Princess Marie Louise Children's Hospital were screened for both norovirus infection and their secretor status. Eighty-one percent (81.0%) of these samples were collected from the period August 2015 to December 2015 with the remaining 19.0% collected in January and February 2016. Both genders were well represented, 49.0% (71/145) were females while 51.0% (74/145) were males. Children aged between 6 to 24 months represented 67.0% of the study population (median age of 11 months; age range of 0.5-52 months). After diarrhoea (mean of 5 episodes/day), the most common symptoms were vomiting (86.9%; mean of 4 episodes/24hrs), moderate to severe dehydration (73.1%), and fever (58.6%). Of the 145 enrolled cases, 92.4% (134/145) were categorized as severe acute gastroenteritis, defined as a Vesikari score $\geq$11, whereas 11 of the cases (7.6%) were mild to moderate diarrhoea (Vesikari score: 7- 10). Table 5.3 summarizes the clinical features of the study subjects.

Table 5.3: Demographic and clinical profile of norovirus infected and non-infected study participants

	Total Subject	Nov Pos (%)	Nov Neg (%)	P-value
Subject Number	145	79 (54.5)	66 (45.5)	
Age (months)				0.577
> 6	32	20 (62.5)	12 (37.5)	
6-24	97	51 (52.6)	46 (47.4)	
>24	16	8 (50.0)	8 (50.0)	
Clinical Profile				
Diarrhoea Duration/day				0.272
1-4	144	79 (54.9)	65 (45.1)	
5	1	0 (0.0)	1 (100)	
≥6	0	0 (0.0)	0 (0.0)	
Max No. of Diarrhoeal stools/day				0.916
≥6	45	24 (53.3)	21 (46.7)	
4 - 5	76	41 (53.9)	35 (46.1)	
1 - 3	24	14 (58.3)	10 (41.7)	
Vomiting				0.504
Absent	19	9 (47.4)	10 (52.6)	
Present	126	70 (55.6)	56 (44.4)	
Vomit Duration/day				0.906
0	19	9 (47.4)	10 (52.6)	
1	43	24 (55.8)	19 (44.2)	
2	32	17 (53.1)	15 (46.9)	
≥3	51	29 (56.9)	22 (43.1)	
Max No. of Vomiting episodes/day				0.255
0	19	9 (47.4)	10 (52.6)	
1	1	0 (0.0)	1 (100)	
2 - 4	54	26 (48.2)	28 (51.8)	
≥5	71	44 (62.0)	27 (38.0)	
Temperature (Fever)				0.672
37.1-38.4	44	22 (50.0)	22 (50.0)	
38.5-38.9	85	47 (55.3)	38 (44.7)	
≥39.0	16	10 (62.5)	6 (37.5)	
Dehydration				0.005
None	17	11 (64.7)	6 (35.3)	
Mild	22	5 (22.7)	17 (77.3)	
Moderate - Severe	106	63 (59.4)	43 (40.6)	
Treatment				0.368
Rehydration	0	0	0	
Hospitalization	17	11 (64.7)	6 (35.3)	
Rehydration/Hospitalization	128	68 (53.1)	60 (46.9)	
Vesikari Score				0.532
Non-Severe (<11)	11	5(45.5)	6 (54.5)	
Severe (>=11)	134	74 (55.2)	60 (44.8)	

5.3.2. Molecular Epidemiology of Norovirus Gastroenteritis

Noroviruses were detected in 54.5% (79/145) of the samples tested. Overall, 64 (81.1%) of 79 positive samples belonged to norovirus genogroup II (GII), 14 (17·7%) to GI, and 1 (1.3%) was a mixed infection of both GI & GII. Noroviruses were detected all through the months of study (August 2015 to February 2016) albeit at varying rates. Detection rates of the viruses were however found to be similar between males and females (male: female; 49.4% vs. 50.6%, P=0.660). Infection occurred in all the different age groups at rates that did not vary significantly (Table 5.3). Compared to older children (>2 years), children aged less than 24 months recorded the most norovirus cases (10.1%; (8/79) vs 89.9%; (71/79) respectively, P= 0.577).

Forty-four (44) of the 79 positive samples (55.7%) were successfully genotyped. A total of 9 capsid and 7 polymerase genotypes were detected among the genotyped samples (Table 5.4). Sequences for both capsid and polymerase genes were obtained for 28 of the sequenced samples whilst 18 of the sequenced samples produced only capsid genes. The most prevalent genotype combinations were GII.4P[4] (50.0%; 14/28) and GI.4P[4] (21.4%; 6/28) and the four most common capsid genotypes were GII.4 (50.0%; 22/44), GI.4 (13.6%; 6/44), GII.3 (9.1%; 4/44) and GII.5 (9.1%; 4/44). GI.1, GI.3 and other GII genotypes, including GII.2, GII.9, and GII.10 were detected sporadically. Norovirus GII.4 cases were recorded all through the period of study and consistently circulated as the most predominant genotype through all months of study (Fig 5.1). 54.5% (12/22) of the detected GII.4 genotypes could be assigned variant strains while 45.5% (10/22) remained unassigned. In total, four GII.4 variant strains were found to be co-circulating with the New Orleans_2009 variant (7) being the most prevalent, followed by Sydney_2012 (2), Osaka_2007 (2), and Osaka_2016 (1).

Table 5.4: Distribution of detected norovirus capsid and polymerase genotypes of positive samples collected from children with acute gastroenteritis

Capsid (ORF2) Genotype	Polymerase (ORF1) Genotype								Total
	GI.P1	GI.P3	GI.P4	GII.P21	GII.P4	GII.P7	GII.P30	P[NT]	
GI.1	1	0	0	0	0	0	0	0	**1 (2.3)**
GI.3	0	1	0	0	0	0	0	0	**1 (2.3)**
GI.4	0	0	5	0	0	0	0	1	**6 (13.6)**
GII.2	0	0	0	0	0	0	1	0	**1 (2.3)**
GII.3	0	0	0	3	0	0	1	0	**4 (9.1)**
GII.4	0	0	1	0	14	0	0	7	**22 (50.0)**
GII.5	0	0	0	0	0	0	0	4	**4 (9.1)**
GII.9	0	0	0	0	0	1	0	0	**1 (2.3)**
GII.10	0	0	0	0	0	0	0	1	**1 (2.3)**
Other Calici	0	0	0	0	0	0	0	2	**2 (4.5)**
Total	**1 (2.3)**	**1 (2.3)**	**6 (13.6)**	**3 (6.8)**	**14 (31.8)**	**1 (2.3)**	**2 (4.5)**	**15 (34.1)**	**44 (100)**

P[NT]: Polymerase gene not typed; **Other Calici:** other calicivirus types

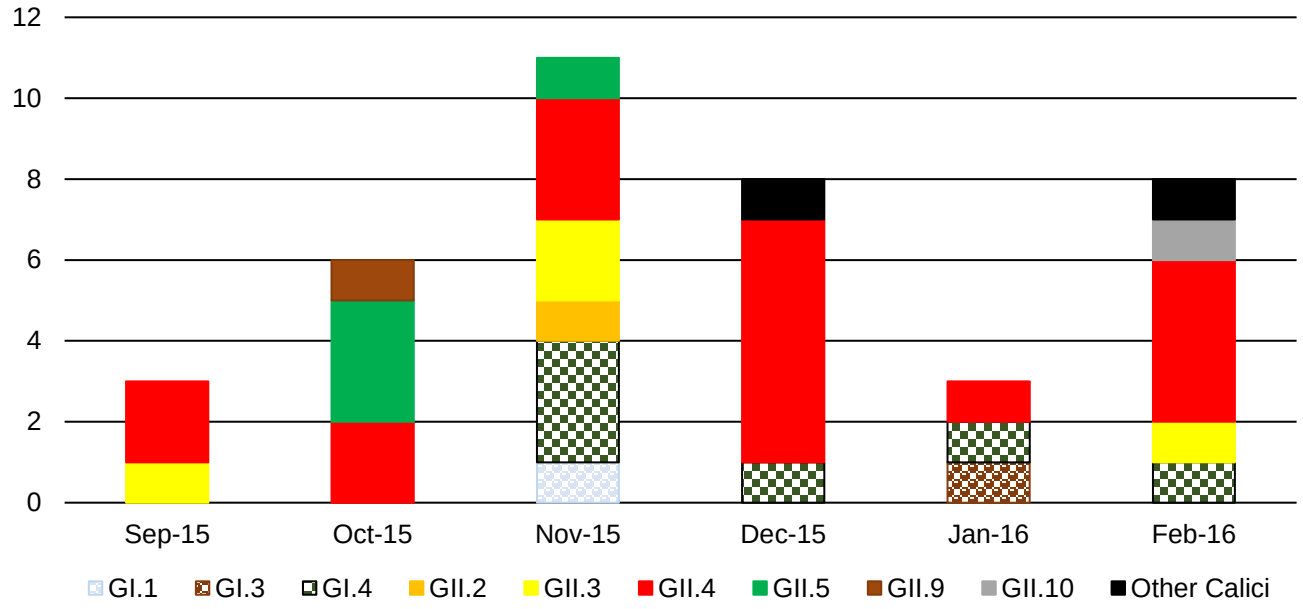


Fig 5.1: Monthly distributions of detected norovirus genotypes in infants and children <5 years of age hospitalized with acute diarrhoea from August 2015 through to February 2016

5.3.3. Genotypic Characterisation of FUT2 genes

In all 145 samples screened, *FUT2* genes were successfully amplified. Most of the study children (87.0% (126/145) were found to be secretor-positive whilst 9.0% (13/145) were secretor-negative (Figure 5.2). RFLP for *FUT2* genotyping was successful in 96% (139/145) of the samples subjected to the restriction digest analysis in the following proportions; 88 (60.7%) heterozygote secretor-positive children (Sese^{428}), 38 (26.2%) homozygote secretor-positive children (SeSe) and 13 (9.0%) homozygote secretor-negative children (se^{428}se^{428}) [Table 5.5].

5.3.4 Association of secretor status and norovirus infection

Of the norovirus symptomatic individuals, 50.0% (19/38) were homozygous secretors and 56.8% (50/88) were heterozygous secretors. More than half (62.0%; 8/13) of symptomatic patients were found to be carriers of the G428A mutation for inactivation of the FUT2 enzyme and hence non-secretors. Among the asymptomatic individuals, 50.0% (19/38) were homozygous secretors, 43.2% (38/88) were heterozygous and 38.5% (5/13) were non-secretors. No significant association was however found between secretor status and susceptibility to norovirus infection (Table 5.6).

In order to compare strain-dependent differences to HBGA susceptibility patterns, we further stratified the results according to genogroups (GI and GII) and genotypes (GII.4 and non-GII.4 genotypes) [Table 5.6; Appendix 5.1]. Children with the non-secretor phenotype were observed to be at a lower risk of being infected with norovirus genogroup I. Among the 14 children infected with GI noroviruses, only two children (14.3%) were non-secretors. Genogroup II norovirus-associated gastroenteritis was 2.4 fold more likely to occur in secretors compared to non-secretors.

For the 62 children with GII infection, 93.5% (n=58) were secretor positives, whereas 6.5% (n=4) were secretor negative children although the difference was not statistically significant ($p = 0.339$).

Also, compared to the homozygous secretor and recessive non-secretor groups, children with the heterozygous allele of the *FUT2* gene were more likely to be infected with GII norovirus (Table 5.6). All GII.4-positive samples (21/21; 100%) were isolated from symptomatic children who were FUT2 secretors. The remaining norovirus genotypes did not display any secretor phenotype specificity (Appendix 5.1).

Comparing norovirus GII.4 with non-GII.4 genotypes, it was observed that the GII.4 norovirus genotype infected more secretor-positive children who possessed the heterozygous allele of the *FUT2* gene (60.0% vs 40.0%, p=0.035) whereas the non-GII.4 norovirus strains had a preference for secretor-positive children with a homozygous allele of the gene (62.5% vs 37.5%, $p<0.05$) in the study population.

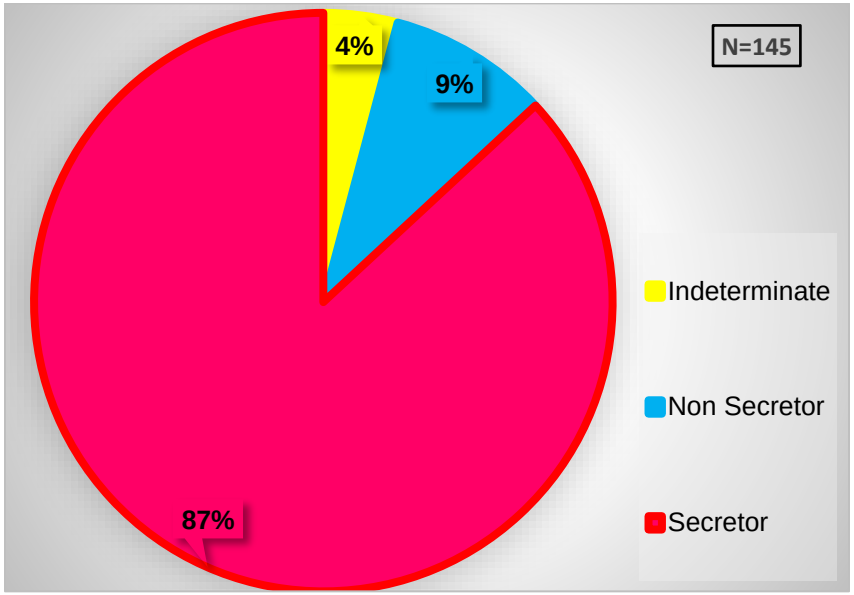


Fig 5.2: Secretor phenotype distribution among study Participants

Table 5.5: FUT2 Genotype Distribution among the study participants

Secretor Phenotype	FUT2 genotype based on G428A SNP			
	AA	GA	GG	Total
Non Secretors	13	0	0	**13 (9.0)**
Secretors	0	88	38	**126 (86.9)**
***ID**	0	0	0	**6 (4.1)**
Total	**13 (9.0)**	**88 (60.6)**	**38 (26.2)**	**145**

***ID:** Undetermined secretor type; **Genotype AA**: Non secretor; **GA**: Heterozygous secretor; **GG**: Homozygous secretor

Table 5.6: Host genetic factors and association to susceptibility to norovirus infections in the Ghanaian pediatric population

	Total	Symptomatic (%)	Non-exposed (%)	OR (CI)	P-Value
No. of patients (%)	**139***	**77 (54.5)**	**62 (45.5)**		
Secretor Phenotype					
Non-Secretor	13	8 (61.5)	5 (38.5)		
Secretor	126	69 (54.8)	57(45.2)	0.9 (0.26 - 2.91)	0.83
Genotyping for FUT2 SNP (G428A)					
GG	38	19 (50.0)	19 (50.0)		
GA	88	50 (56.8)	38 (43.2)	1.3 (0.61 - 2.82)	0.481
AA	13	8 (61.5)	5 (38.5)	1.6 (0.44 - 5.79)	0.471

	Total	GI Exposed (%)	GII Exposed (%)		
No. of patients (%)	**76**	**14 (18.4)**	**62 (81.6)**		
Secretor Phenotype					
Non-Secretor	6	2 (33.3)	4 (66.7)		
Secretor	70	12 (17.1)	58 (82.9)	2.4 (0.40 - 14.73)	0.339
Genotyping for FUT2 SNP (G428A)					
AA	7	2 (28.6)	5 (71.4)		
GA	19	4 (21.1)	15 (78.9)	2.1 (0.35 - 12.78)	0.421
GG	50	8 (16.0)	42 (84.0)	1.5 (0.21 - 10.82)	0.688

	Total	GII.4 Exposed (%)	Non-GII.4 Exposed (%)		
No. of patients (%)	**43**	**21 (48.8)**	**22 (51.2)**		
Secretor Phenotype					
Non-Secretor	4	0	4 (100)		
Secretor	**39**	21 (53.8)	18 (46.2)		0.040
Genotyping for FUT2 SNP (G428A)					
AA	**5**	0	5 (100)		
GA	30	18 (60.0)	12 (40.0)		
GG	8	3 (37.5)	5 (62.5)		0.035

***Analysis excludes Six (6) samples for which secretor status was not determined; ****AA:** Recessive Secretor; **GG:** Homozygous Secretor; **GA:** Heterozygous Secretor

5.4 Discussion

The link between histo-blood group antigen (HBGA) binding patterns to different norovirus genotypes albeit limited, has been extensively studied using virus-like particles (Huang et al., 2005a; Tan & Jiang, 2005a; Tan & Jiang, 2008) to better understand viral immune evasion. Limited studies have however been conducted on the binding patterns of wild type norovirus to HBGAs and susceptibility to natural infections. In this study we investigated whether histo-blood group antigens (HBGA's) were susceptibility makers for norovirus disease in the Ghanaian pediatric population. The results show a similar rate of norovirus infection in both secretor-positive (SeSe/Sese^{428}) and secretor-negative (se^{428}se^{428}) children. The norovirus genotypes causing the infections were however markedly different between the two secretor status groups. Significantly, all the GII.4 infections were found only among secretor-positive individuals. However, secretor-positive children possessing the heterozygous allele (Sese^{428}) of the *FUT2* gene were found to be highly susceptible to infections with both norovirus GII.4 genotype as well as non-GII.4 genotypes.

Norovirus infection was common and occurred in 54.5% of the study population. GII noroviruses were the most commonly detected infecting strains. Among the GII noroviruses, the GII.4 genotype (50.0%) was the most frequently detected, followed by GII.3 (9.1%) and GII.5 (9.1%) genotypes. GI.4 was also the most predominant GI norovirus genotype detected and it was responsible for 13.6% of the AGE cases. It has been proposed that the observed predominance of other norovirus genotypes other than that the predominance of GII.4 in the pediatric population may be because the intestinal tract of children are not very developed and populated with immature carbohydrate-like type 1 chain precursors during the first years of life. This is thought to provide

a favorable environment for opportunistic infections caused by such genotypes as the GII.3, GII.5 and GI.4 noroviruses (Ayouni et al., 2015).

In our present study, infection with either GII.4 or non-GII.4 norovirus genotypes occurred most frequently in secretor-positive children. The GII.4 norovirus strains have been associated in other studies with more severe symptoms, poorer clinical outcomes, and are more likely to be transmitted from person to person (Desai et al., 2012). It, therefore, came as no surprise that they were the most predominant genotype (50%) detected across our study population. Also, our finding that all GII.4 norovirus symptomatic children were *FUT2* secretors suggests that the non-secretor genetic status offers near-total protection against symptomatic GII.4 norovirus infection as have been proposed elsewhere (Carlsson et al., 2009; Costantini et al., 2016; Nordgren et al., 2013).

Despite the susceptibility of secretor-positive children to norovirus infection, the study also confirmed the susceptibility of secretor-negative children to a wide array of other norovirus strains (Table A3; Appendix 5.1). The number of infected secretor-negative children was however lower compared to secretor-positive children. Studies describing non-secretors infected with noroviruses are few (Carlsson et al., 2009; Lopman et al., 2015; Nordgren et al., 2010), however, to date no single genotype have been found to exclusively infect non-secretors.

In this current study, both norovirus GII.4 and non-GII.4 genotypes were observed to have significantly infected more secretor-positive children who possessed heterozygous allele (Sese^{428}) of the *FUT2* gene. A study by Tong and colleagues (Tong et al., 2014) showed that there are differences between the gut microbiome composition of homozygote and heterozygote secretors. Since most enteric viral infections have been proposed to be simultaneously dependent on both gut microbiota composition and secretor status (Jones et al., 2014b; Uchiyama et al., 2014), we

hypothesize that the composition of the gut microbiome, as well as its richness, found existing among heterozygous secretors greatly favor and aid in the attachment and entry of noroviruses into infecting cells.

As most of the norovirus vaccine candidates are VLP-based and designed for parenteral administration (Lucero et al., 2018), thereby bypassing the mucosa, the issues of HBGA expression in the small intestine are unlikely to have a great impact on vaccine immunogenicity (Nordgren & Svensson, 2019). However, the recent success in *vitro* propagation of the noroviruses makes it possible and feasible now to develop live oral norovirus vaccines. If such vaccine candidates are developed, it would be vital to characterize HBGA expression in the recipient in relation to the genotype(s) of the vaccine composition. The vaccine-uptake rate and subsequent protection are likely to be influenced if the vaccine recipient is resistant to the live vaccine strain(s), as has been observed for the live oral rotavirus vaccines (Armah et al., 2018; Bucardo et al., 2018). An ideal live oral norovirus vaccine candidate must contain virus genotypes that provide protection for both infecting genogroups as well as secretor profiles.

This study was limited by hospital-based case enrolment, hence results discussed here factors in only symptomatic norovirus cases. Given the different observations of *in vitro* studies and varying results obtained from limited epidemiological studies, large-scale community-based research may further aid in our understanding of the dynamism that exists between norovirus infection and the host genetic status.

5.5 Conclusion

In conclusion, this study has shown that the predominant GII.4 norovirus display clear secretor specificity. Nonetheless, a wide array of the other different norovirus genotypes were able to infect both secretor positive and secretor negative children to a similar extent. The reasons for this remain unclear, it has however been suggested that other unidentified host genetics and microbiological factors come into play during norovirus infection. The synergistic and antagonistic effects of these host genetics and microbiological interactions remain to be determined in future studies.

CHAPTER SIX

General Discussion and Conclusions

6.1 Overview

Human noroviruses have become the leading cause of medically attended AGE in most countries with national rotavirus vaccination programs (Lopman & Grassly, 2016) and are also a common cause of gastroenteritis outbreaks globally (CDC, 2018b). The high morbidity and mortality (Lopman et al., 2016b) as stated earlier coupled with the high cost (Bartsch et al., 2016) associated with the management of the associated disease burden especially amongst the most vulnerable in the population highlights the need for the development of strategies such as an effective vaccine for the prevention of infection and minimization of disease burden (Lopman, 2015).

The overall aim of this thesis was to get a better understanding of norovirus infection dynamics, strain diversity, evolutionary dynamics, and the host genetic factors associated with the risk of norovirus infection in the Ghanaian pediatric population. The outcome of this work would: (1) contribute to the understanding of the epidemiology, the natural history of norovirus infections, and the seasonality of norovirus infection (2) provide information important for the development of infection control guidelines and (3) provide the critical baseline data important for the future development and testing of norovirus vaccines in Ghana.

6.2 Findings and Implications

6.2.1 A virus with a high prevalence rate and large genetic strain diversity

In this thesis, a comprehensive molecular epidemiological study was performed (Chapters 3 and 4) to characterize circulating norovirus strains in the pediatric population in Ghana between 2008 and 2017. Norovirus was shown to be a significant cause of AGE in the pediatric population with an overall prevalence of 36.2 %, clearly higher than what has been reported globally (18.0%; 95% CI 17 - 20). Infection was most commonly (82.7%) observed in children aged between 6-24 months in the study population (Table 3.3), suggesting that 0-6 months would be the most appropriate age range for effective norovirus vaccination, as early prevention is most critical. Vaccination at this age would imply approximately 80.0 % of the child population would be protected. Contrary to what has been observed in the temperate regions where the norovirus disease displays distinct seasonality (Rohayem, 2009), infections occurred all year round in Ghana (a tropical country) with two seasonal peaks, the major peak between dry cool months of November to January and a minor peak in the wet months of May-July (Fig 3.5).

An extremely large norovirus genetic diversity was observed within our study population. In addition, the distribution and incidence of norovirus genotypes also changed over time and by geographic locations (Fig 3.6 – Fig 3.8). Changes in the epidemiology of noroviruses over time have been shown to impact the emergence of new strains or novel variants often resulting in changes in disease severity and increases in the number of norovirus-associated hospitalizations and deaths (Kowalzik et al., 2015a). This observation could account for the yearly changes in prevalence rates observed in this study.

Despite the changes observed in genotypes over time and across the different study belts, the GII.4 norovirus genotype remained predominant (54.1%) all through the study years (2008 to 2017; (fig 3.7). The high prevalence of the GII.4 genotype was not surprising as this genotype has been the globally predominant cause of norovirus-associated AGE and outbreaks over the last two decades (Tohma et al., 2019; van Beek et al., 2018b). Further analysis of these Ghanaian GII.4 sequences (Chapter 4) revealed the epidemic Yerseke_2006b, and Cario_2007 variants along with the pandemic GII.4_Sydney 2012 variants were predominant during the 2008-2009 and 2011- 2017 timeframes respectively (Table 4.3; Fig 4.1). Other pandemic variants including Asia_2003, FarmingtonHills_2002, Den-Haag 2006b, and New Orleans_2009 were also found in the study, although they mostly co-circulated at very low magnitude. The detection and temporal distribution as well as dominance of these variants coincided with global reports (van Beek et al., 2018b; White, 2014a).

Closer inspection of the study GII.4_Sdyney 2012 variants by phylogenetic analysis revealed them segregating into two clusters (Fig 4.2a). Cluster I contained norovirus strains with the GII.4_Sydney 2012 capsid genotype which had recombined with a [P16] polymerase virus. This strain was initially detected at lower rates between 2013 and 2015. However, between 2016 and 2017 it had become the most dominant genotype associated with infection and hospitalizations. Cluster 2, on the other hand, contained norovirus stains also with the GII.4_Sydney 2012 capsid genotype which had however recombined with a virus strain containing the [P4_New Orleans 2009] polymerase genotype as well as earlier GII.4_Sydney 2012 variants for which their ORF1 region could not be genotyped. In this study, strains within cluster 2 circulated and were predominant between 2011 and 2015.

The co-circulation of these two norovirus strains, both containing the GII.4 Sydney capsid but different polymerase genotypes show the significance of the virus biology in the exchange of the non-structural regions among the GII.4 Sydney strains as it provides them with an added advantage in the persistence of their capsid sequence within the norovirus population (Barclay et al., 2019; Hasing et al., 2019). It has also been suggested that the acquisition of a new ORF1 could affect the replication activity of the virus (Parra et al., 2017b) which may have caused increased viral fitness and/or transmissibility (Barclay et al., 2019; Lun et al., 2018b).

Norovirus genotype GII.3, commonly associated with childhood infections was the second most common genotype detected. Between 2014 and 2017, this genotype was responsible for 6.3 % of all norovirus infections in the Ghanaian pediatric population. Some studies in Europe and Asia during the 2015-2016 norovirus season reported the emergence and predominance of a new GII.3[P12] strain (Fu et al., 2019; Kuang et al., 2019; Lun et al., 2018a). This strain was however not detected in our study, instead, all the study GII.3 strains possessed the P[21] polymerase type.

The novel GII.P17-GII.17 (GII.17 Kawasaki 2014) strain was observed to replace the predominant GII.4 strains as the most important cause of norovirus associated acute gastroenteritis in many parts of the Asian region during the winter of 2014/2015 norovirus season (Chan et al., 2015a; de Graaf et al., 2015a). This new viral strain was detected a year later with wide circulation in many European countries (Chan et al., 2017a). In this study, the novel GII.17 noroviruses identified first in 2008, was the fourth most common strain (~ 5.0 %) causing clinical norovirus-associated gastroenteritis cases in the pediatric population during the period of study.

The rapid changes observed in the genetic diversity of noroviruses raise concerns about the anticipated effectiveness of the current vaccine candidates under development. With the GI and GII strains sharing less than 50 % in capsid protein homology (Vinjé et al., 2000) implies this vast

genetic diversity also translates into an extensive antigenic variation which could be a major obstacle to broad-based protective immunity following vaccination (Stern & Andino, 2016). It is only by comprehensive molecular epidemiological studies of all norovirus-associated acute gastroenteritis that we can gain an understanding of how norovirus evolves and adapts to immunological pressures which are critical for the development of effective vaccines and antiviral therapy. These studies would also provide the opportunity to well characterize viral proteins and document changes that occur in the neutralizing epitopes of the noroviruses over time.

6.2.2 Recombination is a driving force for norovirus diversification

RNA viruses generally generate diversity either by recombination or rearrangement of their genome (Lai, 1992). This thesis shows the important role recombination plays in norovirus genetic diversification (Chapter 4). Recombination events have been commonly reported to frequently occur between the overlapping junction of the ORF1 and ORF2 regions of the norovirus genome (Bull et al., 2007), thus enabling the virus to continually evolve and sometimes able to evade the already established immunity within the population, thereby causing epidemics and pandemics (Eden et al., 2013a; Lam et al., 2012; Motomura et al., 2010). The recently introduced dual typing system makes it perfect for the easy identification of circulating recombinant forms (Kroneman et al., 2013). The present study identified the emergence of 17 inter-genotypic and intra-genotypic recombinant types (Table 4.1). Two (2) recombinant forms were isolated from the GI genogroup while the remaining 15 recombinant types were from the GII genogroup.

Of these 17 recombinant forms, GII.4_Sydney 2012[P16] (26.6%, n=16/54) was the most predominant recombinant strain identified. Since 2015, the [P16] polymerase genotype has been globally reported and found in combination with several other capsid genotypes including GII.1,

GII.2, GII.3, GII.10, GII.12 (Barclay et al., 2019; Cannon et al., 2017b), and more recently GII.5 (from this study). Interestingly, work by Cannon et al., reported this novel [P16] polymerase type to be genetically distinct from those that had been in low circulation since 1975 (Cannon et al., 2017b). The acquisition of the [P16] polymerase genotype by several other capsid genotypes did not appear to have influenced virulence nor explored the immunological naivety of the pediatric population (Barclay et al., 2019). Instead, this polymerase genotype seemed to have introduced mutations that have improved viral fitness and transmissibility of the dominant norovirus genotypes and hence the increased number of cases as observed in this study as well as globally.

GII.3[P21] was the second most predominant recombinant strain detected amongst our study population. Ever since their identification, the GII.3 noroviruses have always been reported as recombinant strains possessing non-GII.3 RdRp genotype. Since 2000, the common types of RdRp genotypes associated with the GII.3 capsid have been GII.P12, GII.P21, and more recently GII.P16 (Mahar et al., 2013). The nucleotide substitution rate of the study GII.3 VP1 gene sequences calculated in this study (3.6–9.2 x 10^{-3} nt substitutions/site/year) were found to be comparable to that of that study GII.4 sequences (3.7–7.2 x 10^{-3} nt substitutions/site/year). Polymerase gene switching has been reported as providing an efficient mechanism that might have allowed the GII.3 viruses to acquire increased rates of mutations thereby improving the replicative ability of the virus (Mahar et al., 2013). The GII.3 genotype, regardless of its RdRp type, has always shown a strong association with pediatric infection (Mahar et al., 2013). Studies by Boon and colleagues suggest that the naïve immune system displayed by young children may serve as an added advantage for GII.3 viruses (Boon et al., 2011a). Additionally, the immature gut, as well as the differences in gut microbiome composition of infants and young children, are likely to present different binding opportunities for the GII.3 strain to establish infection and cause disease (Ayouni et al., 2015).

In this current study, though the GII.7 norovirus genotype was less frequently detected within our population, its polymerase genotype [P7] seemed to be more prone to recombine with other capsid genotypes including GII.4 GII.6 and GII.9 (Table 4.1).

The GI noroviruses were responsible for approximately 8.9% of infections within our study population. Norovirus GI cases compared to the GII's are less frequently detected from clinical samples (Kazama et al., 2017; Vega et al., 2014). The majority of GI's analyzed (Table 4.1) in this study were wild-type strains (possessing the same capsid and polymerase genotype) except for the two (2) (GI.1[P13], GI.1P[14]) recombinant strains identified. It has been suggested elsewhere that, of the two genogroups, the GI noroviruses which are more common in environmental samples (Gentry et al., 2009; Lee & Kim, 2008; Lysen et al., 2009) tend to cause mostly asymptomatic infections or present with mild clinical symptoms not requiring hospitalization (Kitajima et al., 2012). Since this study was hospital-based surveillance that enrolled children presenting with moderate-to-severe diarrhoea, a lot of the GI cases would have been missed and hence the small numbers of GI infections observed.

Though further studies are needed to identify the regions of breakpoint of the detected recombinants strains, work from this thesis has shown that norovirus capsids which subsequently acquired a new ORF1 region, were most likely to have had a genetic and selective advantage for continued human infection. These strains also likely had an increased ability to evade host immunity, as population immunity was directed towards the previous circulating antigenic proteins (Stern & Andino, 2016). This work further highlights the importance of dual norovirus genotyping by both regions B (Polymerase) and C (capsid) for full strain identification.

6.2.3 No specific strain could be associated with severe AGE

Importantly, we investigated the correlation between circulating genotypes and clinical outcomes using the Vesikari Clinical Severity Scoring System (Chapter 3). Only a few studies have correlated the association between norovirus infection to circulating genotypes among children (Harris et al., 2019; Mathew et al., 2019). Although the relationship between pathogenicity, virulence, and symptoms of infection by either GI or GII norovirus strains remains speculative, these studies have suggested that the GII.4 norovirus genotype had greater virulence when compared to other non-GII.4 genotypes (Desai et al., 2012; Huhti et al., 2011). Results from our study showed that both genogroups (GI & GII) were able to cause severe infections within the pediatric population. (Appendix 3.4). Unlike the previous reports, we commonly observed that children infected with non-GII.4 norovirus strains recorded equally severe clinical illness comparable to that caused by GII.4 norovirus strains. More interesting was the observation that all the top five commonly detected norovirus genotypes in our study population (GII.4, GII.3, GII.6, GII.5, and GII.17) were associated with severe AGE. While prolonged diarrhoea and vomiting were more frequently observed in children infected with GII.3 and GII.4 norovirus strains, severe dehydration was more commonly associated with GII.3 strain.

These findings may have implications for the selection of norovirus vaccine candidates. While a monovalent GII.4 vaccine could cover the majority of hospitalized norovirus AGE cases, a polyvalent vaccine complemented with other circulating non-GII.4 genotype(s) such as the GII.3 genotype may offer additional protection, especially for young children, and should be considered.

6.2.4 Other Host genetic factors may play a role in norovirus infection

Research on host genetic factors and their association to disease susceptibility has rapidly increased over the last few years (Burgner et al., 2006; Chapman & Hill, 2012). Knowledge gained from such research works has generated insights into the pathophysiology of micro-organisms (Asner et al., 2014), immune responses required for protection (Scepanovic et al., 2018) as well as possible targets for vaccines or antimicrobial treatment (Castiblanco & Anaya, 2015). The epidemiology of norovirus is complex with over 35 genotypes known to cause infections in man (Chhabra et al., 2019). In chapter 5 of this thesis, the role of host genetic effect on susceptibility to norovirus infection in the pediatric population was investigated. The study showed a similar rate of norovirus infection in both secretor-positive and secretor-negative children. What differed significantly between these two groups was the difference in norovirus genotypes infecting each group. All detected GII.4 norovirus symptomatic children were *FUT2* secretors. This observation suggests that the non-secretor genetic status offered near-total protection against symptomatic GII.4 norovirus infection as have been proposed elsewhere (Costantini et al., 2016; Nordgren & Svensson, 2019). Non-secretors were, however, susceptible to a wide array of other non-GII.4 norovirus genotypes though no single genotype was found to be exclusive to this group. The surprising observation that both GII.4 and non-GII.4 norovirus genotypes commonly infected heterozygous secretor-positive children compared to children possessing the homozygous allele of the gene. The full significance of this observation remains unclear and would require further research.

6.3 Limitations and Future direction of Norovirus Research

An important limitation to this study was the lack of non-diarrhoeal healthy controls. The studies conducted in this research work only used clinical samples collected from hospitalized children with severe AGE, thus the norovirus epidemiology described in this thesis only represents the circulating strains from hospitalized children with gastroenteritis and symptoms of norovirus infection. There is also the possibility that the clinical surveillance missed patients with mild uncomplicated diarrhoea. In addition, because this current study did not include an age-matched control group we were unable to compare the prevalence of norovirus infection as well as circulating genotype diversity in symptomatic against asymptomatic healthy children.

For a more comprehensive norovirus epidemiological picture in the Ghanaian population, community-based in addition to environmental studies would be needed. Such studies would provide us with a better understanding of norovirus disease burden, transmission patterns, as well as the complex interplay that exists in the evolutionary dynamics between environmental, community carriage, and clinical strains. Though presently several sensitive and specific diagnostic techniques are available for the detection and characterization of the noroviruses, there is still an urgent need for the optimization of these techniques.

6.4 Conclusion

The results obtained from this study show that norovirus infections are a common cause of pediatric gastroenteritis within the Ghanaian population. The complexity of norovirus epidemiology, evolution, and the indispensable role that genetic recombination play in the emergence of new strains and variants of the noroviruses are further highlighted by the results. The study also showed that between the years 2008 and 2017, noroviruses with the GII.4 lineage were the major cause of norovirus-associated AGE in the pediatric population in Ghana. In

addition, the non-GII.4noroviruses were also very frequently detected in stools of infected children and strongly associated with the generation of the observed widespread genetic diversity of circulating strains. These non-GII.4 noroviruses were able to infect both secretor positive and secretor negative children to a similar extent. Furthermore, clinical symptoms were observed in children infected with non-GII.4 norovirus strains were similar to those observed in children infected with GII.4 norovirus strains. The evolution of the norovirus capsid gene seemed to be dependent on its polymerase gene. Of the GII.4 variants detected, the pandemic GII.4_Sydney 2012 variant appeared to have had an increased capacity for evolutionary changes in response to population immunity. This variant also showed higher transmissibility rates because of its ability to recombine with other co-circulating strains. The irregular emergence and seasonal fluctuations of circulating norovirus strains within the population imply the identification and characterization of conserved neutralizing epitopes of the numerous norovirus capsid proteins is of utmost importance for the development of a successful vaccine that could generate broad protective immunity.

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APPENDICES

Appendix 3.1

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
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On 13th July, 2017, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) conducted expedited review and approved your protocol titled:

TITLE OF PROTOCOL : **Molecular Epidemiology of Noroviruses in Ghanaian Children**

PRINCIPAL INVESTIGATOR : **Belinda Naa L. Lartey, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 12th July, 2018. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

Appendix 3.2:

In-House Ethanol/Sodium Acetate Precipitation Method

1. In a 1.5 ml Eppendorf tube, add 2 volumes of cold absolute ethanol and 1/10 volume of 3M sodium acetate (pH 5.2) i.e. 20.0 µl absolute ethanol and 1.0 µl sodium acetate to 10.0 µl of sequenced DNA.
2. Mix well by pulse vortexing for 30sec, briefly centrifuge down the contents and incubate mixture on ice for 30 minutes.
3. Spin in a micro-centrifuge for 3 minutes at maximum speed (13,500 rpm) and discard the supernatant
4. Add 0.5 ml (500 µl) of cold 70% ethanol to the pellet and spin in a micro-centrifuge for 1 minute at maximum speed (13,500 rpm).
5. Discard the supernatant and dry the pellet in a vacuum dryer or on a heat block.
6. Keep dry pellet in the dryer until loading onto the Genetic analyzer or store at -20 ^{O}C until ready to use.

Appendix 3.3

Table A1: The Vesikari Scoring System

Parameter	Severity Score		
	1	**2**	**3**
Diarrhea	-	-	-
Maximum number of stools (per day)	1 – 3	4 - 5	≥6
Diarrhea duration (per day)	1 – 4	5	≥6
Vomiting	-	-	-
Maximum # of vomiting episodes (per day)	1	2–4	≥5
Vomiting duration (per day)	1	2	≥3
Maximum body temperature (℃)	37.1–38.4	38.5–38.9	≥39.0
Severity of dehydration	N/A	mild	Moderate-Severe
Treatment	Rehydration	Hospitalization	Rehydration/ Hospitalization
Severity rating scales	**<7 (mild)**	**7–10 (moderate)**	**≥11 (severe)**

Table adapted from Ruuska and Vesikari, 1990; *Severity of dehydration was as assessed by the clinician

Appendix 3.4:

Table A2: Association between genotypes and clinical severity of illness in norovirus infected children as measured with the Vesikari Scoring System

Norovirus (NoV) Type	Total	Vesikari Scores (VS)			P-Value
		Mild (VS <7)	Moderate (VS = 7 - 10)	Severe (VS ≥11)	
NoV Genogroup					0.755
GI	**66**	0	12 (18.2)	54 (81.8)	
GII	**326**	5 (1.5)	51 (15.6)	270 (82.8)	
GI/GII	**42**	0	7 (16.7)	35 (83.3)	
Total	**434 (100)**	**5 (1.2)**	**70 (16.1)**	**359 (82.7)**	
NoV Genotype					0.000
GI.1	**1**	0	0	1 (100)	
GI.3	**4**	0	0	4 (100)	
GI.4	**6**	0	2 (33.3)	4 (66.7)	
GI.7	**6**	0	1 (16.7)	5 (83.3)	
GII.1	**1**	0	1 (100)	0	
GII.2	**5**	0	2 (40.0)	3 (60.0)	
GII.3	**13**	0	0	13 (100)	
GII.4	**86**	0	10 (11.6)	76 (88.4)	
GII.5	**7**	0	1 (14.3)	6 (85.7)	
GII.6	**9**	0	2 (22.2)	7 (77.8)	
GII.7	**1**	0	0	1 (100)	
GII.8	**2**	0	1 (50.0)	1 (50.0)	
GII.9	**2**	0	0	2 (100)	
GII.10	**1**	0	0	1 (100)	
GII.12	**3**	0	0	3 (100)	
GII.13	**2**	1 (50.0)	0	1 (50.0)	
GII.17	**7**	0	0	7 (100)	
GII.21	**4**	0	0	4 (100)	
Total	**160 (100)**	**1 (0.6)**	**20 (12.5)**	**140 (87.5)**	

Appendix 5.1

Table A3: Distribution of Secretor status among the different norovirus genotypes detected

Norovirus Type	N	Secretor Phenotype		FUT2 Genotype for (G428A) SNP		
		Secretor	Non secretor	sese	Sese	SeSe
Genogroup I	**8**	**7**	**1**	**1**	**4**	**3**
GI.1	1	1	0	0	1	0
GI.3	1	1	0	0	0	1
GI.4	6	5	1	1	3	2
Genogroup II	**35**	**32**	**3**	**4**	**26**	**5**
GII.2	1	1	0	0	0	1
GII.3	4	3	1	2	2	0
GII.4	21	21	0	0	18	3
GII.5	4	3	1	1	2	1
GII.9	1	0	1	1	0	0
GII.10	1	1	0	0	1	0
Other Calici	3	3	0	0	3	0