MOLECULAR DETECTION OF NOROVIRUS GI AND GII GENOTYPES IN CHILDREN LESS THAN TWO YEARS OF AGE AND IMPACT ON CHILD GROWTH.

BY

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A dissertation Submitted in Fulfillment of the Requirements for the Master of Science (MSc) Degree in Microbiology.

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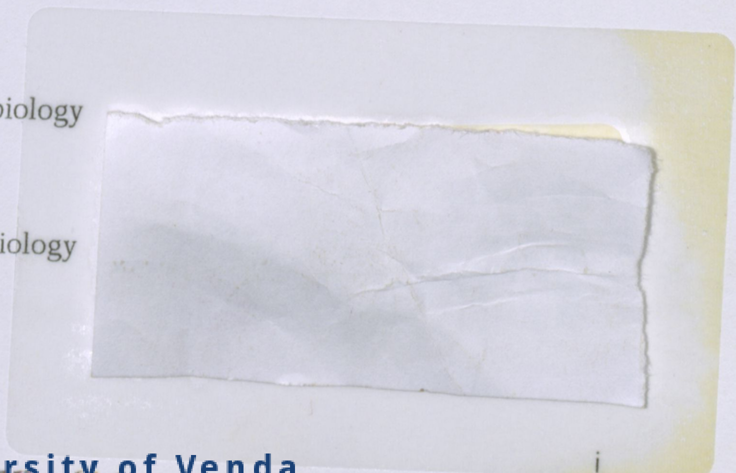

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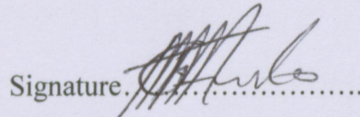
STATEMENT OF ORIGINALITY

I Glenton Thabo Moloro, hereby declare that this dissertation for the award of Masters Degree in Microbiology (MScMB), University of Venda has not previously been submitted for a degree at this or any other University, and all references contained therein have been duly acknowledged.



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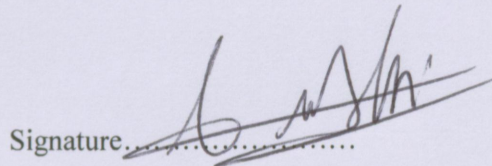

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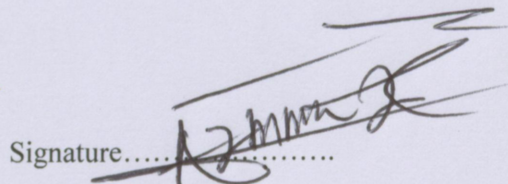

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DEDICATION

I dedicate this work to the people who mean a lot in my life and gave me courage to continue. To my parents who stood by me at all times as mentors, pillars of strength and shoulders I always leaned on.

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I thank God for giving me strength to hold on. I thank my supervisor and other senior staff members in the school of Mathematics, Microbiology and Biochemistry Departments. I would also thank friends who were colleagues in the same department as me, knowing you added more to my learning. I thank you for the work you helped me with, advises and discussions we had.

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LIST OF ABBREVIATIONS

$3CL^{pro}$:	3C-like protease
CP:	Crossing Points
Cryo-EM:	Cryo-electron microscopy
CsCl:	Cesium-Chloride
EM:	Electron Microscopy
FCV:	Feline calicivirus
FUT:	Fucosyltransferase
GI:	Genogroup 1
GII:	Genogroup 2
HBGAs:	Human Histoblood Group Antigens
HIV:	Human Immunodeficiency Virus
ICTV:	International Committee on Taxonomy of Viruses
ICTV db:	International Committee on Taxonomy of Viruses Data Base
JV:	Jena Virus
MNV:	Murine Norovirus
Na*Cl*:	Sodium Chloride
NCBI:	National Centre for Biotechnology Information

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N/S: N-terminal/Shell capsid

NLV: Norwalk-Like Virus

NoV: Norovirus

ORF: Open Reading Frame

OTUs: Operational Taxonomic Units

p22: Norovirus Protein 22 (*p37, p40, p41, p48*)

RdRp: RNA-dependent RNA-polymerase

RHDV: Rabbit hemorrhagic disease virus

RNA: Ribonucleic Acid

RoV: Rotavirus

rtRT-PCR: Reverse Transcriptase Real- Time Polymerase Chain Reaction

SaV: Sapovirus

SD: Standard Deviation

SPSS: Statistical Package for the Social Sciences

SRSVs: Small-Round-Structured Viruses

ssRNA: single-stranded-RNA

VLPs: Virus-like particles

VP: Viral Protein

WHO: World Health Organization

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ABSTRACT

Background: Noroviruses (NoVs) are, after Rotaviruses, the second most common causative agents of acute gastroenteritis in young children. In South Africa, NoV was first reported in 1993 in gastroenteritis associated with the consumption of salad. NoV antibody prevalence was later reported in both urban and rural South African populations, and Norwalk and Hawaii strains were detected. However very few studies have been conducted to identify strains of NoV in the country and their impact on child growth is not well understood. This study aims to identify common NoV genogroups and the strains that are more prevalent in diarrhoeal and non-diarrhoeal stool samples of children less than 2 years of age in rural areas in Vhembe district, South Africa, using reverse transcriptase Real Time PCR. Moreover, an investigation of the impact of different genotypes on child group was determined.

Methodology: In the present study, 185 children were recruited of whom 88 were males and 97 females. Of all the children 141 had experienced diarrhoea at least once while 44 never had diarrhoea. Samples were treated with Sodium Chloride (NaCl), and RNA was purified from them using the QiaAmp viral RNA purification kit and stored at -20°C. Following RNA purification, samples were subjected to One step reverse transcriptase real-time PCR to detect NoV genotypes. Positive samples were further run in reverse transcriptase PCR using specific primers that amplify genogroup-specific sequences of the N-terminal and shell (N/S) region of the NoV VP1 gene, and the cDNA synthesized was run in a conventional PCR. Successfully amplified conventional PCR products for NoV GI and NoV GII were sequenced and the sequences aligned and compared with the existing sequences in the GeneBank, in order to determine the genetic relationship and variability of strains of NoV in Vhembe district, South Africa.

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Results: In the study, 708 samples were tested, of which 256 (36.2%) were diarrhoeal samples and 453 (63.8%) were non-diarrhoeal samples. Norovirus GII was the most common genogroup detected in the present study. Among the 256 diarrhoeal samples, 34 (24.1%) were GI, and 93 (66.0%) were GII. Five (13.2%) of the children who were born stunted presented with NoV GI, whilst 22 (57.9%) of them presented with NoV GII at 12 months. The number of infection increased with the children's age. About 12 (61.7%) of the children who were stunted presented with NoV GI and 29 (61.7%) of the children who were stunted presented with NoV GII. Norovirus GII infections showed to be the highest in June (48.1%), October (48.0%) and November (50.6%) whilst infection with Norovirus GI was the highest in October (20.0%). Analysis of sequences showed that the NoV strains differed in their sequences up to 40%. Comparison of study strains with reference has shown the difference in the sequences of the strains, indicating a high mutation rate among Norovirus strains.

Discussion: The present study shows that infection with GII is the most common variant among children and contracted easily during the winter months of the year. Reverse-transcriptase RT-PCR can be recommended as a rapid and sensitive method that can be used to detect NoV, in order to give a quick response to eliminate and prevent infection. Though sequences detected are of similar strains, their nucleotide alignment varies due to the high mutation rate of NoV. Mutations in NoV can cause a problem with vaccine development or an alteration to confer vaccination in order to prevent infection, and spread of NoV.

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Conclusion: The present study shows how NoV affects the children's health and has an effect on the child's nutritional state and growth. It also shows that Norovirus strains differ from one country to another, this also include seasonal variation of Norovirus genogroups, which calls for a lot of attention on vaccine development. Norovirus has shown to be more active during winter season and has NoV GII as a predominant strain of which other studies also agree with these results.

Keywords: Norovirus, GI genotype, GII genotype, reverse-transcriptase RT-PCR, Vhembe, South Africa

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CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

Norovirus (NoV), also known as Norwalk virus, are after Rotaviruses, the second most common causative agents of acute gastroenteritis in young children (Puustinen *et al.,* 2012). In 1991 NoV was associated with an outbreak of gastroenteritis from consumption of salads in South Africa, and it was then later reported in other studies conducted in the country (Netshikweta *et al.,* 2011; Ramudingana, 2009). In one of the studies NoV antibody prevalence levels of 94 - 96% were detected in both urban and rural South African populations, including Norwalk and Hawaii strains (Mans *et al.,* 2010).

Acute diarrhoea is one of the most common pediatric illnesses worldwide. The four significant viral causes of diarrhoea in children under the age of 5 years are Rotaviruses, Caliciviruses (Norovirus and Sapovirus), Adenoviruses and Astroviruses (Moyo *et al*., 2007). Amongst these viral agents, Norovirus (NoV) and Rotavirus account for most sporadic gastroenteritis and outbreaks (Wu *et al.,* 2008).

Noroviruses (NoVs) and Sapoviruses are genetically and antigenically diverse single-stranded RNA viruses that belong to 2 different genera *Norovirus* and *Sapovirus* in the family *Caliciviridae.* They are collectively referred to as human Caliciviruses. Unlike Rotaviruses, Norwalk virus infection is more common in older children and adults than in infants and toddlers (Patel *et al.,* 2008). NoVs have shown to be a significant agent of sporadic gastroenteritis in the developed part of the world (Ayolabi *et al*., 2010). They are also the leading cause of non-bacterial acute gastroenteritis outbreaks worldwide, responsible for more than 86.0% of all outbreaks caused by viruses; and have been associated with chronic diarrhoea among transplant patients (Ramudingana, 2009; Widdowson *et al.,* 2005).

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NoVs are genetically classified into 5 genogroups, GI–GV, in which GI and GII strains are responsible for most human disease. Over the past 15 years, new GII.4 variants have been identified; some have been associated with a global increase in the number of outbreaks. Attempts to grow human NoV in cell culture have largely failed, leaving many details of the replication and life-cycle unclear (Vega *et al*., 2011; Furman *et al*., 2009).

Electron Microscopy (EM) was the first tool used for identifying NoVs. Immunologic assays developed during the late 1970s and 1980s improved detection rates but lacked broad reactivity to detect the full spectrum of NoVs, therefore, the cause of most gastroenteritis outbreaks could not be determined. Development and subsequent widespread use of molecular diagnostic assays have led to significant improvements in understanding the role of NoVs in gastroenteritis outbreaks. In 2007, the first NoV vaccine from Ligocyte Pharmaceuticals, together with a team of environmentalist from Baylor College of Medicine started its phase 1 trial. By 2011, a monovalent nasal vaccine completed phase I/II trials, while bivalent intramuscular and nasal vaccines are still at earlier stages of development (Hall *et al.,* 2011).

In the present study, reverse transcriptase Real Time PCR was used for the detection of Norovirus GI and GII from diarrheal and non-diarrheal stool samples from children less than 2 years old. Positive samples were further sequenced at the capsid N/S domain of the VP1 gene, and the sequences were compared to existing data base for the identification of further variations in the NoV genome. The impact of NoV infection on child growth was also evaluated.

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1.2 RATIONALE AND OBJECTIVES OF THE STUDY

1.2.1 Study Rationale

Enteric viruses are a major cause of infectious diarrhoea in children less than five years old (Mans *et al*., 2010). Although many pathogens can cause diarrhoea, more than 75% of the cases are caused by viruses (Ren *et al*., 2013). In the field of enteric microbiology, every major advance in diagnostics has enhanced our understanding of the etiology of gastroenteritis, the role of each pathogen, the different modes of transmission, and control methods that should be considered. Before 1970, more than 80% of gastroenteritis episodes did not have an etiologic diagnosis; these cases were associated to weaning, and malnutrition or most often, idiopathic causes. However by 1972 a collection of novel enteric viruses were identified by electron microscopy, and these include the Norwalk Virus (Norovirus), Rotavirus, Astrovirus, enteric Adenovirus, and Sapovirus just to name a few (Glass, 2013).

Noroviruses are identified as etiologic agents of acute gastroenteritis across all age groups worldwide, although their role as agents of gastroenteric diseases has long been unrecognized and under-appreciated because diagnostic tools were not commonly available. The application of new molecular diagnosis tools such as Real-time RT-PCR has shown that they are significant contributors to diarrhoeal disease burden in both children and adults (Ayolabi *et al*., 2010). The role of Noroviruses in developing countries has been less firmly established. However, in many Asian and African countries, most children appear to acquire serum antibodies to Noroviruses early in life, suggesting that the virus probably plays a pre-eminent role in pediatric diarrhoea (Aw *et al.,* 2009).

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Norovirus as a cause of gastroenteritis outbreaks is not well documented in Africa and in South Africa in particular. Its role in sporadic acute gastroenteritis is not well characterized in many countries (Kawada *et al.,* 2012). South Africa lacks NoV outbreak reporting systems and therefore the number and impact of NoV infections is underestimated (Mans *et al*., 2010). Lack of routine tests prevents health care providers from making etiologic diagnosis and limits our understanding of transmission within hospitals and among staff, and in the community (Glass, 2013).

Immunological protection from NoV infection and disease is poorly understood. Immunity is likely to be short-lived and primarily genogroup, if not genotype-specific (Gallimore *et al.,* 2007). The progress in the characterization and control of Norovirus has been hampered by the lack of a rapid and sensitive assay for use in clinical settings and the inability to cultivate human Noroviruses in cell culture (Hall *et al.,* 2011).

Ability to use a small animal model for the study of Murine Norovirus (MNV) provides an ideal opportunity to explore important aspects of NoV pathogenesis, virus dissemination, and host immunity that would be otherwise difficult to study (Hyde and Mackenzie, 2012). In spite of the evident importance of NoV infections, NoVs are not routinely tested for in diagnostic laboratories and NoV infections are probably underreported (Mans *et al.,* 2013). No simple immunological or biological methods are available to characterize human NoVs; therefore, sequence analysis has become the method of choice (Zheng *et al*., 2006).

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1.2.2 General Aim

The aim of the study was to determine the prevalence and genetic characteristics of Noroviruses among children less than two years of age in the Vhembe district, South Africa, and its association with malnutrition.

1.2.3 Study Objectives

The objectives of the study were:

- To determine the prevalence of Norovirus genogroups I and II in diarrhoeal and non diarrhoeal stools from children less than two years of age in the Vhembe District.
- To determine the genetic diversity of Norovirus at a portion of the capsid N/S (N-terminal/Shell capsid) domain, and assess their relatedness to sequences in GeneBank.
- To determine seasonal distribution of NoV infection in symptomatic and asymptomatic children under the age of two years.
- To determine the impact of NoV infection on child growth and malnutrition.

1.2.4 Research Questions

- Which NoV genogroup is the most common cause of diarrhoea in children under the age of two years in the Vhembe District?
- What is the impact of NoV infection on child growth in the Vhembe District?
- Are there any differences in the sequence of Noroviruses nucleocapsid N/S (N-terminal/Shell capsid) gene found in the Vhembe District and those found elsewhere?

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CHAPTER 2: LITERATURE REVIEW

2.1 HISTORY OF NOROVIRUS

Norovirus was originally named the "Norwalk agent" after Norwalk, Ohio, in the United States, where an outbreak of acute gastroenteritis occurred among children at Bronson Elementary School in November 1968. The prototype virus of the NoVs, Norwalk virus, was identified by 1972 in stored human stool that was observed under electron microscope. Numerous outbreaks with similar symptoms have been reported since then (Appleton, 1987; Patel *et al.,* 2008).

Cloning and Sequencing of the Norwalk virus genome showed that these viruses have a genomic organization consistent with viruses belonging to the family Caliciviridae. The name was shortened to "*Norovirus*" after being identified in a number of outbreaks on cruise ships and received attention throughout the United States. The name Norovirus was approved by the International Committee on Taxonomy of Viruses in 2002 (Norovirus. In: ICTVdB — The Universal Virus Database, version 4, 2006). Figure 1 and 2 shows the Norwalk virus under electron microscope.

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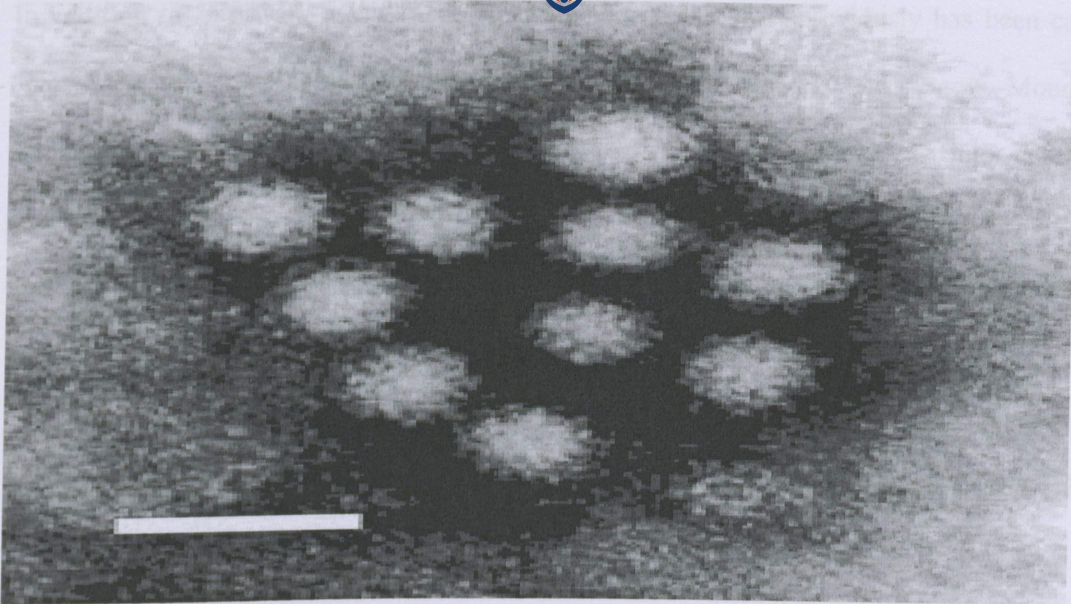

Figure 1: Norovirus from the faeces of an infected child (Gray, 2009).

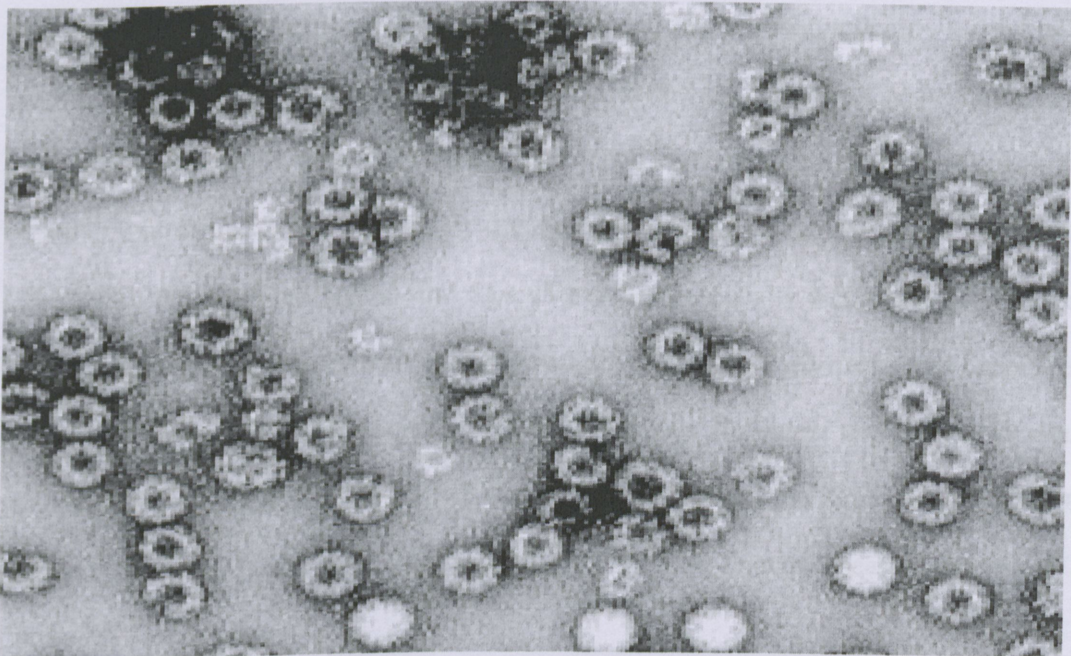

Figure 2: EM images of CsCl-purified NoV VLPs negatively stained with 2% uranyl (Hansman *et al*., 2006).

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In addition to "Norwalk agent" and "Norwalk virus," the virus previously has been called "Norwalk-like virus," "small-round-structured viruses" (SRSVs), and "Snow Mountain virus." Common names of the illness caused by NoVs still in use include "winter vomiting disease," "winter vomiting bug," "viral gastroenteritis," "acute nonbacterial gastroenteritis," and also "stomach flu," but this actually is a broad name that refers to gastric inflammation caused by various viruses and bacteria (Appleton, 1987).

2.2 CLASSIFICATION, STRUCTURE AND ANTIGENIC COMPOSITION OF NOROVIRUS

2.2.1 Classification of Norovirus

The family Caliciviridae is comprised of four genera, Norovirus, Sapovirus, Lagovirus and Vesivi. NoV and SaV are found in the genera Norovirus and Sapovirus, respectively, whilst other Caliciviruses of veterinary importance, such as rabbit hemorrhagic disease virus (RHDV) and feline calicivirus (FCV), are found in Lagovirus and Vesivirus, respectively (Tu, 2008). Noroviruses (NoVs) and Sapoviruses are genetically and antigenically diverse single-stranded RNA viruses that belong to two different genera *Norovirus* and *Sapovirus* in the family *Caliciviridae* (Patel *et al.,* 2008). The genus *Norovirus* currently comprises 5 genogroups, designated GI - GV, which can be grouped into at least 32 genetic clusters. Only genogroups GI, GII, and GIV have been associated with human disease (Ramirez *et* al., 2008).

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Furthermore, numerous genetic lineages and variants can be distinguished within genotypes, some of which have epidemiological significance (Gallimore *et al.,* 2007). Based on their capsid gene (VP1) sequence, GI genogroup is further subdivided into at least nine genotypes and GII genogroup into at least twenty genotypes, of which GII.4 is responsible for more than 85% of outbreaks reported (Yoon *et al*., 2008). However in Asia; Hong Kong in particular, GII.4 strain was detected only among sporadic NoV cases, not in outbreaks (Puustinen *et al*., 2012; Vega *et al.,* 2011; Siebenga *et al*., 2009). Figure 3 shows the classification of NoV into its five genogroups, though, not much research has been done on animal NoVs.

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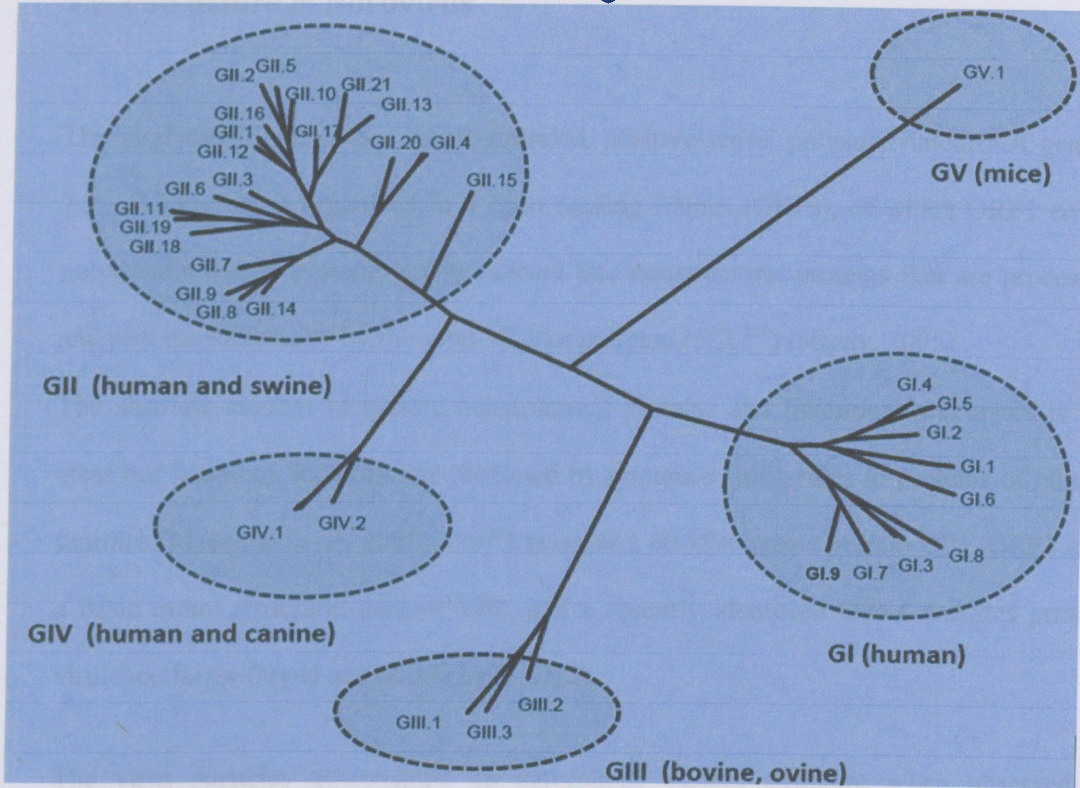

Figure 3: Genetic classification of Norovirus into five genogroups (Centre for Disease Control and prevention).

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2.2.2 Structure of Norovirus

The viral capsid encloses a single-stranded, positive-sense, polyadenylatedRNA genome of 7.3 - 7.7 kb that is organized in 4 open reading frames (ORFs), of which ORF1 encodes a polyprotein that is proteolytically cleaved into nonstructural proteins that are processed co- and post translationally by the viral 3C-like protease ($3CL^{pro}$) (Hardy, 2005).

The absolute number of mature nonstructural proteins and functional precursors is not yet clear but functions for some are predicted by sequence similarities to proteins of other virus families (Mesquita *et al.*, 2010). ORF2 encodes a 60-kDa capsid protein VP1, ORF3 encodes a basic minor structural protein VP2, and a recently identified ORF4 encodes proteins of virulence factor (Hyde and Mackenzie, 2012).

The virus particles demonstrate an amorphous surface structure when observed under electron microscopy and are between 27-38 nm in size (Victoria *et al.*, 2009). The most variable region of the viral capsid is the P2 domain, which contains antigen-presenting sites and carbohydrate-receptor binding regions (Cao *et al.*, 2007). The estimated mutation rate (1.21×10^{-2} to 1.41×10^{-2} substitutions per site per year) in this virus is high even compared with other RNA viruses (Prasad *et al.*, 2001).

The X-ray crystal structure of the viral-like particle from the prototypic GI.1 NoV identifies two domains, the shell and protruding (P) domains (Hansman *et al.*, 2006). Cryo-electron microscopy (cryo-EM) and X-ray crystallography analyses of NoV viral-like particles (VLPs) have determined the shell (P2) and protruding domains (P1) (subdomains P1-1, P1-2) of the capsid protein, with the P1 subdomain interacting with the shell and the P2 subdomain residing on the outer surface of the capsid and likely containing the determinants for antigenicity and receptor binding (Hansman *et al.*, 2011).

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2.2.2.1 Structural viral proteins

The NoV VP1 and VP2 both are synthesized from a protein-linked subgenomic RNA containing both ORF2 and ORF3. VP1 ranges from ≈ 530-555 amino acids with molecular weight of 58-60 kDa. VP1 assembles into viral like particles (VLP$_{s)}$ when expressed in insect cells by a recombinant baculovirus (Prasad *et al*., 2001).

These particles structurally and antigenically mimic native virus except that they do not contain RNA. VLPs can be expressed and purified in relatively high yield, and are the sources of most data regarding structural and functional domains of VP1 and NoV capsids. The structure of the NoV capsid (genogroup I) has been solved to near atomic resolution by X-ray crystallography. VP1 folds into two major domains designated S for the shell domain and P for the protruding domain. Figure 4 shows the arrangement of the VP1 domains and ribbon representation of a VP1 monomer, including the sizes of these domains (Prasad *et al*., 2001).

The N-terminal 225 amino acids constitute the S domain and contain elements essential for formation of the icosahedron. The P domain comprised of the remaining amino acids and it is divided into two sub-domains, P1 and P2. The P domains interact in dimeric contacts that enhance the stability of the capsid and form the protrusions on the virion. The P2 domain, a 127 amino acid insertion, is thought to play a vital role in receptor binding and immune reactivity, and possibly is primarily responsible for ABO histo-blood group antigen interactions associated with susceptibility to Norovirus infections (Gray, 2009).

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VP2 ranges from 208-268 amino acids with a molecular weight of ≈ 22-29 kDa and exhibits extensive sequence variability between strains. Roles of VP2 in the replication cycle are unclear, but it is known that VP2 is a minor structural protein present in one or two copies per virion. VP2 is not necessary for $VLP_{s,}$ assembly but is important for production of infectious virus when evaluated in a feline calicivirus (FCV) reverse genetics system (Hardy, 2005).

VP2 is a basic protein with a calculated isoelectric point of >10.0. This chemistry has led to the idea that VP2 binds RNA and may have a role in RNA genome packaging. There are as yet no experimental data that describe RNA binding activity of VP2, however, the lack of RNA binding data does not exclude the possibility that VP2 plays a role in packaging the viral genome (Glass *et al.,* 2000).

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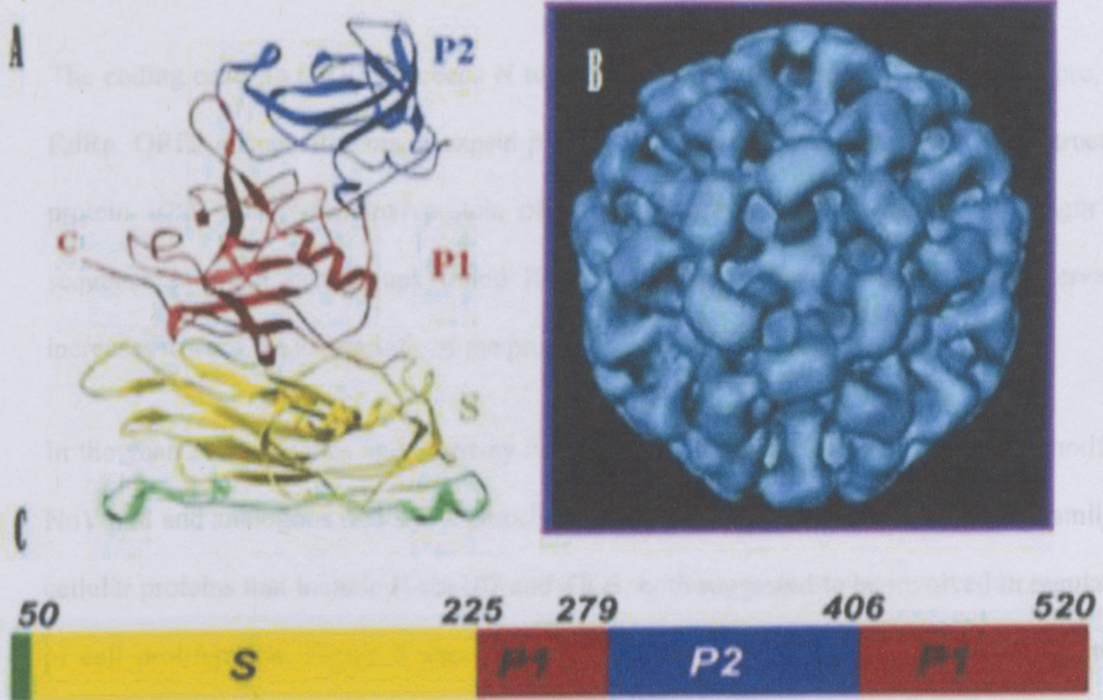

Figure 4: A: VP1domains and ribbon representation of a VP1 monomer. The schematic illustrates domains of VP1 and the colors corresponding to the ribbon structure. B: 3-Dimension structure of NoV, showing the protein formation of NoV (Gray, 2009). C: The small N-terminal domain (aa 10-49) faces the interior of the particle. The shell domain (S) is colored in yellow and extends from amino acids 50-225. The P1 subdomains are colored red and comprise amino acids 226-278 and residues 406-520. The P2 domains (blue) are an insertion in the P2 domains and consist of amino acids 279-405. The hypervariable region of VP1 is found in the P2 domains (Hardy, 2005).

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2.2.2.2 Nonstructural viral proteins

The coding order in ORF1 proceeds N to C terminus, p48, NTPase, p22, VPg, 3CLpro, and RdRp. ORF2 encodes the major capsid protein VP1 and ORF3 encodes the minor structural protein VP2. The N-terminal protein of ORF1 is to some extent variable in length and sequence between genogroups I and II viruses, and amino acid sequence conservation increases toward the C terminus of the protein (Victoria *et al*., 2009).

In the year 2000, Hughes and Stanway noted the presence of H box/NC sequence motifs in NoV p48 and analogous regions of parechoviruses. H box/NC motifs are found in a family of cellular proteins that include H-rev107 and TIG3, both suggested to be involved in regulation of cell proliferation. Figure 5 shows a labelled Norovirus genome organization (Norwalk strain), which gives an actual idea of how the structural and non-structural proteins of Norovirus are assigned (Hughes and Stanway, 2000).

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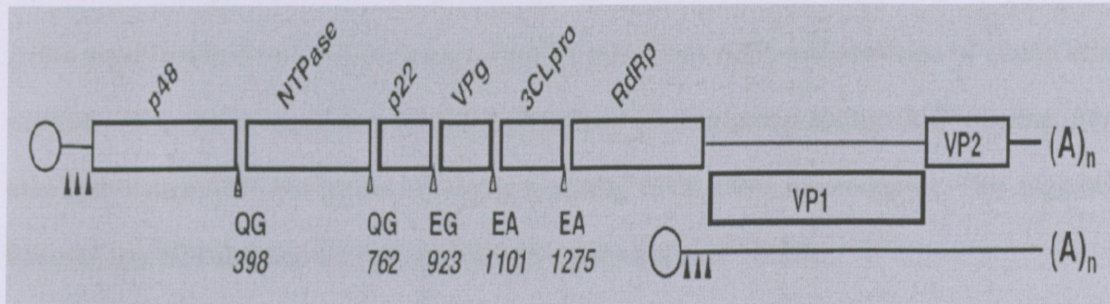

Figure 5: Labelled Norovirus genome organization (Norwalk strain). The diagram shows Nonstructural proteins in ORF1 and protease cleavage sites, indicated by open arrowheads. Amino acids numbers below the cleavage sites are the P1 residues of the recognition dipeptides. Filled arrowheads indicate translation initiation codons. The VPg-linked subgenomic RNA encoding VP1 and VP2 is indicated below the ORFs. VPg is depicted as a circle linked to both genomic and subgenomic RNAs (Hughes and Stanway, 2000).

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Norovirus protein 48 (*p48)* similar to *p37*, coordinates localization properties of VAP-A in intracellular membranes to anchor membrane-bound replication complexes during virus RNA replication. The presence of three specific motifs, A, B, and C, classify *p41* (*p40)* (NTPase) in the superfamily 3 of RNA helicases. Purified *p41* binds ATP, and mutation of amino acid residue 168 in the A motif that ablated a phosphate binding group abolish ATP binding. *p41* also hydrolyzes ATP, but cannot unwind a synthetic RNA: DNA heteroduplex. This suggests that *p41* has NTPase, but not helicase activity (Almanza *et al*., 2008).

p22 (or *p20* depending on the genogroup) occupies a position in the Norovirus genome similar to the position of the 3A protein in picornaviruses genomes (Glass *et al.,* 2000). There is yet no data on feasible functions of *p22*, with the exception of its presence in a p22-VPg-3CL^{pro} precursor in the proteolytic processing pathway. *p22* shares only limited sequence similarity with *p30* and 3A. Consistent with the replication-associated properties of 3A, the feline calicivirus (FCV) *p30* equivalent of *p22* is found in polymerase competent membrane complexes isolated from FCV-infected cells. A lot remains to be learned about this protein and how it functions compared to those of 3A, given the comparable relative positions of 3A and *p22* in their respective polyproteins (Hardy, 2005).

VPg is 15 kDa and covalently linked to genomic and subgenomic mRNAs. Evidence for this relation is known only from the animal Caliciviruses, however it is likely that the same holds true for Noroviruses. VPg plays diverse functions in replication cycles, some of which are common between families. It has been known for some time that calicivirus genomic RNA devoid of VPg is not infectious (Herbert *et al*., 1997).

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Direct experiments suggesting a role for VPg in translation of the viral RNA showed that removal of VPg from FCV genomic RNA extracted from purified virions vividly reduced the levels of viral protein synthesized in vitro, though the fidelity of initiation appeared to remain intact, thus it was deduced that VPg could function in ribosome recruitment to the viral RNA (Herbert *et al*., 1997). A strong support for the hypothesis that VPg recruits translation machinery to viral RNA was provided by data that showed that VPg interacts directly and specifically with translation initiation factor eIF3 and with 40S ribosomal subunits. The data described, imply a novel mechanism of translation initiation mediated by viral protein-protein interactions with cellular translation machinery (Daughenbaugh *et al*., 2003).

Noroviruses encode a single protease called 3C-like ($3CL^{pro}$) because of its similarity to the picornavirus 3C. The most extensive analysis of the Norovirus $3CL^{pro}$ enzyme was performed with the genogroup I Chiba virus. Norovirus $3CL^{pro}$ contains at minimum, a functional catalytic dyad comprised of His30 and Cys 139, similar to the hepatitis A virus $3CL^{pro}$ (Herbert *et al*., 1997). Analysis of the Norovirus $3CL^{pro}$ identified a nucleophilic residue and further suggested a glutamic acid residue at position 1154 relative to the entire polyprotein important for efficient processing activity. The significance of this residue as a structural or catalytic residue has not been determined. Further studies are needed to find out what cleavage activities are attributable to $3CL^{pro}$ and its $3CL^{pro}$-RdRp precursor (Gallimore *et al.*, 2007).

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Norovirus RdRp extends from amino acid 1281 to the C terminus of ORF1 (Norwalk strain numbering). The NoV RdRp overall has catalytic and structural elements characteristic of RdRp of other positive strand RNA viruses as the fingers, palm and thumb domains common to all polymerases were evident. Significant difference in the C terminal segment of the NoV RdRp was observed; it consisted primarily of the location of this domain in the active site cleft near the catalytic aspartic acid residue. The contributions of this unique domain of the NoV RdRp to RNA replication deserve further attention since the structural details that confer functional enzymatic activities may shed light on targets for antiviral design to inhibit NoV replication (Hardy, 2005).

Enzymatic studies with recombinant protein confirmed that the 3CL^{pro}-RdRp precursor is a bifunctional protein with both protease and polymerase activity, and the polymerase alone is recently reported to have similar activity to the precursor when assayed in vitro. The template requirements of the NoV RdRp and the 3CLpro-RdRp precursor are important to interpret as infectious clones become available (Belliot *et al.*, 2005).

2.3 NOROVIRUS CAPSID N/S (N-terminal/Shell capsid) GENE.

NoV strains can be further divided into genotypes based on more than 80% sequence identity in the complete capsid protein VP1. Conversely, for molecular epidemiological investigation, tentative genotyping methods based on partial genomic sequencing of the RNA-dependent RNA polymerase (RdRp) and capsid genes are commonly used. Previous studies have used the RdRp region for the amplification because this region was believed to contain the most conserved nucleotide sequence in the NoV genome (Kojima *et al.*, 2002).

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The capsid N/S domain is the most suitable region for defining the genotypes (Katayama *et al*., 2002). Like other enteric viruses that lack a lipid envelope, NoVs have a durable capsid structure allowing them to withstand temperature ranges from -80°C up to ~60°C and survive at low pH (2.7 for 3 hr) (Green, 2007; Iritani *et al*., 2008). A large number of the polymerase and capsid N/S domain sequences have been reported previously (Katayama *et al*., 2002). On the basis of the X-ray crystallographic structure of Norwalk virus, capsids are composed of 90 dimers of VP1 (Prasad *et al*., 1999). The VP1 gene is the most hypervariable part of the genome as it encodes the domain of the virus involved in receptor binding (Chen *et al*., 2013). Each VP1 monomer (530 amino acids [aa]) contains a short N-terminal region (aa 1 to 49), followed by a shell (S) domain. The N-terminal/shell (N/S) domain forms the inner core of the capsid and is the most conserved part of VP1 (Almanza *et al*., 2008). The polymerase and capsid N/S domains appeared to be suitable for the genotyping based both on topology and on pair-wise distance analyses (Katayama *et al*., 2002).

The P domain proteins lacking the N-terminal S domain are helpful for use in structural studies compared with VLPs, because the P domain proteins are easily purified from extracts of transformed *Escherichia coli* cells and crystallographic analyses of them are conducted in a short period of time. Success in amplifying many strains with genogroup-specific primers, designated to amplify the N terminal domain and a part of the shell domain of the capsid protein (capsid N/S domain) of NoV, led to use of this primer set for the detection of NoV (Kojima *et al*., 2002; Prasad *et al*., 1999).

An accumulation of mutations in the capsid region was reported in a persistently NoV infected patient who excreted the virus for more than 2 years. Most of the mutations were accumulated in the hypervariable domain (the P2 domain), which is the most exposed part of the structure and has shown to contain determinants for strain specificity (Obara *et al*., 2008).

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There are a variety of sets of primers used in RT-PCR assays to detect and group NoV. With the aid of the primers; G1SKF (5'-CTGCCCGAATTYGTAAATGA-3'), G1SKR (5'-CCAACCCARCCATTRTACA-3', G2SKF (5'-CNTGGGAGGGCGATCGCAA-3') and G2SKR (5'CCRCCNGCATRHCCRTTRTACAT-3') designated to detect NoV in RT-PCR by amplifying a capsid N/S domain, NoV can now be successfully grouped into respective genogroups and strains in most recent studies (Kojima *et al.*, 2002).

Two sites within the P2 domain have shown high degrees of variation over time, which is significant in that these sites are likely epitopes for immune responses. Named Site A and Site B, the two sites are linked to epochal antigenic shift and seasonal outbreaks, respectively. Site A specifically has been linked with the binding of HBGA, crucial for infection, while Site B helps determine the strength of the bond between the virus and the host HBGA cell (Zakikhany *et al.,* 2012).

The primers mentioned above amplify a segment known as "Region C" within the NoV genome. Correspondingly, Regions A and B are within ORF1, while Region D is downstream of Region C within ORF2 (Mattison *et al.*, 2009). Most researches have shown that different assays have varying sensitivities to different NoV genotypes, with overall sensitivities ranging from 52-73% depending on the strain. Even though, these primers had likely provided a low estimate of the NoV prevalence in Ghanaian children. Overall, of the protocols capable of genogrouping viruses, the Region C primers used in the present study yield the highest sensitivity assays (Vinje *et al.*, 2003). Figure 6 shows in detail the regions of the sequences on ORF2.

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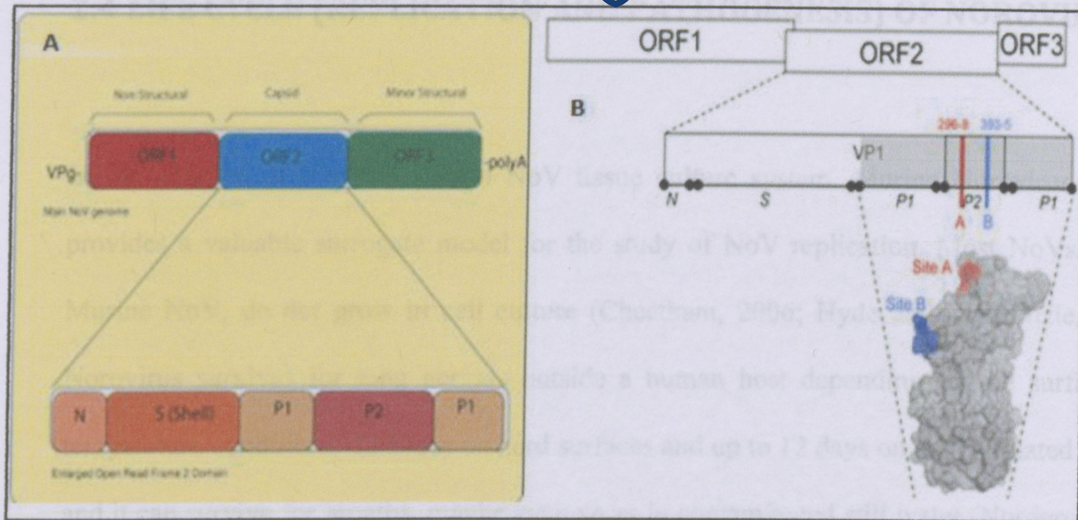

Figure 6: **A**: Basic Norovirus genome structure. The ORF2 capsid region has been enlarged to illustrate protruding domains. **B**: Location of Site A and Site B in the GII-4 NoV P2 domain. The NoV genome has three open reading frames (ORF1, 2, 3) and the major capsid protein (VP1) is encoded by ORF2. The VP1 protein has three main domains: an N-terminal domain (N), the highly conserved shell domain (S), and the protruding domain (P) which forms surface exposed spikes on the virus surface, which is further subdivided into hypervariable P2 domain (P2) and the more conserved P1 domain (P1). There are two antigenic sites in the hypervariable P2 domain of the GII-4 NoV capsid protein. Site A (shown in red) is comprised of consecutive amino acid residues 296-298. Site B (shown in blue) is comprised of continuous amino acid residues 393-395. Amino acid position numbering relative to prototype strain Lordsdale/1993/UK (Accession Number: X86557), residues mapped onto the surface of GII-4 NoV strain VA387 P-domain crystal structure (PDB Number 2OBS) described by Cao *et al* (Cao *et al*., 2007; Allen *et al*., 2008).

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2.4 LIFE CYCLE (REPLICATION AND PATHOGENESIS) OF NOROVIRUS

In the absence of a viable human NoV tissue culture system, Murine Norovirus (MNV) provides a valuable surrogate model for the study of NoV replication. Most NoVs, except Murine NoV, do not grow in cell culture (Cheetham, 2006; Hyde and Mackenzie, 2012). Norovirus survives for long periods outside a human host depending on the surface and temperature conditions: 12 hours on hard surfaces and up to 12 days on contaminated fabrics, and it can survive for months, maybe even years in contaminated still water (Nordgren *et al*., 2010). During infection, the virus begins to multiply within the small intestine. After approximately one to two days, Norovirus symptoms appear (Hyde and Mackenzie, 2012).

2.4.1 Norovirus Receptors

Human NoVs recognize histo-blood group antigens (HBGAs) that are expressed on the surface of mucosal epithelial cells and also present on the surfaces of red blood cells. The glycosyltransferases that regulate their synthesis are encoded by the highly polymorphic ABO, Lewis, and secretor gene families (Tan *et al*., 2006; Karst, 2010). NoV recognizes α1,2-linked fructose residues whose expression on gut epithelial cells and in body fluids is dependent on a wild-type *FUT2* gene. Individuals with a wild-type *FUT2* gene, referred to as secretors are susceptible to Norwalk virus infection, whilst individuals that contain a null *FUT2* allele, referred to as nonsecretors are completely resistant (Lindesmith *et al*., 2003).

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The VP1 subunit of the Norovirus consists of a shell (S) and a protruding (P) domain that is made up of middle P1 and distal P2 subdomains. The S domain is responsible for the icosahedral shell structure, the P1 and P2 subdomains have been implicated in antigenicity and cellular receptor binding of these viruses (Karst, 2010). Binding of the VP1 proteins occurs through human histoblood group antigens (HBGAs) as receptors. The P2 domain was reported to be the most protruding and is diverse among different norovirus groups, indicating its critical function in interacting with host (Chen *et al*, 2004). P2 being the most hypervariable region of the genome it is responsible for HBGA receptor binding. Several animal caliciviruses also bind HBGAs; they include the highly virulent rabbit hemorrhagic disease virus that segregates within the Lagovirus genus (Chen *et al*., 2013).

There are genogroup-specific differences in the receptor binding interface of the Norovirus capsid that interacts with the HBGA. Norovirus strain-specific binding can be segregated generally into those that bind A/B epitopes and those that bind Lewis epitopes (Tan *et al*., 2009). GI and GII strains are contained within both binding categories; hence, even if a GI and a GII NoV interact with the same HBGA, the precise residues involved in this binding will most likely be different (Shirato *et al.,* 2008).

The HBGA binding interface on NoV particles is genogroup-specific. While NoV strains exhibit different HBGA binding properties, together they can infect with nearly all individuals due to their high genetic variability (Ruvoen-Clouet *et al*., 2000). This highlights the highly adaptive nature of Noroviruses and the likelihood of a long co-evolution of human NoVs with their human host (Karst, 2010).

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2.4.2 Intracellular Replication

Viruses infect enterocytes of the upper small bowel, causing inflammation and transient blunting of the villi. Following attachment and entry, the viral genome is released out from the capsid. The positive sense RNA genome acts as a messenger RNA which is translated by host machinery in the cytoplasm into single polypeptide encoding all viral non-structural proteins (NS1-NS7) (Hyde and Mackenzie, 2012). Figure 7 shows the schematic representation of the single polypeptide. The polypeptide is co- and post-translationally cleaved by viral protease (NS6) to release functional polyprotein species and mature viral proteins that facilitates subsequent rounds of replication, mediated via the viral RNA-dependent RNA-polymerase (RdRp or NS7). When initial translation and the proteolytic release of viral proteins have occurred, the positive-sense genome serves as a template for negative-strand RNA synthesis (Hyde and Mackenzie, 2012).

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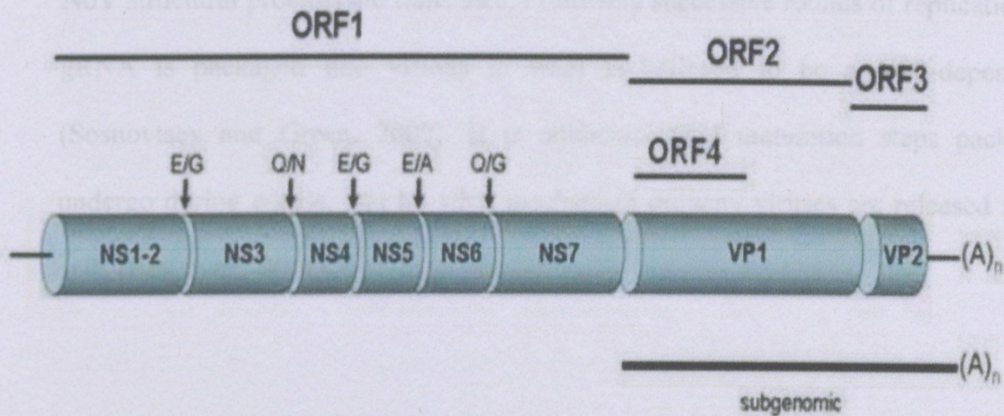

Figure 7: Genome structure and organisation of NoVs. The NoV genome is polyadenylated at the 3' end and protein linked at the 5' end to the viral encoded protein NS5 (VPg). Open reading frame 1 (ORF1) encodes six non-structural proteins which are translated as a single polypeptide and co- and post-translationally cleaved by the viral encoded protease (NS6) at the cleavage sites indicated. ORF2 and ORF3 encode the structural proteins VP1 and VP2 respectively and are translated from a subgenomic RNA (sgRNA) species which is also polyadenylated and NS5-linked. ORF4encodes for a recently identified protein thought to aid in immune evasion (Hyde and Mackenzie, 2012).

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Negative-sense RNA transcripts are then used as templates for the synthesis of more full-length genomic RNA (gRNA) and positive-sense subgenomic RNA (sgRNA) from which the NoV structural proteins are translated. Following successive rounds of replication, full length gRNA is packaged into virions in what is believed to be a VP2-dependent manner (Sosnovtsev and Green, 2000). It is unknown what maturation steps packaged virions undergo during egress, and by what mechanism progeny viruses are released from infected cells (Bok *et al*., 2011).

2.5 TRANSMISSION OF NOROVIRUS PARTICLE

It is evident that NoV infections in South Africa occur early in life and affect all groups of the population (Mans *et al.,* 2010). Transmission of Norovirus occurs through ingestion of contaminated water, food (particularly oysters), and person to person by the fecal-oral route, airborne transmission, and contact with contaminated surfaces (Bull *et al.,* 2006). Outbreaks of Norovirus infection often occur in closed or semiclosed communities, such as long-term care facilities, overnight camps, hospitals, dormitories, and prisons (Noda *et al*., 2007).

The source of waterborne outbreaks includes water from municipal supplies, wells, recreational lakes, swimming pools and ice machines. Shellfish and salad ingredients are the foods which are mostly implicated in NoV outbreaks. Ingestion of shellfish that have not been sufficiently heated poses a high risk for Norovirus infection. Majority of those infected make a full recovery within two days, but much care must be taken with the very young and older people who catch NoV, as they are at higher risk of dehydration (Parashar and Monroe, 2001).

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2.6 INTERSPECIES TRANSMISSION AND REASSORTMENTS EVENTS IN NOROVIRUS GI AND GII GENOTYPES

Though interspecies transmission of Noroviruses has not been documented, strains that infect pigs are found in GII, and a GIV Norovirus have been discovered recently as a cause of diarrhoea in dogs, which suggest the potential for Zoonotic transmission (Hall *et al.,* 2011). It has recently been thought that animals did not carry human Noroviruses, since generally species barriers seem to be rather strong for viruses. However, with the great number and variability of human Norovirus strains the idea of animal being reservoirs for NoV has become more interesting. A study from Finland has shown that pet dogs can carry human strains of Norovirus and pass them on to people in the household, though it seems very unlikely that the transmission would be as easy between man and dog since viruses are generally species specific (Goetz., 2012).

Zoonotic transmission between dogs and humans is not new, and the close and often intimate interactions between these 2 species have been suggested as a major disease risk for humans (Mesquita *et al*., 2010). Also close genetic relatedness of swine NoV with human NoVs of GII showed the potential for transfer of NoV from animals to humans. Additionally, current findings of viruses genetically related to human NoVs, as well as to animal NoV sequences in pigs and calves, have raised concerns about the possible emergence of recombinant viruses (Mesquita *et al*., 2010).

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2.7 EPIDEMIOLOGY OF NOROVIRUS

NoV causes viral gastroenteritis predominately during winter in temperate climates.The virus is resistant to pH levels between 5 to 10, acid, ether, and is able to survive temperatures of 60°C for 30 minutes. *Winter vomiting bug* is a common term for Noroviruses in the UK, because the virus tends to spread more easily in winter, when people tend to spend more time indoors and near to each other (Karst, 2010).

Little data is available on molecular epidemiology of NoV in the rural areas of South Africa as in other African countries (Mans *et al*., 2013). NoVs are genetically classified into 5 genogroups, GI–GV, with GI and GII strains responsible for most human disease. NoV genogroup GII is the most prevalent in human infections, and causes most outbreaks. The known host range of NoVs has recently expanded, the virus is now found in mice, cows, and pigs (Widdowson *et al*., 2005). NoV is genetically diverse and has broad host specificity, infecting a wide range of mammalian species including humans (GI, GII, and GIV), swine (GII), bovine (GIII), canine and lion (GIV), and Murine (GV). Genogroup three (GIII) has also been studied in calves (Hyde and Mackenzie, 2012).

NoV infections are naturally acute and self-limited, but, disease can be much more severe and prolonged in infants, elderly, and immunocompromised individuals (Karst, 2010). A number of factors contribute to the nature of NoV outbreaks. This includes the high infectivity of NoV particles, the persistence of NoVs in the environment, prolonged shedding of virus from both symptomatic and asymptomatic individuals, and a lack of lasting immunity (Rockx, 2002).

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Researchers have confirmed that GII.4 strains resulted in pandemics in 1995–1996, 2002, 2004 and 2006 (Siebenga *et al*., 2009). The GII.4 variants spread rapidly and globally and are thought to be responsible for 70-80% of all NoV outbreaks, at least since 2002 (Tu *et al*., 2008). A current study demonstrates that GII.4 strains associated with severe illness were circulating since 1974, and that the ancestral strain possibly emerged in the 1960s. It is not clear why the GII.4 NoV strains are so predominant, but possibilities include increased environmental stability, transmissibility, and virulence (Bok *et al*., 2009).

NoV GIII has not been found in humans; therefore these viruses do not appear likely to cause human disease. Zaafrane has shown the possibility of GIII infections in calves, on a study based in Tunisia (Zaafrane *et al*., 2012). Within GIII, two genotypes of bovine NoV exist; these are represented by Jena Virus (JV), which was isolated from cattle in Germany, and Newbury 2 virus, which was identified in the faeces of diarrhoeagenic calves in the United Kingdom (Karst, 2010). A closely related bovine GIII NoVs were recently identified in faecal samples from pigs and sheep in New Zealand, possibly demonstrating a third GIII genotype (Otto *et al*., 2011).

Though NoV GIV rarely infect human, NoVs GIV (Alphatron-like) have been recently identified in small and large carnivores and classified as GIV.2. Furthermore, a recombinant canine NoV strain was found to have GIV.2 ORF1 (polymerase complex) but novel ORF2 (capsid protein) dissimilar to any known NoV genogroups and genotypes (Ntafis *et al*., 2010). The first NoV to infect mice was described in 2003. The NoV GV has been proposed to accommodate the recently identified Murine Norovirus 1 on the basis of its distinct capsid protein sequence (Karst *et al*., 2003).

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2.8 GEOGRAPHIC DISTRIBUTION OF NOROVIRUS GENOGROUPS IN SOUTH AFRICA AND SOME AFRICAN REGIONS

NoV GI, GII and GIV (rarely), in order of greatest to lowest numbers, are responsible for human outbreaks. Within the GII genogroup, GII.4 strains are responsible for most human NoV outbreaks, including pandemics (Matthews *et al*., 2012). In2008 GII.4 variant was the most frequently detected strain in South Africa (Mans *et al*., 2010).

A seroepidemiological study in the Pretoria area of South Africa showed that 57% of children were exposed to NoV GII.4 at the age of 1–2 years. A later study revealed NoV antibody prevalence levels of 94–96% in urban and rural South African populations (Mans *et al*., 2010). A more recent study in Gauteng province, SA indicated that after rotavirus (24% prevalence), NoV is the most frequently detected viral pathogen in paediatric patients hospitalized with viral gastroenteritis (Mans *et al*., 2013).

Previous studies using baculovirus-expressed Norwalk virus (NV) and Mexico virus (MxV) capsid antigens have revealed that human calicivirus infection is common in South Africa. In the study, surveillance was extended to different populations, as well as to four other Southern African countries which are Namibia, Angola, Zimbabwe, and Mozambique (Smit *et al*., 1999). Norovirus GI and GII strains were identified in Botswana (Mattison *et al*., 2010). In Lagos, Nigeria a study by Ayolabi confirmed NoV genogroup GII as the predominant type in circulation (Ayolabi *et al*., 2010).

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Similar findings were done by Oluwatoyin *et al*, in a study confirming that NoV GII infections is more common than GI in Nigeria (Oluwatoyin *et al* 2012). An epidemiological study was conducted in Kenya to clarify the prevalence of Human Calicivirus infections in infants, since systematic survey of the Human Calicivirus infections and associated gastroenteritis in infants has not been conducted (Nakata *et al*., 1998). Zaafrane *et al*., has shown that NoVs GIII and Nebovirus are endemic in diarrheic calves in Tunisia (Zaafrane *et al*., 2012).

There are limited findings on the Norovirus Genogroup GIII, GIV, and GV, as they rarely infect humans and are mostly found in animals. GIV rarely infect human, the remaining genogroups known to infect animals have not been much documented, due to lack of detection methodologies, or can be the fact that NoV infections in Africa are less than other countries, such as Europe and Switzerland (Hall *et al.,* 2011).

2.9 DETECTION AND DIAGNOSIS OF NOROVIRUS

Electron microscopy was the first tool used for identifying Noroviruses but proved to be a time-consuming and insensitive method that was rarely available outside of research settings. Immunologic assays developed during the late 1970s and 1980s improved detection rates but lacked broad reactivity to detect the full spectrum of Noroviruses and were not extensively available, preventing their utility for routine use in outbreak investigations, Therefore, the cause of the most gastroenteritis outbreaks could not be determined (Hall *et al.,* 2011).

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Since the 1990s, the development and subsequent widespread use of molecular diagnostic assays have led to significant improvements in understanding the role of Noroviruses in gastroenteritis outbreaks. Nonetheless, progress in the characterization and control of NoV has been hampered by the lack of a rapid and sensitive assay for use in clinical settings and the inability to cultivate human NoVs in cell culture (Hall *et al.,* 2011). Routine protocols to detect NoV (Norovirus RNA) in clams and oysters by Reverse Transcription Polymerase Chain Reaction are being employed by governmental laboratories such as the FDA in the USA (Shieh *et al.,* 2000).

Since a large proportion of NoV illness results from foodborne exposures, great effort has gone into the development of methods for detecting and eliminating virus contamination from food items, particularly shellfish and fresh produce (Monroe, 2011). In the past, NoV genotyping was performed solely on the RNA-dependent RNA polymerase (RdRp) region of open reading frame ORF1 of the single stranded, positive-sense NoV RNA genome. Later studies showed better segregation of the different strains into their respective genotypes by phylogenetic analysis of nucleotide sequences within the capsid region of ORF2 (Puustinen *et al*., 2012).

However, genotyping based solely on the capsid sequence would miss the naturally occurring recombinant NoVs which cluster into two distinct groups of NoV strains when regions RdRp and capsid are subjected to phylogenetic analysis (Puustinen *et al*., 2012). Expression of the major capsid protein (VP1), which usually results in the formation of virus-like particles (VLPs) that are morphologically similar to the native virus, has permitted a better understanding of antigenicity of NoV (Hansman *et al.,* 2006).

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Two types of assay have been used to examine cross-reactivity among these VLPs; they are antibody ELISA and antigen ELISA. The antibody ELISA is broadly reactive, but the antigen ELISA is highly specific, however, detailed information on the cross reactivity among many of the genetically distinct NoV strains is limited (Hansman *et al.,* 2006).

A key breakthrough came around 1990 when the first genetic sequences of enteric viruses were decoded, opening the way for a new molecular diagnostics and reverse transcription PCR (RT-PCR) (Glass, 2013). It was then by 2002 when Kojima *et al* (2002) designed primers that specifically target NoV genotypes. Primer design involved different pairs of genogroup specific primers, which were subjected to specific reverse-transcriptase PCR, each with its own conditions. Reverse-transcriptase PCR identified the primers which showed to be sensitive in detecting Norwalk viruses' genogroup. The primers target the 5' end of ORF2, amplifying genogroup specific sequences of the N-terminal and Shell (N/S) region of the VP1 gene (Kojima *et al*., 2002).

In the present study we employed a One-step reverse transcriptase and primers defined by Kojima *et al* (2002), to identify NoV GI and NoV GII in stools samples of children who are less than 2 years of age. Detection done by Kojima *et al* (2002) has also shown that for high detection Nested PCR is recommended, this is also effective when the bands of the interest are not very clear on the agarose gel (Kojima *et al*., 2002).

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2.10 TREATMENT AND MANAGEMENT OF NOROVIRUS INFECTION

There's no specific medicine to treat people with NoV infection. Norovirus infection cannot be treated with antibiotics because it is a viral infection. If a person is infected with NoV, he or she should drink a lot of liquid to replace fluid lost from vomiting and diarrhoea. This will help prevent dehydration. Sports drinks and other drinks without caffeine or alcohol can help with mild dehydration, but they may not replace important nutrients and minerals. The required supplements for replacing nutrients and minerals can be found over the counter, and are mostly helpful for mild dehydration. Severe dehydration can lead to serious problems which may require hospitalization for treatment with fluids administered intravenously (Siebenga *et al*., 2009).

Hand washing with soap and water shows to be an effective method for reducing the transmission of Norovirus pathogens. Alcohol rubs (≥62% ethanol) may also be used as an adjunct, but are less effective than hand-washing, as NoV lacks a lipid viral envelope. Sanitizing surfaces where NoV particles may be present, using a solution of 5 to 25 tablespoons of household bleach per gallon of water (15 to 75 ml per 1 litre; 1.5 to 7.5%), is highly recommended or other disinfectants approved by the Environmental Protection Agency (EPA) for effectiveness against NoV (Center for Disease Control and Prevention, 2011).

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2.11 VACCINE DEVELOPMENT FOR NOROVIRUS

Scientists are developing new weapons to fight NoV years after they successfully produced vaccines against RoV. The delay reflects the difficulty of researching NoV, which can't be grown outside the human body. Early human volunteer studies demonstrated that challenge with Norwalk virus (NV) conferred short-term immunity (>2 yrs) to reinfection with the same virus (Bok *et al*., 2011). In the current issue of the New England Journal of Medicine, Dr. Robert Atmar from Baylor College of Medicine, elaborated that, given the number of NoV infections that occur annually and the health costs associated with these infections, the study shows feasible to make vaccine that will protect against NoV infection and the illness it causes (Atmar *et al*., 2011). LigoCyte Pharmaceutical Inc announced in 2007 that it was working on a Norovirus vaccine and had started phase 1 trials. As of 2011, a monovalent nasal vaccine completed phase I/II trials, while bivalent intramuscular and nasal vaccines are at earlier stages of development (Dolin, 2007).

After Takeda Pharmaceutical Co has bought LigoCyte Pharmaceutical Inc, Head of Japanese drug maker's unit Rajeev Venkayya, has confirmed development of a drug by Takeda Pharmaceuticals, the farthest drug along of several immunization candidates. A course of shots may confer lifelong protection against ninety five percent of NoV strains. Takeda's vaccine candidate combined viral components from two NoV types, that laboratory studies has shown that they can fight all strains known to have circulated during the past twenty years. The vaccine will be of benefit to individuals in cruise ships, schools and nursing homes that struggle to deal with highly contagious, untreatable scourge (Matsuyama, 2013).

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CHAPTER 3: MATERIALS AND METHODS

3.1 ETHICAL CLEARANCE

This study was part of the Mal-ED project that was approved by the Research Ethics Committee of the University of Venda. Permission to carry out the study was obtained from the Provincial Department of Health, Polokwane. The objectives and concepts of the study were clearly explained to the potential participants and signed consent forms were obtained before a participant was registered in the study. Confidentiality of the participants was kept by giving each participant a code and the consents were the only documents containing identifiers of the participants and they were kept under lock and key.

3.2 STUDY SITE

This study was conducted in rural developing areas of Dzimauli community that is composed of several villages, such as Pile, Tshibvumo, Tshapasha, Matshavhawe and Thongwe in the Vhembe district, Limpopo Province, South Africa. The Vhembe district is situated in the northernmost region of the country and shares its northern border with Beitbridge district in Matabeleland South, Zimbabwe. Figure 8 shows the location of Vhembe district, situated north of South Africa. The areas have people with different religious, educational and socio-economic backgrounds, living in neighborhoods with distinctly different levels of sanitation, such as clean drinking water and flushing or well-built pit toilets.

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3.3 SAMPLE COLLECTION

A total of 185 children were recruited through their mothers in the study and followed up every month from birth to the age of 18 months. The Healthcare workers were responsible for sample collection from children on a monthly basis. Both diarrhoeal and non diarrhoeal samples were collected. Diarrhoeal samples were collected as soon as the parents or care takers of the child reported the diarrhoeal cases in children irrespective of whether a sample was collected from the participant for the month. A diarrheic case in this study was defined as a child passing loose, liquid, and watery or a bloody loose stool three or more times in a 24-hour period with or without mucus, as reported by parents. The samples were stored at 4°C to 8°C in cooler boxes and delivered to the laboratory. Upon delivery at the lab, 0.8g of fecal sample was aliquoted and stored at -70°C for future analysis. All the sample collection and processing forms were checked and completed in the lab upon arrival.

3.4 DATA COLLECTION

Demographic information such as age and sex of each child was collected and recorded from the field. Anthropometric data such as height, weight and head circumference were also collected on a monthly basis by field workers. The health status of each child was also checked by field workers every month during sample collection exercise.

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3.5 LABORATORY TESTING

3.5.1 Extraction of viral single-stranded RNA (ssRNA) for RT-PCR

Viral RNA extraction was carried out using the viral QIAamp viral RNA Mini kit., 2007 (Qiagen, Hilden, Germany), and was performed according to the manufactures protocol. Briefly, 0.1g of stool specimen was aliquoted into 1.5ml tube and diluted by 1ml of 0.86% NaCl, then centrifuged at 6000rpm for 20min. 560µl of carrier RNA with buffer AVL was added to 1.5ml tube, then 140µl of faecal suspension was added to the tube and the samples were handled according to the manufacturers' protocol. At final centrifugation 60ul of Norovirus RNA was eluted into a clean Eppendorf tube. Norovirus positive controls, for genogroup I and genogroup II, from University of Virginia was used as a positive control and treated water was used as a negative control.

3.5.2 One step reverse-transcriptase Real time polymerase chain reaction for the detection of Norovirus

A one step reverse-transcriptase real time-PCR described by Trujillo *et al.,* 2006, was used for the detection of Norovirus with the primers and probe described by Kageyama *et al*., 2003.

For the detection of Genogroup I Norovirus: The reaction mixture contained 12.5µl of 2X RT-PCR buffer, 1.00µl of each of the following; 25X RT-PCR buffer, Forward primer-Cog1F (10 µM), Reverse-Cog 1R (10 µM). Then 0.15µl of probe 1-Ring 1a (10 µM) and probe 2-Ring 1b were added followed by addition of 1.67µl of detection enhancer. The mixture was adjusted to 23µl by adding 5.53µl of nuclease free water. Then 2µl of the RNA was added to the labeled wells respectively to final volume of 25µl. The plate was centrifuged at 500 x g for 1min to remove bubbles or drops on wells. The cycling conditions were used as described in Table 3.1.

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For the detection of Genogroup II Norovirus: The reaction mixture contained 12.5µl of 2X RT-PCR buffer, 1.00µl of each of the following; 25X RT-PCR buffer, Forward primer-Cog2F (10 µM), Reverse-Cog 2R (10 µM). Then 0.30µl of probe 1-Ring 2 (10 µM) was added and followed by addition of 1.67µl of detection enhancer. The mixture was adjusted to 23µl by adding 5.53µl of nuclease free water. Then 2µl of the RNA was added to the labeled wells respectively to final volume of 25µl. For both mixtures, Light Cycler 480 form Roche (Roche Applied Science, Germany), was used. The cycling conditions were used as described in Table 3.1.

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Table 3.41 : PCR primers, probes and cycling conditions used for G1 and GII ORF ½ regions of the Noroviruses

PCR	Cycling conditions	Primers	Primer sequence	Position	Amplicon size
GI	45˚C-10 min 95˚C-10 min 95˚C-15 sec } X40 60˚C-1 min } 4°C cooling	COG1F	5´CGYTGGATGCGNTTYCATGA3´	5291	84bp
		COG1R	5´CTTAGACGCCATCATCATTYAC3´	5375	
		RING1 a	FAM-AGATYGCGATCYCCTGTCCA-TAMRA	5340	
		RING1b	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA		
				5340	
G II		COG2F	5´CARGARBCNATGTTYAGRTGGATGAG3´	5003	97bp
			5´TCGACGCCATCTTCATTCACA3´		
		COG2R	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	5100	
		RING2		5048	

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3.5.3 Quality Control

To ensure that the results observed for one step reverse-transcriptase real time-PCR were not contaminated; working areas were swabbed and tested as well for norovirus. Clean sterile swabs were cut by breaking them into pieces that are not longer than 3cm. All working areas and materials were swabbed. The swabs were then placed in Eppendorf tubes that contained 200μl sterile distilled water. The tubes with the swabs were shaken for 30 seconds and vortexed for 1 minute. The swabs were removed from the tubes, and the tubes were placed in the centrifuge. The tubes were centrifuged at 10 000rpm for two minutes. 200 µl of supernatant was transferred into a clean Eppendorf tube and treated the same way as the ssRNA for one step reverse-transcriptase real time-PCR. Results were observed on computer screen using the Light Cycler 480 II (Roche Applied Science, Germany).

3.5.4 Avoiding PCR contamination

To avoid false positive results which may be due to cross contamination of samples and products from previous PCR amplifications, the following measures were taken into consideration during the procedure: New thin-walled PCR tubes and two milliliters Eppendorf tubes were sterilized by autoclaving. Working areas were decontaminated with 70% ethanol. Calculations and table of templates were made before preparation of templates, and the book of calculations was kept outside the lamina air flow, on the bench where it is visible to see. Reagents, primers and controls were always kept on ice as recommended.

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The light and fan of the Lamina air flow was kept on at all times during template preparations for all PCR amplifications. New fresh gloves were worn at all times when performing the procedure and were changed frequently from one area to another. To pipette DNA, primers and reagents, a fresh tip was used for each, only with the master mix was one pipette used, but with careful attention not to touch the tube it is being transferred to. If it touched the tube, the tip was changed immediately. After, preparation of templates, the plates were at all times immediately taken to the respective thermocycler.

3.5.6. Genotyping of Norovirus Capsid N/S (N-terminal/Shell capsid) gene

3.5.6.1 Reverse transcriptase PCR

PCR amplification of the NS region was performed using primers described by Kojima *et al.*, (2002). These primers (G1SKF and G2SKF forward primers) and (G1SKR and G2SKR reverse primers) target the 5' end of ORF2, amplifying genogroup-specific sequences of the N-terminal and shell (N/S) region of the VP1 gene (Chen *et al.*, 2013). In the present study, we took into account the genogroup specificity of these sets of primers to detect the NoV genogroups found in selected rural areas of research in Vhembe district. A reverse transcriptase PCR was carried out to amplify the samples that tested positive with one step reverse-transcriptase real-time-PCR. The reverse transcriptase PCR products and amplified DNA, were then prepared for conventional PCR. The cycling conditions for the reverse transcriptase PCR were as shown in Table 3.2.

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Table 3.52: Primers and their band sizes, used to amplify rtRT-PCR products for sequencing

PCR	Cycling Conditions	Primer	Primer Sequence	Amplicon size
GI	45°C- 45 min 95°C- 10min 95°C-30s ⎫ 50°C-30s ⎬ X40 72°C-1 min ⎭ 72°C-7min 4°C cooling	G1SKF	5′-CTGCCCGAATTYGTAAATGA-3′	330bp
		G1SKR	5′-CCAACCCARCCATTRTACA-3′	
GII		G2SKF	5′-CNTGGGAGGGCGATCGCAA-3′	387bp
		G2SKR	5′-CCRCCNGCATRHCCRTTRTACAT-3′	

3.5.6.2 Conventional PCR

A conventional PCR described by Kobayashi *et al*., 2000, was used to amplify DNA by reverse transcriptase PCR, with the primers described by Kojima *et al.,* 2002. Conventional PCR was performed with the G-storm, GS 482 dual block thermal cycler (Vacutec®, Johannesburg, South Africa) for DNA amplification. The products of the conventional PCR were resolved by agarose gel electrophoresis, of which 3*ul* of the amplified product was used to run a (1% w/v) agarose gel. The remaining conventional PCR products were sent to Stellenbosch University for sequencing. The band sizes of the Conventional PCR products are also genogroup specific, with GI at 330bp and GII at 387bp. All gels were view under UV-Transilluminator from Bio-Rad Laboratories, the Bio-Rad Image Lab 4.0.1, which also allowed picture capturing. The set of primers and cycling conditions for conventional PCR are described in Table 3.3.

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Table 3.6~~3~~: Primers and their band sizes, used to amplify Conventional PCR products

PCR	Cycling Conditions	Primer	Primer Sequence	Amplicon size
GI	95°C-10min 95°C-30s, 50°C-30s, 72°C-1 min (X40) 72°C-7min 4°C cooling	G1SKF	5′-CTGCCCGAATTYGTAAATGA-3′	330bp
		G1SKR	5′-CCAACCCARCCATTRTACA-3′	
GII		G2SKF	5′-CNTGGGAGGGCGATCGCAA-3′	387bp
		G2SKR	5′-CCRCCNGCATRHCCRTTRTACAT-3′	

3.5.7 Sequencing of the N-terminal/Shell capsid gene.

Viral RNA was extracted from stool suspensions by using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). A One step reverse-transcriptase RT-PCR was carried out in a Light Cycler 480 (Roche Applied Science, Germany), with the reaction mixtures and enzymes that include primers and probes described by Kageyama *et al*., (2003). A reverse-transcriptase PCR and Conventional PCR was performed using pairs of PCR primers described by Kojima *et al.,* (2002) with the G-storm, GS 482 dual block thermal cycler (Vacutec®, Johannesburg, SA). DNA sequencing was performed by Stellenbosch University, (Stellenbosch University, Matieland, 7602, Stellenbosch, South Africa).

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The nucleotide sequences were determined on both strains using the PCR primers described by Kojima *et al.,* (2002). DNA sequencing was done using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems) using the manufacturers protocol with slight modifications. For fragment analysis, two microliters of cleaned PCR product was mixed with the appropriate internal size standard (Applied Biosystems) and Hi-Di prior to denaturing for 5 minutes at 95°C. Directly after heating the samples were placed on ice for 5 minutes. Electrophoresis was performed on either an ABI3130xl or an ABI3730xl using a 50cm Capillary array and POP7 (all supplied by Applied Biosystems).

3.5.8 Phylogenetic Analysis of the N-terminal/Shell capsid gene.

Phylogenetic tree analysis is the study of estimated evolutionary analysis. The evolutionary history inferred from phylogenetic analysis usually depicted as branching, treelike diagrams that represent an estimated backgrounds of the inherited relationships among molecules (gene trees), organisms, or both. Four steps are involved in Phylogenetic tree analysis; they include alignment of the sequence, determination of the substitution base or nucleotide, tree building and evaluation.

Nucleotide sequences from the study (N-terminal/Shell capsid gene sequence) were aligned and edited using Staden Package programme (Staden-2.0.0b10-windows-x86_64.exe) (Bonfield, 1995) and Bioedit (Hall, 1999) and blasted on the web based NCBI tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare them with the strains in the GeneBank. A neighbor-joining method (Saitou and Nei, 1987) which is pair-wise distance was used to calculate maximum-likelihood to construct a tree with boot-strap of 1000 replicates to measure the accuracy of the tree (Felsenstein, 1985).

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It included two reference strains of NoV, from Russia and one Japan (NoV) for NoV GI, and for GII. The neighbor method was also used with several reference strains from Australia, Vietnam, United Kingdom, Canada, Japan, and one strain form South Africa, all acquired from the GeneBank. All reference strains were acquired from a web-based NCBI tool. The bootstrap values of the operational taxonomic units (OTUs) of NoV GI and NLV GII, which represent the branches on the phylogenetic tree, were calculated, and the topological errors of the tree were also edited. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analysis of the sequences was conducted in MEGA5 software (Version 5.0., 2011).

3.6 DATA ANALYSIS

3.6.1 Nutritional data analysis

The field teams measured and record the anthropometric data of each child from 1 month. To indicate a child's nutritional status, anthropometric indices (height-for age (stunting), weight-for-height (wasting), and weight-for-age (underweight), was expressed as z-scores [z-scores: departure in standard deviations (SD) units of a weight and/ or height measurement from the mean of an age and sex-specific reference population] (WHO, 1986). Anthropometric data from reference populations published by the NCHS and United States centers for disease control was used as standards (Dibley *et al*., 1987). Anthropometric measures were converted to z-scores using the EPINUT component of the EPI-INFO computer program version 6 (Dean *et al*., 1995). The z-score cut-off used to classify moderate malnutrition was SDs below the reference median (<-2 SD). The cut-off for severe malnutrition was considered in SDs below the median (<- 3 SD).

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3.6.2 Statistical analysis

The results were entered into an excel sheet (Microsoft office package) and analyzed using Statistical Package for the Social Sciences (SPSS for WINDOWS version 18.0). Assuming the data will follow a normal distribution, comparison of proportions and statistical significance was tested using the Chi-square test. All data was expressed as mean standard deviations. Student's tests were used to compare means between qualitative data and one-way analysis of variance to compare means between quantitative data. Correlations between qualitative variables were assessed using Spearman's correlation coefficient. Statistical analyses using 2 or Fisher's exact test was performed for comparisons of percentages. All tests were two-tailed; p value less than 0.05 were considered statistically significant.

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CHAPTER 4: RESULTS

4.1 REAL TIME POLYMERASE CHAIN REACTION FOR THE DETECTION OF NOROVIRUS

Norovirus single stranded RNA was extracted from selected diarrhoeal and non-diarrhoeal (monthly) samples. Purified RNA samples were tested with a One step reverse-transcriptase real time PCR to detect the presence of Norovirus. Results were observed using LightCycler® 480 (LCS480 1.5.0.39) from Roche. A total of 708 stool samples were tested, of which 256 were diarrhoeal samples and 452 were non-diarrhoeal samples. Of the 708 samples tested 160 samples were positive with Norovirus GI and GII. Ninety one (14 GI and 77 GII) were diarrhoeal samples and 69 (19 GI and 50 GII) were monthly samples. Figures 9 and 10 shows the amplification curves as observed, of which the average Cp-value [crossing points (CP-value) is defined as the point at which the fluorescence rises appreciably above the background fluorescence] for positive sample ranged from 32.15 and above. As shown in the Figures, the detection of NoV GI gave one positive sample in many, whilst for NoV GII, more than two samples per run tested positive. For the quality control test, none of the swabs obtained from the laboratory surfaces and equipment showed positive for any of the two norovirus genogroups tested. The CP values varied from 16 to 39 from the Genogroup I and from 8 to 39 for Genogroup II. Figures 11 and 12 show the variation of the CP values for both Norovirus Genogroups I and II.

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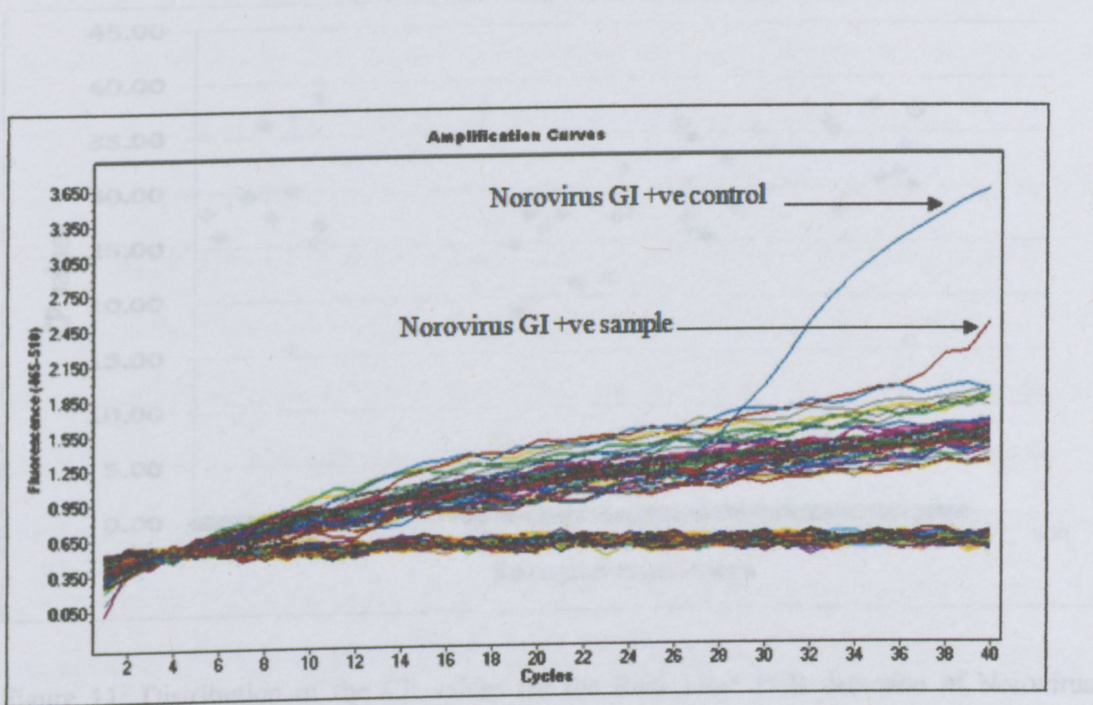

Figure 9: Line graph presentation, showing amplification curves for NoV genogroup I.

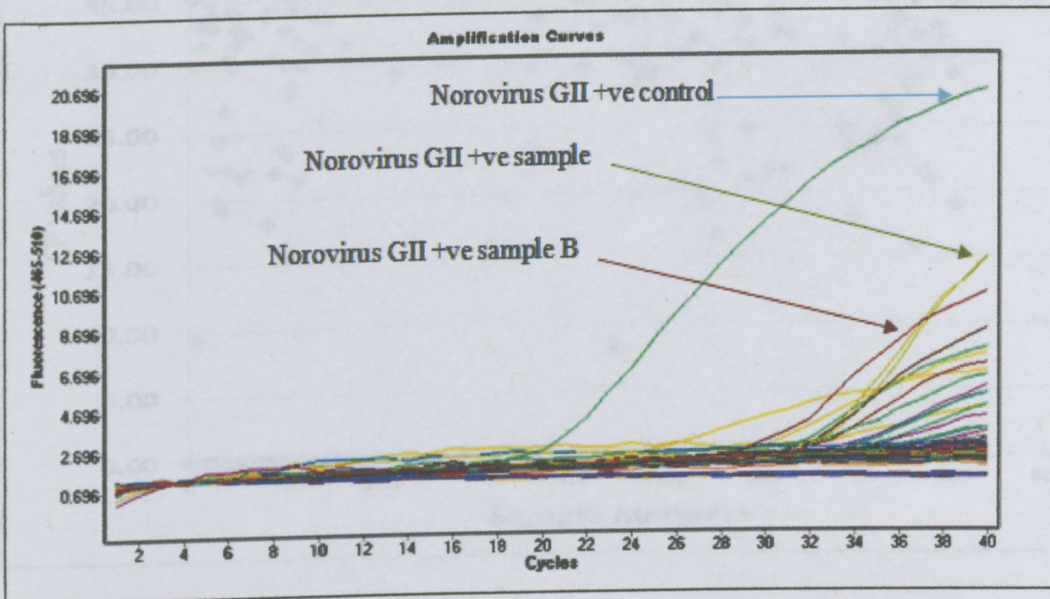

Figure 10: Line graph presentation, showing amplification curves for NoV genogroup II (GII).

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Positive samples were then selected for reverse transcriptase PCR.

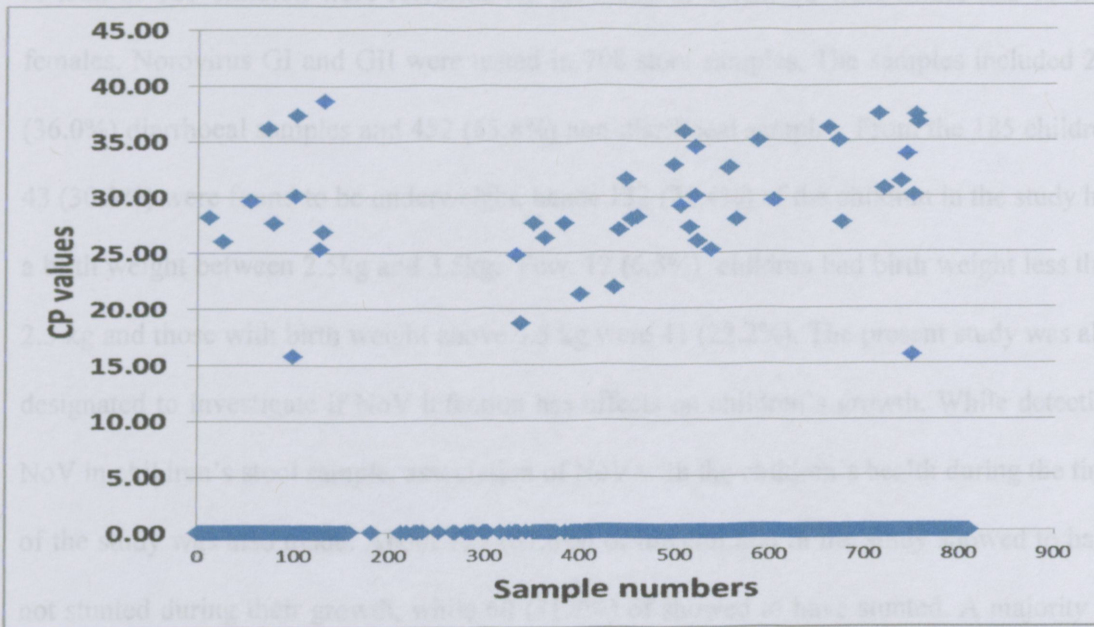

Figure 11: Distribution of the CP values for the Real Time PCR detection of Norovirus Genogroup I obtained from the light cycler 480.

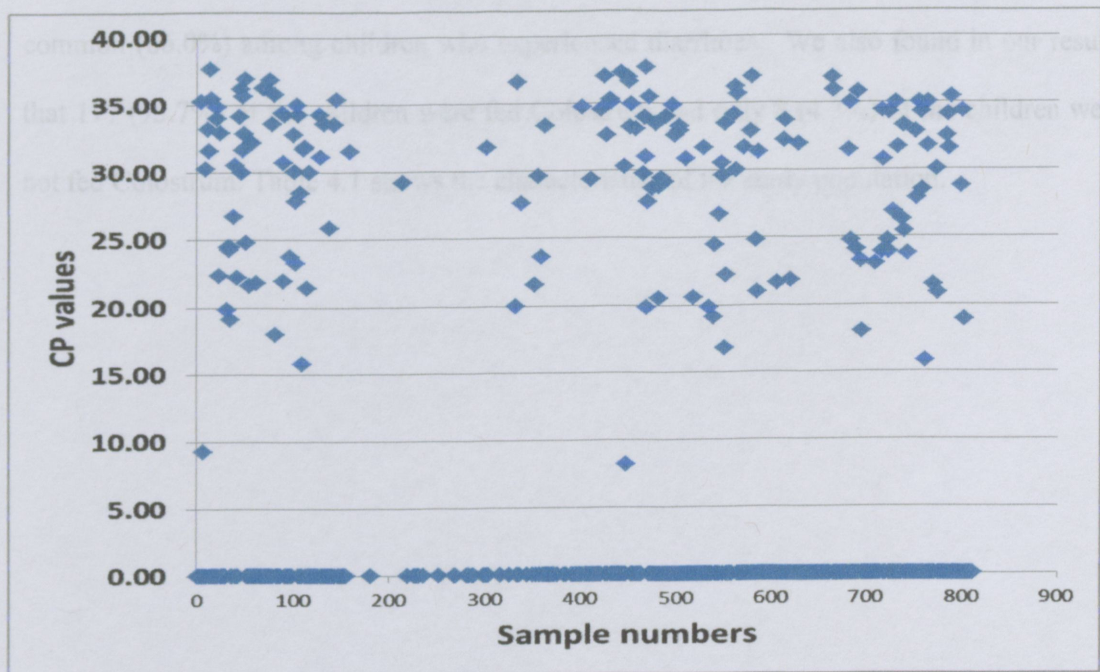

Figure 12: Distribution of the CP values for the Real Time PCR detection of Norovirus genogroup II obtained from the light cycler 480.

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4.2 DEMOGRAPHIC CHARACTERISTICS OF THE STUDY PARTICIPANTS

A total of 185 children were recruited for the study of whom 88 were males and 97 were females. Norovirus GI and GII were tested in 708 stool samples. The samples included 256 (36.0%) diarrhoeal samples and 452 (63.8%) non-diarrhoeal samples. From the 185 children, 43 (30.3%) were found to be underweight, hence 132 (71.4%) of the children in the study had a birth weight between 2.5kg and 3.5kg. Few, 12 (6.5%) children had birth weight less than 2.5 kg and those with birth weight above 3.5 kg were 41 (22.2%). The present study was also designated to investigate if NoV infection has effects on children's growth. While detecting NoV in children's stool sample, association of NoV with the children's health during the time of the study was also made. About 125 (67.6%) of the children in the study showed to have not stunted during their growth, while 60 (31.7%) of showed to have stunted. A majority of the children, 141 (76.2%), showed to have experienced diarrhoea during their growth, and 44 (23.8%) of these children never experienced diarrhoea. Norovirus GII appeared to be common (66.0%) among children who experienced diarrhoea. We also found in our results that 177 (95.7%) of the children were fed Colostrum and only 8 (4.3%) of the children were not fed Colostrum. Table 4.1 shows the characteristics of the study population.

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Table 4. 6~~1~~: Anthropometric characteristics of the children in the study

Anthropometric Characteristics		Frequency	Percent (%)
Gender	Male	88	47.6
	Female	97	52.4
Participants Experiencing Diarrhoea.	No Diarrhoea Experienced	44	23.8
	Experienced Diarrhoea	141	76.2
Types of samples	Diarrhoeal	256	36.1
	Non-Diarrhoeal	452	63.8
Birth weight	Less than 2.5kg	12	6.5
	2.5kg-3.5kg	132	71.4
	Above 3.5kg	41	22.2
General Body weight with growth.	Underweight	43	23.3
	Not Underweight	142	76.8
Health status	Wasted	31	16.8
	Not Wasted	154	83.2
	Stunted	60	31.7
	Not Stunted	125	67.6
	On Colostrum	177	95.7
	Not on Colostrum	8	4.3

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4.2.1 Consistency of collected from samples from the children

We used the data provided by field workers to monitor the children's health status, and did analysis from data given by the field workers. Stool samples, monthly or diarrhoeal, were tested for NoV presence in association with their consistency, and whether the stool samples had mucus or blood. Table 4.2 shows the frequency of stool samples in association with their consistency, and having blood or mucus in them. A majority of the stool samples collected was soft, about 397 (56.1%) in total compared to 23 (31.5%) samples that were formed, the remaining samples included 65 (9.2%) liquid samples and 10 (1.4%) watery samples. Also, from the 708 stool samples collected 13 (1.8%) presented with blood and 176 (24.9%) presented with mucus.

Table 4. 72: Consistency of all stool samples

Consistency	Frequency	Percentage
Watery	10	1.4
Liquid	65	9.2
Soft	397	56.1
Formed	223	31.5
Blood	13	1.8
Mucus	176	24.9
TOTAL	708	100.0

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4.3 OCCURRENCE OF NOV IN ASSOCIATION TO CHILDREN'S ANTHROPOMETRIC CHARACTERISTICS

We observed children's health statuses and found that NoV GII to be a more common variant than NoV GI, especially in children who had experienced diarrhoea (76.2%), and those who did not breast feed, but were fed colostrums 177 (95.7). NoV infection showed less infectivity in the children who are breast feeding; although NoV GII is most common in those children. Table 4.3 provides the overall characteristics of the children in the study, their health status and birth weight.

Table 4.83: Occurrence of NoV genogroup in the study population.

No. of children	Genogroup	Frequency	Percentage
185	Norovirus GI	49	26.5
	No Norovirus GI	136	73.5
185	Norovirus GII	125	67.6
	No Norovirus GII	60	32.4
No. of samples	**Genogroup**	**Frequency**	**Percentage**
708	Norovirus GI	67	9.4
	No Norovirus GI	641	90.5
708	Norovirus GII	244	34.5
	No Norovirus GII	464	65.5

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4.3.1 Occurrence of NoV genogroups in association to the children's health status

From the 185 participants, NoV distribution was also assessed according to the children's sex, amongst the 88 males and 97 females. Although both norovirus genogroups were more common among males, there was no significant association ($p>0.05$) between NoV infection and the sex of children (Table 4.4). Analysis for NoV GI gave the significant value $\chi^2= 0.053$ $p = 0.817$ and NoV GII gave the values $\chi^2 = 1.240$; $p = 0.266$ on analysis of norovirus infections associated with the sex of the children, with GII being almost at the average value that show study significance ($\chi^2 = 2.000$; $p = 0.050$).

In a total of 256 diarrhoeal samples, 34 (24.1%) tested positive for GI, and 93 (66.0%) samples tested positive to GII. Results showed no significance for both GI infections in children experiencing diarrhoea ($\chi^2 = 1.715$; $p = 0.190$), and no significance in GII infections to children with diarrhoea ($\chi^2 = 0.701$; $p = 0.402$). Table 4.4 shows the distribution of NoV genogroups in accordance to the children's anthropometric characteristics. Results analysis of the children characteristics in association to occurrence of NoV genogroup has shown NoV GII to be most common especially when looking at the children's birth weight.

When comparing with the children's birth weight, 82.9% of the children with a higher birth weight, above 3.5kg, presented with NoV GII, also in both genders, NoV GII showed to be common. Analysis showed significance in the children's birth weight in association with the occurrence of NoV genogroups, the values were $\chi^2 = 3.083$; $p= 0.214$ for NoV GI and $\chi^2 = 6.503$; $p = 0.039$. Not much of a difference was observed with analysis of NoV genogroups in association with children's gender. Analysis of occurrence of NoV genogroup in association to children's gender showed values $\chi^2 = 0.053$; $p = 0.817$ for NoV GI and $\chi^2 = 1.240$; $p = 0.266$ for NoV GII.

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Table 4.94: Occurrence of NoV genogroup in association to children's anthropometric characteristics

Anthropometric Characteristics		GI		GII	
		FREQUENCY	%	FREQUENCY	%
Sex	Male	24	27.3	63	71.6
	Female	25	25.8	62	63.9
Birth Weight	Less than 2.5kg	2	16.7	9	75.0
	2.5kg To 3.5kg	32	24.2	82	62.1
	Above 3.5kg	15	36.6	34	82.9
Colostrum	Children on Colostrum	48	27.1	119	67.2
	Not on Colostrum	1	12.5	6	75.0
Participants Experiencing Diarrhoea.	Experienced Diarrhoea	34	24.1	93	66.0
	No Diarrhoea Experienced	15	34.1	32	72.7%

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We observed Norovirus genogroups in association to development in children and among children who were stunted and those who were not. NoV infection showed a significant variation in genogroup specific infection. Norovirus infections showed to be significant in children who were stunted and who were not (GI: $x^2 = 4.813$; p = 0.028; GII: $x^2 = 2.375$; p = 0.123).

Analysis of NoV Genogroup I infections related to children being underweight, also showed to be significant (NoV GI: $x^2 = 3.874$; p = 0.049), but no significance was observed with NoV Genogroup II in associated with children being underweight (NoV GII the values were very low $x^2 = 0.003$; p = 0.953). Furthermore, the difference in whether the child was wasted or not wasted had no significance in NoV infection (NoV GI: $x^2 = 0.599$; p = 0.439; NoV GII: $x^2 = 1.045$; p = 0.307).

Through monitoring the children's health status monthly, we were able to look at NoV infections in association to the children's health status. Figure 13 shows the frequency in detail, of the study analysis. A majority of the children in the study, about 38 (20.5%), showed to be stunted at birth and the number increased to 49 (26.5 %) when they reach 18 month, with a gradual reduction to 31 (25.4%) at 12 months.

Few children showed to be underweight, estimated at about 16 (8.6%) while majority of the children, about 147 (79.5%), are born almost at the recommended weight. The number of wasted children was low, about 6 (3.8%) children in the study population and their number reduces to 2 (1.1%) when the child is growing. A total of 156 (84.3 %) children who were not wasted at birth their number reduces to 132 (71.4%) as the children grow, which can mean the children cannot be wasted at birth, but can be wasted during development or growth.

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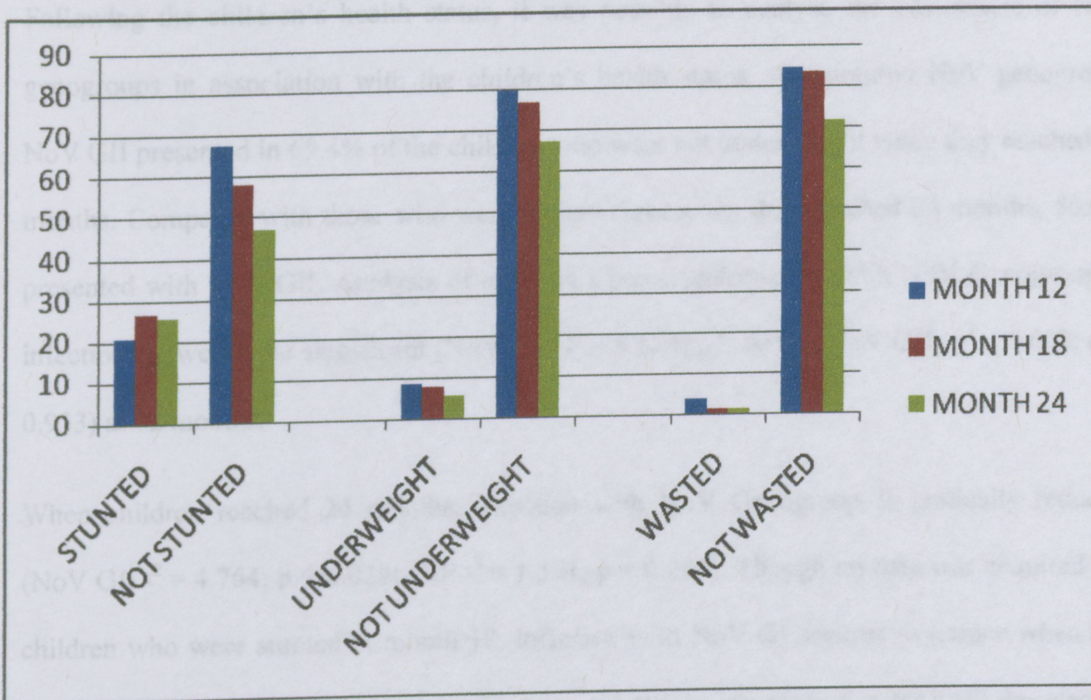

Figure 13: Frequency of children health status shown followed monthly. The figure shows the frequency of children's health status in month 12, month 18 and month 24.

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Following the children's health status, it was possible to analyse the occurrence of NoV genogroups in association with the children's health status. As common NoV genogroup, NoV GII presented in 69.4% of the children who were not underweight when they reached 24 months. Compared with those who were underweight when they reached 24 months, 50.0% presented with NoV GII. Analysis of children's being underweight with NoV Genogroup II infection showed to be significant (NoV GI: $x^2 = 3.874$; p = 0.049; NoV GII: $x^2 = 0.003$; p = 0.953) at 12 months.

When children reached 24 months, infection with NoV Genogroup II gradually reduced (NoV GI: $x^2 = 4.764$; p = 0.029; GII: $x^2 = 1.591$; p = 0.207). Though no data was acquired for children who were stunted at month 18, infection with NoV GI seemed to reduce when the child grew. The number of children who were wasted, in association to NoV GI dropped to 0.0% when the children reached 24 months, and for those who are not wasted at birth, NoV GI infection seemed to be constant at 27.6% when the children are 12 months to 31.1% when the children reach 24 months.

Analysis of results for association of NoV infection with wasted children showed no significance (NoV GI: $x^2 = 0. 599$; p = 0.439; NoV GII: $x^2 = 1.045$; p = 0.307 at 12 months, but infection with NoV in wasted children showed to be significant when the children reach 24 months (NoV GI: $x^2 = 0.895$; p = 0.344; NoV GII: $x^2 = 0.959$; p = 0.327).

Infection with NoV to children who are stunted at birth is low at birth and increases as the children grows to 24 months, with NoV GII being the common genogroup. Children who were not stunted in month 12, month 18 and month 24 presented with a high percentage of NoV. Analysis of results for infection with NoV in children who are stunted at birth showed no significance (NoV GI: $x^2 = 4.813$; p = 0.028; NoV GII: $x^2 = 2.375$; p = 0.123) at 12 months and (NoV GI: $x^2 = 0.875$; p = 0.350; NoV GII: $x^2 = 1.280$; p = 0.258) at 24 months (Table 4.5).

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Table 4.105: Occurrence of NoV genogroups in association with children growth status

GROWTH STATUS	MONTH 12				MONTH 18				MONTH 24			
	GI		GII		GI		GII		GI		GII	
	FREQUENCY	%	FREQUENCY	%	FREQUENCY	%	FREQUENCY	%	FREQUENCY	%	FREQUENCY	%
STUNTED	5	13.2	22	57.9	13	26.5	31	63.3	12	25.5	29	61.7
NOT STUNTED	39	31.2	89	71.2	32	29.9	74	69.2	29	33.3	62	71.3
UNDERWEIGHT	1	6.3	11	68.8	2	14.3	9	64.3	0	0.0	5	50.0
NOT UNDERWEIGHT	43	29.3	100	68.0	43	30.3	96	67.6	41	33.1	86	69.4
WASTED	1	14.3	6	85.7	0	0.0	0	0.0	0	0.0	2	100.0
NOT WASTED	43	27.6	105	67.3	0	0.0	0	0.0	41	31.1	89	67.4

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4.4 OVERALL DISTRIBUTION OF NOROVIRUS IN THE STUDY POPULATION

Statistical analysis software, Statistical Package for the Social Sciences (SPSS for WINDOWS version 18.0), was used to determine the distribution of NoV genogroups in Vhembe district. Figure 14 shows the distribution of NoV from 2010 to 2013 in Vhembe.

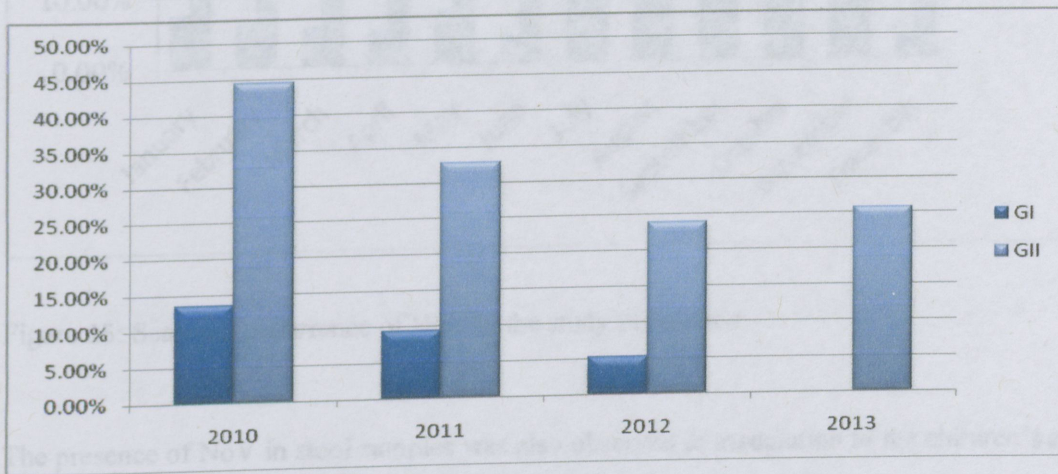

Figure 14: Occurrences of NoV between the year 2010 and 2013 in the study population.

Infections with NoV was also determined using age difference of the children in the study, to see at which age is the child more susceptible to infection with NoV, and which age group amongst the children in the study, is constantly affected by NoV infection. NoV infections occur in different ranges, and the infections also depend on which genogroup it is. Looking at the infections per month, we determined how often does NoV infection occur in Vhembe, therefore, we can know in each season how the NoV infection is like in Vhembe district. Norovirus GII infection was the highest in November (50.6%). Other months include June (48.1%) and October (48.0%). Analysis showed that infection with Norovirus GI was very low in December (2.6%) than in June (3.8%) and was the highest in October (20.0%). Figure 15 shows the distribution of the infection experienced in a season.

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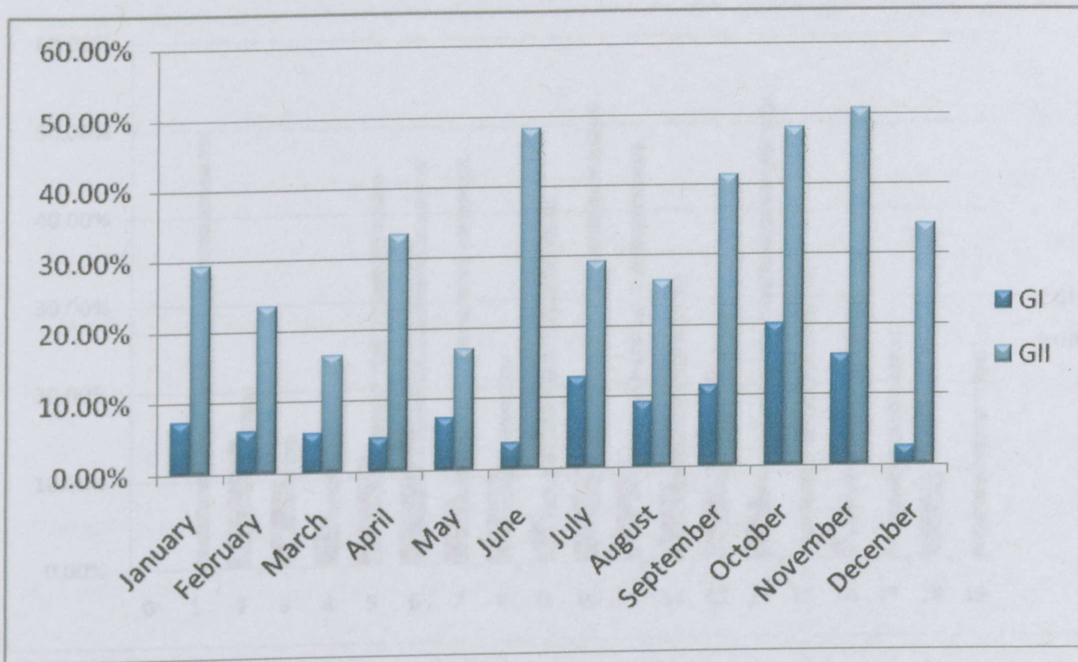

Figure 15: Seasonal occurrence of NoV in the study population.

The presence of NoV in stool samples was also observed in association to the children's age. Most of the children have showed to have NoV GII as a common infection at birth, whilst NoV GI, start to infect children from one month and then the infection decreases when the children grow up. Results show that infection with NoV GI at the seventeen month was lower than infection with NoV GII. This can deduce that as the child grows, infection with NoV gradually reduces, which suggest that when the child is about to reach two years of age, immunity gradually develops. Figure 16 shows in details the difference in NoV infections associated with children's age for both NoV genogroups in the first two years of a new born baby.

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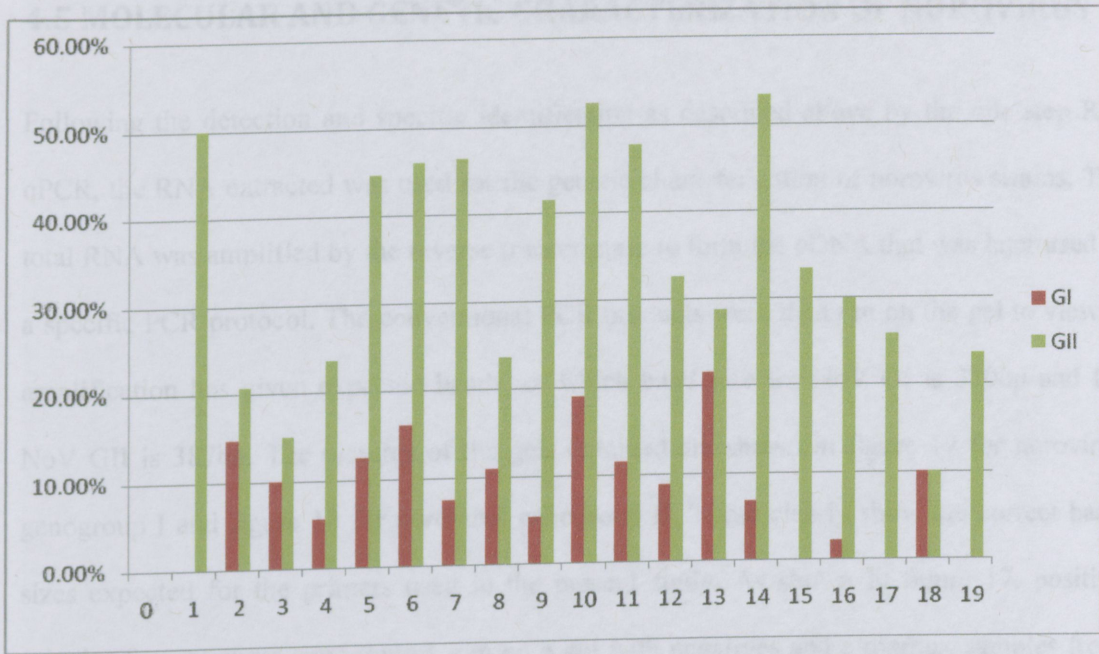

Figure 16: Occurrence of NoV GI and NoV GII in association with the children's age, from 0 month to 24 months.

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4.5 MOLECULAR AND GENETIC CHARACTERIZATION OF NOROVIRUS

Following the detection and specific identification as described above by the one step RT-qPCR, the RNA extracted was used for the genetic characterization of norovirus strains. The total RNA was amplified by the reverse transcriptase to form the cDNA that was later used in a specific PCR protocol. The conventional PCR products were then ran on the gel to view if amplification has given expected bands, of which band size for NoV GI is 330bp and for NoV GII is 387bp. The pictures of the gels obtained are shown in figure 17 for norovirus genogroup I and figure 18 for norovirus genogroup II. These clearly show the correct band sizes expected for the primers used in the present study. As shown in figure 17, positive samples for norovirus genogroup I, run on a gel with negatives and a marker, samples from wells 8, 10, 11, 17, 18, 20, 22, 31 and 34 show clear bands for norovirus genogroup I which means a successful amplification. Samples 4, 5, 7 and 20 may have amplified but the bands are not clearly visible, this means for an effective amplification for this bands they are subjected to nested PCR for successful amplification. In figure 18, most of the samples in the study for norovirus genogroup II show to have successfully amplified without the need for a nested PCR, as seen with norovirus genogroup II positive samples from the wells 8, 10 and 11. Though there were faint bands for some of the amplified samples for norovirus genogroup II, there not faint enough to actually show that amplification was not succeful.

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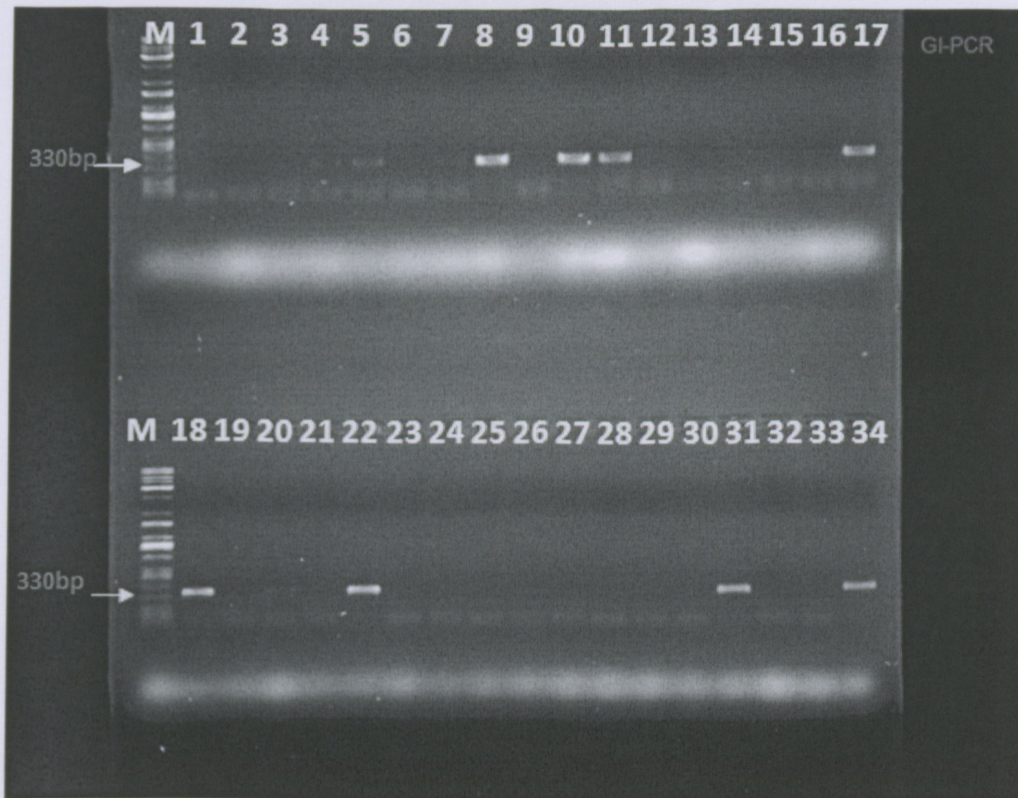

Figure 17: Picture of agarose gel showing NoV GI bands at 330bp. Kapa ladder was used as marker to mark positive bands for NoV GI are seen with samples from well 8, 10, 11, 17, 18, 22, 31 and 34 with faint bands from wells 4, 5, 7 and 20.

M – Marker 1000bp (Kapa)

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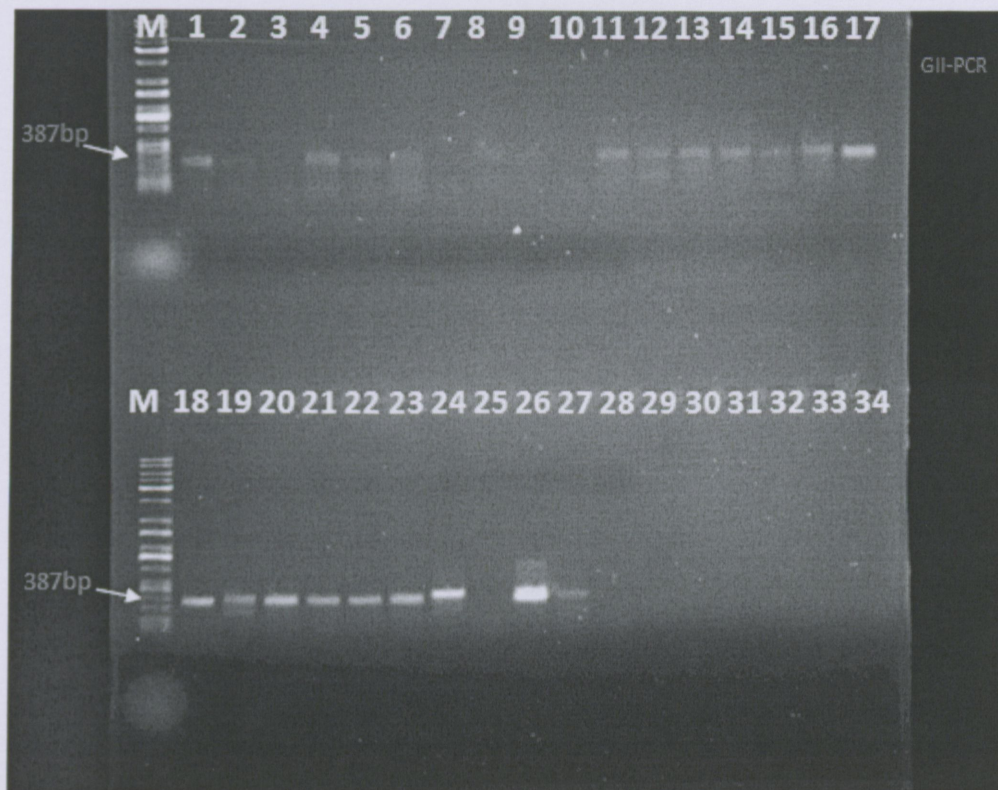

Figure 18: Picture of agarose gel showing NoV GII bands at 387bp. NoV GII gave most of the positive results as observed on a gel with samples from well 1, 2, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 26 and 27. Well 17 was used for positive control and well 34 was used for negative control. With the aid of the marker, the bands can be observed at their respective size on the gel.

M – Marker 1000bp (Kapa)

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4.6 SEQUENCE ANALYSIS OF THE N-TERMINAL/SHELL CAPSID GENE.

The amplicons obtained from the conventional PCR were sent for sequencing to Stellenbosch University, Cape Town (Stellenbosch University, Matieland, 7602, Stellenbosch, SA) and were compared with the strains found in the gene bank. DNA sequencing was done using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems) using the manufacturers protocol with slight modifications. For fragment analysis, two microliters of cleaned PCR product was mixed with the appropriate internal size standard (Applied Biosystems) and Hi-Di prior to denaturing for 5 minutes at 95°C. Directly after heating the samples were placed on ice for 5 minutes. Electrophoresis was performed on either an ABI3130xl or an ABI3730xl using a 50cm Capillary array and POP7 (all supplied by Applied Biosystems).

The phylogenetic tree of the PCR products indicated that the NoV amplified by G1SKF and G1SKR were grouped into GI cluster, whereas NoV amplified by G2SKF and G2SKR were clearly grouped into GII cluster. Therefore, this primer set can specifically amplify the NoV genome according to its genogroup. Furthermore, phylogenetic tree indicated that a broad range of both GI and GII strains can be detected with this primer set. Figure 19 shows the difference in bases of the study sequence and the sequence found in the GeneBank. There was no major difference in the nucleotides pairs of study sequence compared with the reference sequences, the difference included change in not more than three nucleotide per sequence, though this changes in nucleotide sequence are very crucial in genetic studies. Phylogenic tree analysis, show study sequence A09G1SKF and B09G1SKF in the first cluster of sequence for GI, in which it is grouped will all the reference sequence. The remaining study sequence forms a second cluster which is on its own and does not fall with the chosen reference sequence. Figure 20 show in detail, the clusters formed by the strains compared with the strains found in the GeneBank, to see if they fall in the same group.

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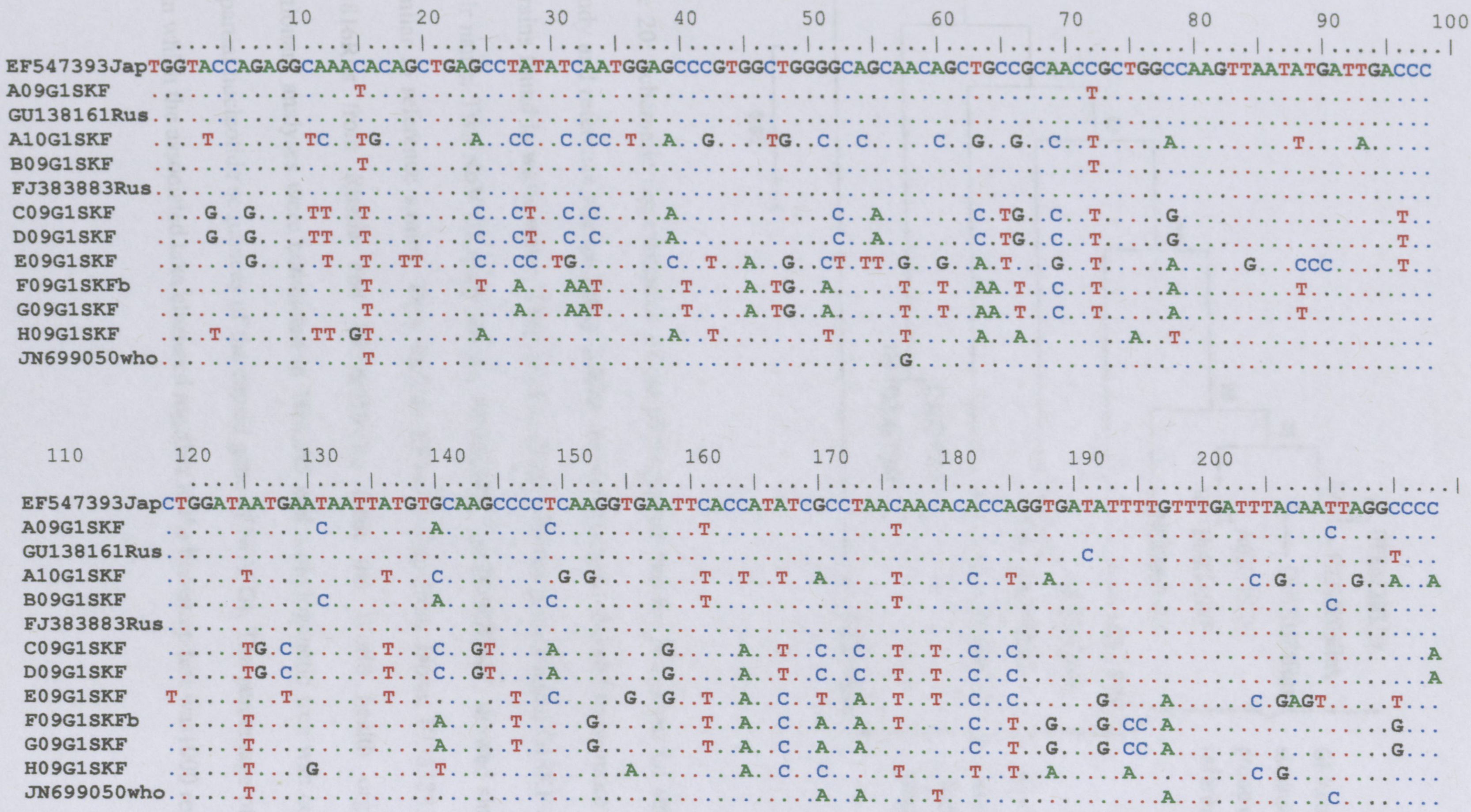

Figure 19: Schematic representation of the alignment of Norovirus GI strains

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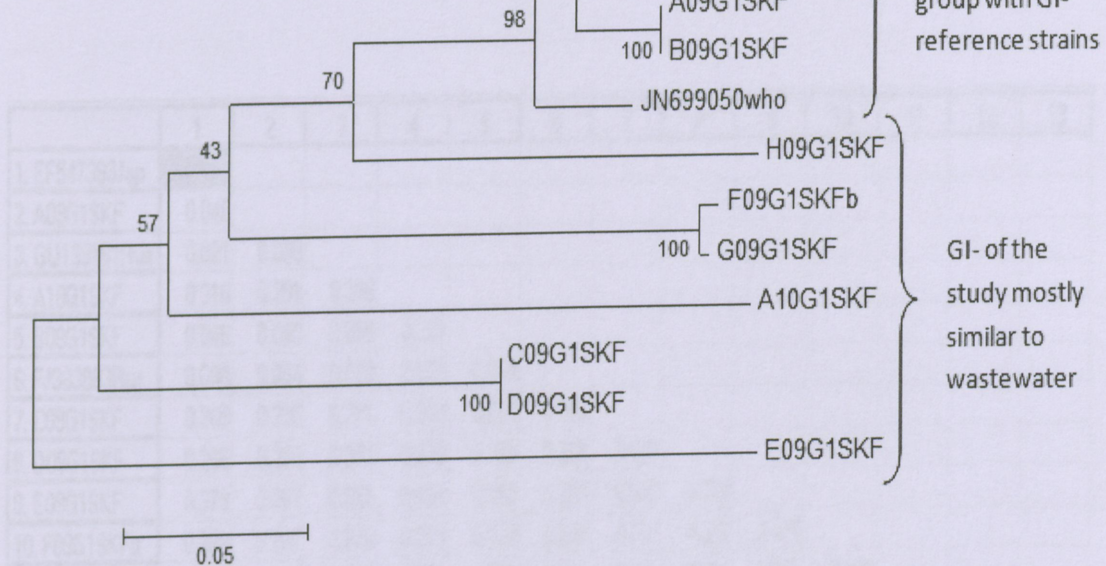

Figure 20: Schematic representation of the phylogenetic tree for Norovirus GI strains from the study and reference strains. Study strains for Norovirus GI showed to be more similar to the strains found in waste water. They are identified with the primer name GISKF at the end of their name. Two NoV GI study strains, A09GISKF and B09GISKF, showed similarity to be similar to reference strains. They include EF547393Jap from Japan, FJ383883Rus, and GU13816Rus from Russia and JN699050who from the World health organization. Evolutionary analyses were conducted in MEGA5 and a phylogenetic tree was constructed from partial nucleotide sequences of the capsid gene of NoV GI. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was1000 replicates.

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The estimates of average evolutionary divergence over all sequence pairs for NoV GI were 0.233. As observed from analysis of the nucleotide, that make up the study strain and the reference strains, the no difference also in the genetic distance, hence the values of the study sequences are low when compared to the reference strains. This shows the small difference in nucleotide of the study sequences and that found in the GeneBank.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. EF547393Jap													
2. A09G1SKF	0.046												
3. GU138161Rus	0.021	0.059											
4. A10G1SKF	0.310	0.291	0.316										
5. B09G1SKF	0.046	0.000	0.059	0.291									
6. FJ383883Rus	0.008	0.054	0.029	0.323	0.054								
7. C09G1SKF	0.266	0.255	0.271	0.278	0.255	0.266							
8. D09G1SKF	0.266	0.255	0.271	0.278	0.255	0.266	0.000						
9. E09G1SKF	0.379	0.367	0.357	0.404	0.367	0.379	0.326	0.326					
10. F09G1SKFb	0.268	0.237	0.274	0.279	0.237	0.268	0.332	0.332	0.375				
11. G09G1SKF	0.254	0.235	0.260	0.285	0.235	0.254	0.341	0.341	0.385	0.008			
12. H09G1SKF	0.196	0.201	0.202	0.279	0.201	0.196	0.267	0.267	0.397	0.302	0.307		
13. JN699050whc	0.060	0.064	0.073	0.313	0.064	0.069	0.247	0.247	0.371	0.234	0.221	0.190	

Figure 21: Estimates of Evolutionary Divergence between Sequences. The figure shows the variations by genetic distance of the study sequences and the reference sequence.

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Unlike NoV GI, phylogenetic analysis of NoV GII showed a significant difference in nucleotide sequence of study strains compared with reference strains. The difference characterize a difference in a large number of nucleotide per sequence, they can be about 4 to 6 nucleotide difference per sequence. Figure 22 show the difference in nucleotide sequence of the study strains with comparison to the strain found in the GeneBank for NoV GII. Only one strain from the study, B11G2SKF had its nucleotide sequence in a position marked in figure 16, similar to all reference strains, except the strain from Japan. Sequence analysis shows a high difference in nucleotide from strain of the study, G10 068G2SKF and H10 066G2SKF compared to reference strain KC10029UK and another from the study. A majority of the reference sequences did not form cluster with the study sequence, but three sequences D10G2SKF, E10G2SKF and H10066G2SKF formed the second cluster with one reference sequence, EF547393Jap from the GeneBank. Figure 23 show phylogenetic analysis of the study strain for NoV GII. The estimated average of evolutionary divergence over all sequence pairs for the first group of NoV GII were 0.910.

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```
EF547393Jap   TGGTACCAGAGGCAAACACAGCTGAGCCTATATCAATGGAG CCCGTGGCTGGGGCAGCAACAGCTGCCGCAACCGCTGGCCAAGTTAATATGAT TGAC
KF060106Aus        ....TC....A    ....GT...GG.TT.....  .....A.T...T..C..T.TT..G..AC.TGTA..G.....GCAA...G.A..  ....
B11G2SKF      .C.A..TTAC   .TCG..A    ....T.CC.GG.TT.....  .......T...T..C..T.TT..G..AC.GGTA..G......CAA...G.A..  ....
EF126963Hol     .C.......TC....A    ....GT...GG.TT.....  .....T.T...T..C..T.TT..G..AC.TGTA..G......CAA...G.A..  ....
KC495641ZAF   .C..C.......TC....A    ....GT...GG.TT.....  .....A.T...T..C..T.TT..G..AC.TGTA..G.....GCAA...G.A..  ....
D10G2SKF      .C...........C....A    ....GT...GG..C.T....  ..G....TG..A..TT...TC..A..TC.TGT..TC.......CAA..C..A..  ....
E10G2SKF      .C..........TC....A    ....A.C..GG..C.T..A  ..G..........TT...T...C...C..CT.A.C..T...AA....G.T..  A...
KC810029UK    .C..C.......TC....A    ....GT...GG.TT.....  .....A.T...T..C..C.TT..G..AC.TGTA..G......CAA...G.A..  ....
G10_068G2SKF  ..ACGT..A.   TC.G...        .G.CC.TA.GTCC.G.G.TACACA.GT.TA..ATGGTT.   .GTTGG.TAC.C.TG...AA....G.T..A.C..
H10_066G2SKF  .C..........TC....A    ....A.C..GG..C.T..A  ..G..........TT...T...C...C..CT.A.C..T...AA....G.T..  A...
KF060079Aus        ....TC....A    ....GT...GG.TT.....  .....A.T...T..C..T.TT..G..AC.TGTA..G.....GCAA...G.A..  ....
AB303932Hol        ....TC....A    ....GT...GG.TT.....  .....T.T...T..C..T.TT..G..AC.TGTA..G......CAA...G.A..  ....
EU876886Egy        ....TC....A    ....GT...GG.TT.....  .....T.T...T..C..T.TT..G..AC.TGTA..G......CAA...G.A..  ....
AY502018CAN   .C..C.......TC....A    ....GT...GG.TT.....  .....T.T...T..C..T.TT..G..AC.TGTA..G......CAA...G.A..  ....
KC409292VNM   .T..C.......TC....A    ....GT...GG.TT.....  .....A.T...T..C..T.TT..G..AC.TGTA..G......CAA...G.A..  ....

                     110       120       130       140       150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
EF547393Jap   CCCTGGATAATGAATAATTATGTGCAAGCCCCTCAAGGTGAATTCACCATATCGCCTAACAACACACCAGGTGATATTTTGTTTGATTTACAATTAGGCC
KF060106Aus   ........T.GA..C....T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
B11G2SKF      ........T.GA.......T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
EF126963Hol   ........T.GA.......T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC..G...
KC495641ZAF   ........T.GA..C....T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
D10G2SKF      ........T.GAG.A....T...C.....A..A..G.....G.......G.C.....G.GG...T.G..T.....G..GC.A..AA..C.TG.......
E10G2SKF      ........T.GATTA..C.T......G.....CA.T..A..G.....AG.T..A..CCG...TT.G..T..G..AG.CC.AC.AA.C...GG.......
KC810029UK    ........T.GA..C....T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
G10_068G2SKF  ........T.GATTA..C.T......G.....CA.T..A..G.....AG.T..A..CCG...TT.G..T..G..AG.CC.AC.AA.C...GG.......
H10_066G2SKF  ........T.GATTA..C.T......G.....CA.T..A..G.....AG.T..A..CCG...TT.G..T..G..AG.CC.AC.AA.C...GG.......
KF060079Aus   ........T.GA..C....T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
AB303932Hol   ........T.GA.......T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC..G...
EU876886Egy   ........T.GA.......T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC..G...
AY502018CAN   ........T.GA.......T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC..G...
KC409292VNM   ........T.GA..C....T...A.........GGT..A..G..T..AG....C....GA...G.T........A..AC.A.GGAGCGCG.CC......
```

Figure 22: Schematic representation of the alignment of first group of Norovirus GII

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The following figure, figure 23, demonstrates the phylogenetic tree that was a result of the analyzed results from the first group of NoV GII.

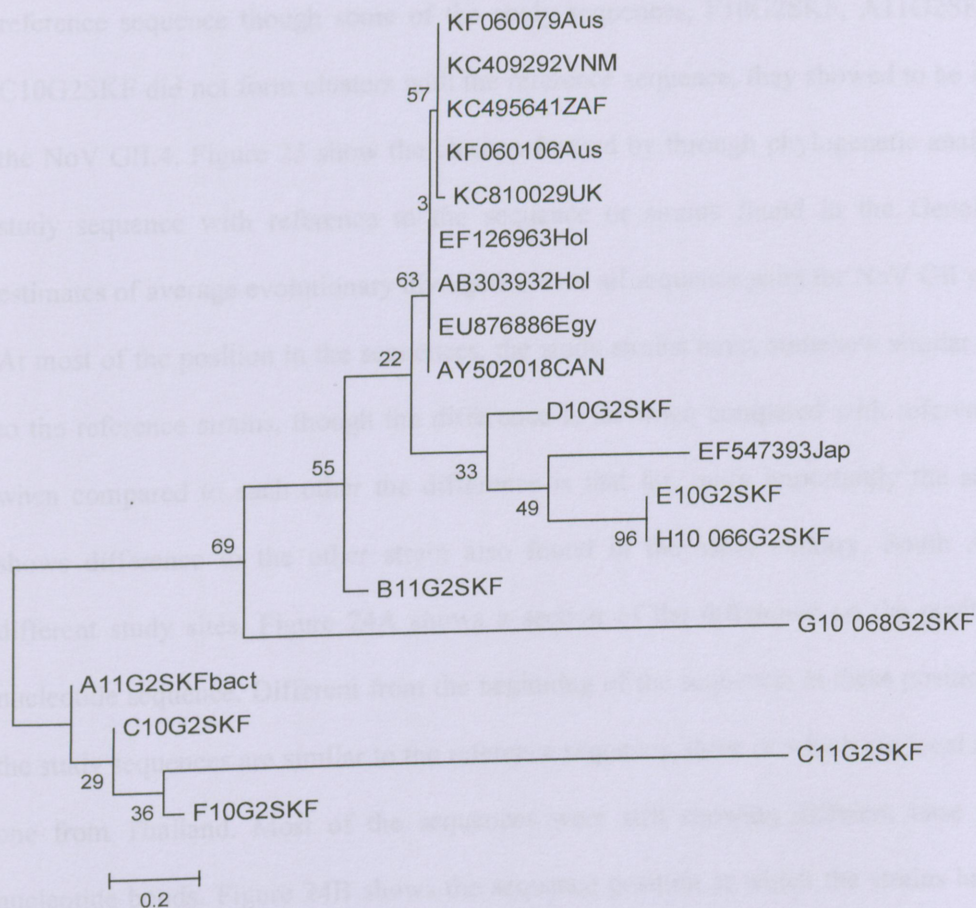

Figure 23: Phylogenetic tree of the first run for Norovirus GII constructed from partial nucleotide sequencing of the capsid gene. The study strains are labeled with the primer name G2SKF at the end. Evolutionary analyses were conducted in MEGA5 in which three study strains; D10G2SKF, E10G2SKF and H10066G2SKF, showed to be similar to one reference strain, EF547393Jap from Japan. The rest of the selected study strains showed no similarity to reference strains; instead they form a second cluster of the phylogenetic tree that includes only the reference strain from Japan.

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The second group of NoV GII also showed a difference in the strains sequences, but it was not as far different as the first group of NoV GII to the reference strains. Phylogenetic analysis of the study sequences showed NoV GII that is more similar to NoV GII.4 from the reference strains. Most of the study sequences formed clusters with the respective identical reference sequence though some of the study sequences, F10G2SKF, A11G2SKFbact and C10G2SKF did not form clusters with the reference sequence, they showed to be identical to the NoV GII.4. Figure 25 show the clusters formed by through phylogenetic analysis of the study sequence with reference to the sequence or strains found in the GeneBank. The estimates of average evolutionary divergence over all sequence pairs for NoV GII were 0.345 At most of the position in the sequences, the study strains have, somehow similar nucleotide to the reference strains, though the difference is far when compared with reference strains, when compared to each other the difference is that far, more importantly the study strain shows difference to the other strain also found in the same country, South Africa, but different study sites. Figure 24A shows a section of the difference on the position of the nucleotide sequence. Different from the beginning of the sequence, at these positions, few of the study sequences are similar to the reference sequence, three of which are local strains and one from Thailand. Most of the sequences were still showing different base pairing or nucleotide bonds. Figure 24B shows the sequence position at which the strains have almost similar nucleotide as the reference strains.

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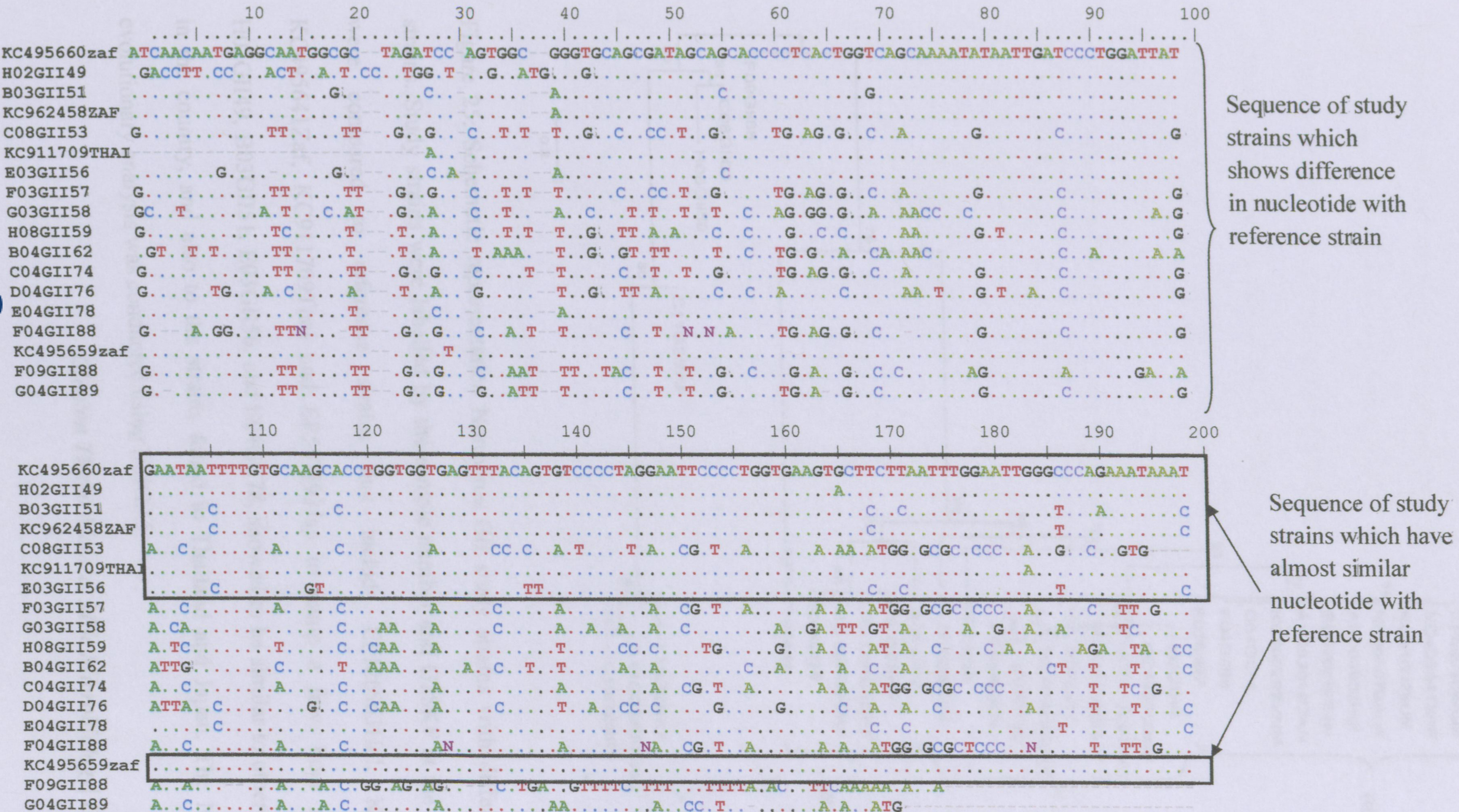

Figure 24: Schematic representation of the alignment of the second group of Norovirus GII

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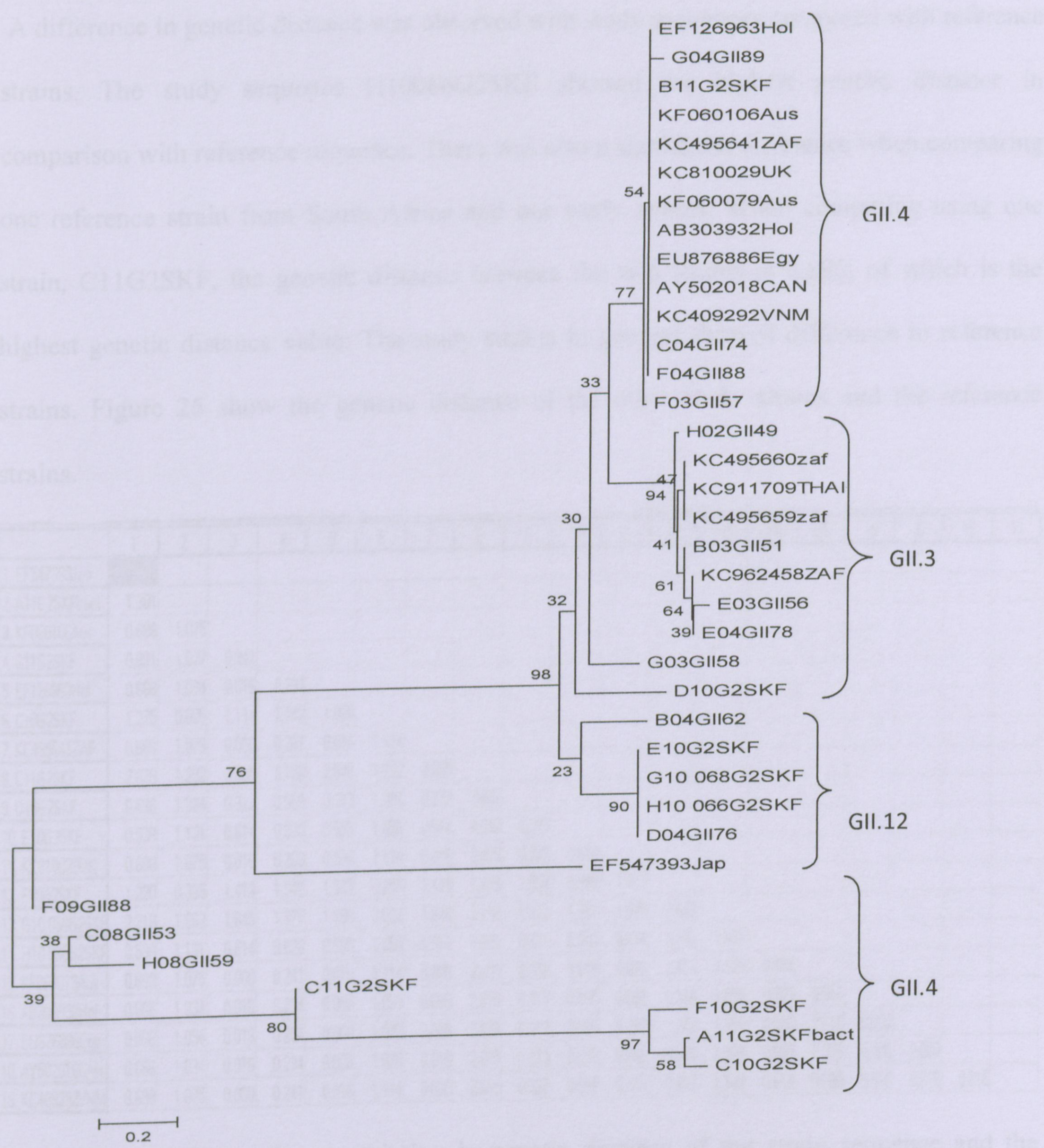

Figure 25: Schematic representation Norovirus GII study strains with different reference strains. Study strains were labelled by the sample number and G2SKF at the end of which were compared to reference strains that include EF126963Hol, KF060106Aus, KC495641Zaf, KC911709Thai and EF547393Jap to name a few. Four study strains; H02GII49, B03GII51, E03GII56, and E04GII78, showed to be similar to other strains found in the country, and also to the strains found in Thailand and Japan. The Norovirus GII evolutionary analysis was conducted using MEGA5.

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A difference in genetic distance was observed with study sequences compared with reference strains. The study sequence H10066G2SKF showed the highest genetic distance in comparison with reference sequence. There was also a significant difference when comparing one reference strain from South Africa and our study strains. When comparing using one strain, C11G2SKF, the genetic distance between the two strains is 2.805, of which is the highest genetic distance value. The study strains in general showed difference to reference strains. Figure 26 show the genetic distance of the other study strains and the reference strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. EF547393Jap																			
2. A11G2SKFbact	1.301																		
3. KF060106Aus	0.688	1.075																	
4. B11G2SKF	0.891	1.077	0.241																
5. EF126963Hol	0.666	1.034	0.016	0.234															
6. C10G2SKF	1.275	0.085	1.114	1.142	1.070														
7. KC495641ZAF	0.688	1.075	0.000	0.241	0.016	1.114													
8. C11G2SKF	2.629	1.292	2.805	2.108	2.670	1.232	2.805												
9. D10G2SKF	0.666	1.384	0.322	0.569	0.313	1.384	0.322	2.601											
10. E10G2SKF	0.538	1.131	0.614	0.820	0.595	1.056	0.614	4.042	0.371										
11. KC810029UK	0.688	1.075	0.016	0.263	0.032	1.114	0.016	2.805	0.322	0.614									
12. F10G2SKF	1.220	0.335	1.413	1.349	1.349	0.255	1.413	1.439	1.598	0.996	1.413								
13. G10 068G2SKF	2.218	1.963	1.649	1.377	1.598	2.034	1.649	2.138	1.922	1.991	1.649	2.629							
14. H10 066G2SKF	0.538	1.131	0.614	0.820	0.595	1.056	0.614	4.042	0.371	0.000	0.614	0.996	1.991						
15. KF060079Aus	0.688	1.075	0.000	0.241	0.016	1.114	0.000	2.805	0.322	0.614	0.016	1.413	1.649	0.614					
16. AB303932Hol	0.666	1.034	0.016	0.234	0.000	1.070	0.016	2.670	0.313	0.595	0.032	1.349	1.598	0.595	0.016				
17. EU876886Egy	0.666	1.034	0.016	0.234	0.000	1.070	0.016	2.670	0.313	0.595	0.032	1.349	1.598	0.595	0.016	0.000			
18. AY502018CAN	0.666	1.034	0.016	0.234	0.000	1.070	0.016	2.670	0.313	0.595	0.032	1.349	1.598	0.595	0.016	0.000	0.000		
19. KC409292VNM	0.688	1.075	0.000	0.241	0.016	1.114	0.000	2.805	0.322	0.614	0.016	1.413	1.649	0.614	0.000	0.016	0.016	0.016	

Figure 26: The figure shows variation in genetic distance of the strain sequence and the reference sequence. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 63 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

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A number of the NoV GII strains were analyzed with reference strains to observe the overall difference in genetic distance of the strains in the present study and the reference strain. A phylogenetic tree was made using the strains and the table showing the overall genetic distance was made from this selected strains of NoV GII, since it will be enable a clear idea as to how much difference is there in the sequence found in the present study, and the strains found in South Africa and other parts of the world, as reference strains. Figure 27 show the analysis made from selected strains with the reference strains. In this group of NoV GII phylogenetic analysis, a number of study sequence B03GII51, E03GII56, E04GII78 and H02GII49 formed a major cluster with the reference sequence, in which it was similar to reference sequences found in other parts of South Africa. The remaining study sequence formed two clusters which varied with reference sequences but were identified as NoV GII.4

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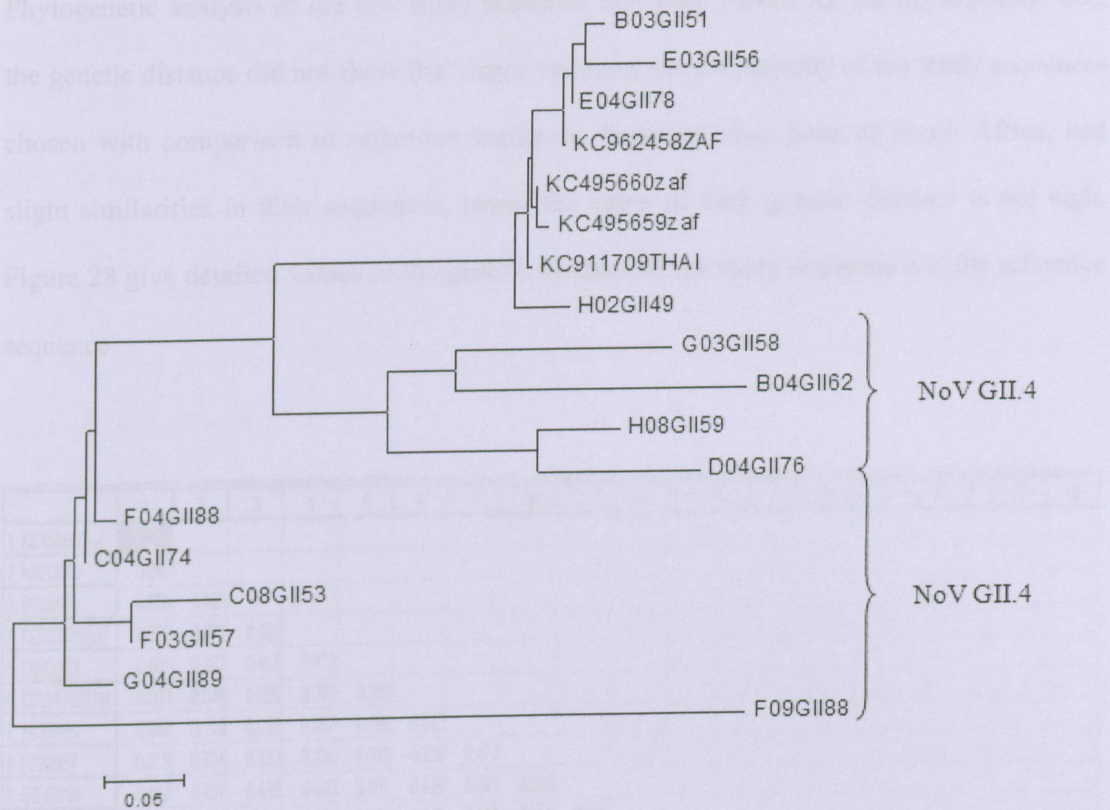

Figure 27: Schematic representation Norovirus GII study strains phylogenetic tree constructed from partial nucleotide sequencing of the capsid gene to determine the overall genetic distance between sequences. The study strains were labelled with the GII after the sample number. Though the study strain formed two different clusters that confirm no similarity of study strains to reference strains, they showed to belong to Norovirus GII.4 strain which is a sporadic strain. Four study strains; B03GII51, E03GII56, E04GII78, and H02GII49 showed to be similar to the three reference strains found in the country and one in Thailand.

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Phylogenetic analysis of the few study sequence that were picked for the phylogenetic tree, the genetic distance did not show that major variation since a majority of the study sequences chosen with comparison to reference sequences found in other parts of South Africa, had slight similarities in their sequences, hence the value of their genetic distance is not high. Figure 28 give detailed values of the genetic distance of the study sequence and the reference sequence.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. KC495660zaf																		
2. H02GII49	0.048																	
3. B03GII51	0.056	0.100																
4. KC962458ZAF	0.023	0.073	0.032															
5. C08GII53	0.401	0.387	0.416	0.413														
6. KC911709THAI	0.008	0.048	0.056	0.031	0.399													
7. E03GII56	0.082	0.119	0.057	0.057	0.488	0.082												
8. F03GII57	0.319	0.334	0.333	0.330	0.048	0.318	0.414											
9. G03GII58	0.442	0.491	0.438	0.440	0.401	0.426	0.543	0.340										
10. H08GII59	0.410	0.424	0.438	0.422	0.350	0.395	0.522	0.314	0.349									
11. B04GII62	0.466	0.460	0.483	0.466	0.496	0.449	0.536	0.453	0.325	0.405								
12. C04GII74	0.283	0.297	0.296	0.294	0.082	0.282	0.373	0.031	0.340	0.303	0.455							
13. D04GII76	0.415	0.442	0.418	0.402	0.502	0.399	0.469	0.458	0.360	0.157	0.405	0.438						
14. E04GII78	0.031	0.073	0.024	0.008	0.413	0.031	0.048	0.330	0.440	0.422	0.466	0.294	0.402					
15. F04GII88	0.295	0.309	0.296	0.305	0.100	0.294	0.373	0.048	0.353	0.315	0.455	0.023	0.467	0.305				
16. KC495659zaf	0.008	0.056	0.064	0.031	0.413	0.016	0.090	0.330	0.455	0.422	0.479	0.294	0.427	0.039	0.305			
17. F09GII88	0.788	0.819	0.814	0.806	0.571	0.784	0.899	0.546	0.832	0.749	0.860	0.525	0.920	0.806	0.541	0.806		
18. G04GII89	0.329	0.344	0.343	0.340	0.129	0.328	0.411	0.074	0.393	0.354	0.473	0.048	0.481	0.340	0.057	0.340	0.528	

Figure 28: Estimates of Evolutionary Divergence between Sequences for the overall genetic distance for NoV GII. The figure shows in the details the variation of the study sequence and the reference sequence for the second group of NoV GII.

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CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 DISCUSSION

This study demonstrates that NoV is a significant cause of childhood gastroenteritis in Vhembe and is now recognized as an important etiologic agent of diarrheal illness worldwide. In many African countries, molecular diagnostic methods for viral gastroenteritis are difficult to access, and consequently few epidemiological data is available on either viral gastroenteritis in general, or those due to NoV in particular. Reverse-transcriptase RT-PCR can be recommended as one of the rapid and sensitive methods that can detect NoV, in order to give a quick response to eliminate and prevent infection. In the present study, tests by reverse-transcriptase RT-PCR have proven that Norovirus can be present also in asymptomatic children, those with no diarrhoea; hence it was detected in some of the monthly samples.

These can also mean that infection is still at its early stage, which it hasn't manifested to diarrhoea. A majority of symptomatic children's samples, those with diarrhoea, tested positive to detection with reverse-transcriptase RT-PCR, which is expected with viral diarrhoeal infections. The study is in agreement with the findings done by Netshikweta *et al.*, 2011; that NoV is the most common causative agent of gastroenteritis (Netshikweta *et al.*, 2011).

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The test results give a margin of proximity, in concern with the number of infected children in an area of study, irrespective of the child having diarrhoea or not. The number of infections per month can also give a seasonal occurrence of the virus, and the number of diarrhoeal infections experienced can describe the severity of the viral infection in a season. Diarrhoea in children can give an estimation of manifestation of the virus in the body, since symptoms appear after two days of consumption of the virus. It is now recognized that vomiting with airborne NoV dispersion is an important route of virus dissemination. Although aerosol spread may initiate an outbreak, continued propagation of NoV disease outbreak can, in some cases, be associated to the role of subsequent environmental contamination (Said *et al.*, 2014).

Though no child in the study was hospitalized due to diarrhoea, a child can lose water and nutrients in the body, which can lead in disturbance in health and eventually growth is altered. In a more recent study by Mans *et al.*, (2013); in Gauteng province, SA indicated that after Rotavirus (24% prevalence), NoV was the most frequently detected viral pathogen in pediatric patients (14% prevalence) hospitalized with viral gastroenteritis (Mans *et al.*, 2013). Children are usually wean from maternal antibody and introduced to semisolid foods which may be contaminated due to improper handling. At a tender age, it is the period in which they crawl around picking anything they see into the mouth. This finding is consistent with the observation in Ghana and in Bangladesh where infection was found common in children under 2 years of age. NoV genogroups GI, GII and GI+GII were detected, with GII as predominant (Ayolabi *et al.*, 2010).

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A study by Wu *et al*., (2008); has shown that, most children in Taiwan vomit most of the time rather than have diarrhoea during infection (Wu *et al*., 2008). In the present study, infections with NoV mostly were high between birth and by 18 months morbidity reduces. Also in the study by Smit *et al*., (1999); it was shown that in Ga-Rankuwa, Pretoria, SA, 40% of the children less than two years of age, especially between eight and eleven months are susceptible to infection by NoV. This was found through an outbreak of infection with an unknown agent that causes diarrhoea in children, which were eventually hospitalized. Tests from serum and stool sample of the children showed the presence of NoV (Smit *et al*., 1999). Yoon *et al*., (2008), also enlightened that; although NoV infections were detected in all age groups, NoV infections were found most frequently in the less than one year old group (43.0%; 49 of 114) in South Korea, and also determined that most of the NoV infections occurred in children less than two years of age (74.6%; 85 of 114), with highest incidence of NoV infection in the one year old group, and the incidence decreased with increasing age over two years (Yoon *et al*., 2008).

A variant of GII.4 viruses appear to emerge almost annually and some variants spread globally and replace previously dominant GII.4 strains. NoVs are characterized by high environmental stability and a very low infectious dose (Mans *et al*., 2013).Seasonal occurrence of the genogroups show study significance as NoV GII infections are more seen almost year in and year out in Vhembe. The infection with GII shows to remain high even on recurrence of the virus, at which the body should have developed immunity, but since the immunity is weak at a young age, developing immunity to recurring infections with NoV seems to be impossible, and therefore, vaccination can play a role in preventing high morbidity thereby preventing high mortality. As most of the infections occur during winter; NoV GII infections showed to be higher (48.1%) than NoV GI (3.8%) infections.

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In South Korea NoVs were continuously detected throughout the year, but the principal peaks of detection in South Korea were in December (for GI), March (for GII), and October (for GII) (Yoon *et al*., 2008). In the present study NoV GI and GII infections showed very different seasonality characteristics. The occurrence of NoV GII lowered at the beginning of the spring season 26.4% but gradually rises throughout the season 48.0%. During the summer season, both genogroups infections decreases, which later increases when winter begins. Peaks of detection in this study were as follows; November (for GII), December (for GII), and June (for GII); whilst in October infection with GII showed to be high and GI showed to rise, but not too much. In the study by Yoon *et al*., (2008); in South Korea, the peaks of detection were in December (for GI and GII), March (for GII) and October (for GII)The study by Trainor *et al*., (2013) has shown that, same as in South Africa, NoV is detected throughout the year with peaks at the end of the rainy season (March) and towards the end of the dry season (August-November), Norovirus GII.4 was the most commonly detected genotype accounting for 70% of strains characterized, followed by GII.2 (6%), GII.6 (4%) and GII.12 (4%) (Trainor *et al*., 2013).

Mans *et al*., 2013; has also shown that in South Africa, NoV infections are high during winter, the study followed NoV infection from the year 2008 to 2010 in Gauteng, in which there was a difference in genogroup infections between those years. NoV GI showed to be at a high in 2008 and eventually decreased in 2010, while NoV GII has gradually reduced from 2008 to 2010 (Mans *et al.,* 2013). In Vhembe the infection with NoV GI dropped from 13.5% to 0.0%, and NoV GII infections gradual decreased from 44.4% to 25.7% since 2010 to 2013. A greater reduction of NoV GI infections was observed since 2012 to 2013, but with NoV GII infection increased gradually since 2013 and with confirmation of the recent study, in Vhembe NoV is still detected at a high rate, possibly even in other areas of South Africa.

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Accordingly, NoVs are frequently implicated in foodborne and waterborne gastroenteritis outbreaks (Mans *et al*., 2013). In a study by Zhou *et al*., 2012, it is elaborated that detection NoV RNA in water suspected of causing illness helps confirm that a gastroenteritis outbreak is due to NoV (Zhou *et al*., 2012). In the present study stool sample tests were used to confirm infection of NoV, which is ingested from contaminated food or water. There are reports that supports that NoV is found in contaminated food. Several food types, including shellfish, cold foods and fresh produce, have been implicated in foodborne outbreaks of viral disease (Netshikweta *et al.,* 2011).

The most important route of person-to-person transmission is fecal-oral, and vomiting is also implicated as a source of transmission. Ramudingana, (2009); states that NoV was first reported in 1993 in South Africa, and most importantly it was detected in individuals who ate salads. Water also plays a role in the spread of enteric viruses, and contaminated water is an important source of human infection but the true burden of disease is underestimated due to underreporting (Netshikweta *et al.,* 2011). It is quite clear that since salads are mostly raw vegetables or non-cooked food, they are most likely to cause infection by NoV when not cleaned properly or when they are cleaned with contaminated water.

Irrespective of the diarrhoea in NoV infected children, in the present study we also looked at other health problems that can be associated with NoV infection. In the study by Nordgren *et al*., (2013); the sex difference showed no significance although NoV was slightly more prevalent in males (13%) as compared to females (10%). Malnutrition status was also not associated with significant differences in NoV prevalence, nevertheless NoV was frequently observed in malnourished children (Nordgren *et al*., 2013).

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In the present study prevalence of NoV was also seen more frequently in females (52.4%) more than in males (47.6%). Stool samples tested in the present study showed that the difference in the infection in children who are underweight was significant, since a majority of the children's stools with who are underweight showed the presence of NoV GII 68.8% with very few having NoV GI 6.3%.

In stool samples of children who showed stunting 13.3% had NoV GI and 57.9% had NoV GII. NoV GII also seemed to be high, 85.7% when compared with NoV GI 14.3%, in children who were wasted. The difference in the infection in children who are underweight showed significance, since a majority of the children's stools with who are underweight showed the presence of NoV GII 68.8% with very few having NoV GI 6.3%.

At the same time, the infection with NoV GII was also high in children with diarrhoea at 66.0% especially those children who are fed colostrums which make up 67.2% of the study population. The present study showed that children who breast feed are more susceptible to infection with NoV GII 87.5% than infections with NoV GI12.5%.On the other hand, it was to be also true for children who do not breast feed and are given colostrums. 67.2% of the children who were fed colostrums showed to be more susceptible to NoV GII, whilst 27.1% showed the presence of NoV GI.

Due to the known genetic diversity within the genogroups of NoVs, a low detection rate by RT-PCR can be expected. The use of different primer pairs and combinations, as well as the use of primers to the capsid region, can further increase the detection rate of viral antigen in stool specimens in our population (Smit *et al*., 1999). With the aid of reverse-transcriptase RT-PCR, we have detected that NoV genogroup II (GII) is the most predominant genogroup found in Vhembe. Compared with other places in South Africa and in Africa, GII seems to be more epidemic than GI.

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In the study done in Pretoria by Mans *et al*., 2010; NoVs was only detected in children less than 2 years. The GII NoVs predominated (89%) and eight types of the GII were identified with GII.4 (43%) detected most frequently. Furthermore, in years in which a new GII.4 NoV variant emerges, Mans *et al*., (2010) elaborates that an increase in the frequency of detection of GII strains can be detected (Mans *et al*., 2010).

Without a virus culture system, genetic analysis becomes the principal method to classify NoV strains. Currently, classification of NoV strains below the species level has been based on sequences from different regions of the viral genome (Zheng *et al*., 2006). In the present study, samples were sent for sequencing, to determine the genetic diversity of Norovirus at a portion of the capsid N/S (N-terminal/Shell capsid) domain in order to know the prevalent strain in Vhembe district.

When compared to the strains in the gene bank, the NoV GI detected in Vhembe was different from the ones in other countries, namely; Russia and Japan. The difference was estimated at about 40% of the sequence analyzed, since the sequence found from strains from Vhembe has a difference of two to three nucleotides in a sequence.

Also when comparing NoV GII strain in the gene bank, the NoV GII detected in Vhembe was far more different from the ones found in Japan, Australia, South Karolina, Another strain South Africa, United Kingdom, Holland, Egypt, Canada and Vietnam. The difference was estimated at 96%, at which the NoV GII show a high number of difference in their sequence nucleotides.

For NoV GI, fewer bases are different with that found in the gene bank for other NoV strains, for NoV GII switch in bases ranges from a pair to four nucleotides. Change seen in the nucleotide ssequence can give an idea of mutation found in the strain of Norovirus, and can also become more important for vaccine production, in order to produce a target vaccine.

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As seen in the phylogenetic trees the strains of NoV GI and NoV GII found in Vhembe do not match any of the strain found the other countries, two countries Russia and Japan for GI, since infection with NoV GI is low, and in relation to the results observed had no major difference with the NoV GI strains found in the two countries.

For GII, since it is highly infective than NoV GI, it occurs to be different from a majority of nine strains found in other studies, which can show the highly infectivity and rapid spread of NoV GII, since it has different sequence of nucleotide almost in every country, and recently even in the same country the strain can differ. As we see in the present study, a NoV GII strain found in Vhembe, show a significant difference to the other strain found elsewhere in South Africa.

A study by Katayama *et al*., (2002) has shown that the clustering based on the capsid N/S domain successfully distinguished the NoV as well as the grouping based on the antigenicity, as determined by both antigen and antibody ELISAs with recombinant virus-like particles (Katayama *et al*., 2002). Kobayashi *et al*., (2000); identified at least two antigenically different strains in NoV GI, namely Seto virus and Chiba virus, and several strains in NoV GII by an antigen ELISA; these antigenic classifications appeared to be correlated well with that determined by a phylogenetic analysis of the capsid N/S domain (Kobayashi *et al*., 2000). Therefore, it may be possible to predict antigenic types by phylogenetic analysis of the capsid N/S domain. Sporadic variations observed in both the capsid and the polymerase region does not persist during the infectious course. The possibility remains that a small population of NoV with G at the corresponding position already existed at the first exposure. Observations suggest that NoV really evolves in the gastrointestinal tract in a single individual (Obara *et al*., 2008).

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Mutations in NoV can cause a problem with vaccine development or an alteration to confer vaccination in order to prevent infection, and spread of NoV. Multivalent regimens containing vaccine components from several predominant circulating strains of NoV will possibly protect against subsequent homologous infection, as multivalent VLP vaccines have been shown to protect against homologous infection in the mouse and the pig (Lobue *et al*., 2009). Furthermore, it has been shown that increasing the number of strains in multivalent vaccines generates strong and equivalent immune responses to all components of the vaccine without diminishing individual responses compared with the response to a monovalent vaccination.

Recombinant events may create new genotypes or species and cause difficulty in strain classification because the recombinants could be intermediates between genotypes or species. Recombination within the capsid affects correct classification. Partial sequence to classify new NoV strains is discouraged. NoV sequences have great diversity, and hypervariable region is located in the capsid gene (Zheng *et al*., 2006). Multivalent vaccines in the mouse have shown to enhance genogroup-specific, cross-reactive receptor blocking-antibody and T cell responses, and this suggests that multivalent vaccines containing components of both GI and GII strains would probably confer substantial protection against NoV infection (Donaldson *et al*., 2010). Distinct NoV strains have highly variable Histo-blood group Antigen (HBGA-binding patterns (Donaldson *et al*., 2010).

Histo-blood group ABO (H) antigens with a terminal fucose (Hexose deoxy sugar) act as a receptor for human NoV in the gastrointestinal tract. In a study by Nordgren *et al*., (2010); a single nucleotide mutation in the fucosyltransferase (enzyme that transfers an L-fucose sugar from GDP-fucose donor substrate to an acceptor) gene on chromosome 19 provides strong protection from infection in 20% of Caucasians, although some NoV genotypes can infect persons carrying this mutation (Nordgren *et al*., 2010).

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A non-functional fucosyltransferase, FUT2, provides high protection from the most common NoV GII.4 (Carlsson *et al*., 2009). Functional FUT2 transfers fucose sugar to the end of the histo-blood group ABO (H) precursor in the gastrointestinal cells and saliva glands, The ABH antigen is thought to act as receptor for human NoV. FUT2 non-secretors (Homozygous carriers of a non-sense mutation in the FUT2 gene) produce no ABH antigen. The 20% of Caucasians found to be protected from NoV in the study by Nordgren *et al*., (2010); have a non-sense mutation in FUT2 and therefore have a strong although not absolute protection from the NoV GII.4. FUT2 non-secretors can still produce ABH antigen in erythrocytes, as the precursor is formed by FUT1, and can be infected by other genotypes such as GI.3 (Nordgren *et al*., 2010). Secretor positive individuals with blood type O are more likely to be infected than secretor positive individuals of blood type B (Rydell *et al*., 2011).

In the present study, the sequenced GII samples have shown to be similar to the recently termed sporadic GII.4. The study also confirms that NoV GII.4 is now spreading rapidly and at a high rate globally. Probably due to its sporadic characteristic it can cause more infections than other genotypes. We can now confirm as in the other areas of NoV infections, NoV GII.4 is the predominant strain in Vhembe as in other areas.

RNA viruses mostly have a high mutation rate. NoV is seen as one of the viruses which have this particular characteristic. NoV mutation rate is estimated at $1.21x10^{-2}$ to $1.41x10^{-2}$ substitution per base per year. Compared to other RNA viruses, NoV has a high mutation rate (Victoria *et al*., 2009). The present study also confirms the high mutation rate of NoV. Sequence analysis in the present study has shown base difference in the sequence of the known strain in the GeneBank and the strains sequenced from the study.

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Though in some strains base difference is not that significant, Chen *et al*., (2013), also elaborates that given the constant genetic drift and shift of NoVs, many viruses in the study samples may have escaped detection with the specific primers that were also employed in this present study (Chen *et al*., 2013). In addition, many NoV bind to more than one type of HBGA, which suggests that different populations may be susceptible to one strain (Donaldson *et al*., 2010).

The overall genetic distance of the strains sequenced from the study also supported the observation found. For NoV the difference by overall genetic distance was low, at 0.233. Identifying with one of the study strains E09G1skf, for NoV GI, the genetic distance is high, at 0.379, between this strain and the strain found in Japan. When comparing with the reference strains, the genetic distance between the study strains and the strain from Russia the genetic distance is also at 0.379, with the strains found by the World health organization, the genetic distance is 0.371. In general strains in the present study for NoV GI is not far identical from the ancestral strains.

With NoV GII strains in the study, since it was analyzed in two groups, the second group of NoV GII in the study had a low difference by overall genetic distance of 0.345. The first group showed a high difference when analyzed, which had an overall genetic difference of 0.910. NoV GII in the first group had the highest genetic distance at 2.085, when identifying with one of the study strain C11G2SKF, when compared with reference strains from South Africa, Vietnam and one from Australia. When comparing the same study strain with the other reference strains from Holland, Egypt and Canada the genetic distance is 2.670.

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Genetic distance of analyzed strains can confirm the high mutation rate of NoV strains, thus showing how far or big the difference in nucleotide alignment is. With the other study strains such as H10 066G2SKF they show a low genetic distance ranging from 0.538 to 1.034 which is not that low enough to show similarity in the nucleotide sequence of the study and the reference strains.

A decrease in the genetic distance of the second group of NoV GII sequence analysis was observed, but not enough to also confirm similarity in the study strains compared with the reference strains. Comparing the study strain F09 GII88, the genetic distance between this study strain and the reference strain from South Africa was observed to be 0.788, which can confirm that no similarity in two strains though they are found in the same country. This can also show that NoV strains can also differ by area of epidemic than a global difference when considering countries. To support the mentioned statement, the same study strain F09 GII88 was observed in comparison with two other strains from South Africa, of which the genetic distance was the same at 0.806.

The genetic distance was also almost the same when compared with the strain from Thailand, which was 0.784. From the overall observation of the two groups of NoV GII analyzed. It can be concluded and supported that NoV GII is a genogroup that is spreading rapidly in most places, which it is also seen in the Vhembe district, and on the other hand due to high mutation rates of NoV, the GII strains found are different from the strains that have been recently found, when comparing the gene sequence, but are close to the NoV GII.4 found globally.

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5.2 LIMITATIONS AND CHALLENGES

Enough diarrhoeal samples were collected but not all of them showed to have Norovirus during detection with reverse transcriptase Real Time PCR, therefore, the number of RNA to be analyzed reduces according to the number of samples that tested positive with reverse transcriptase. Reduction in the sample number can somehow hinder data analysis for comparison of Norovirus infection among children who have experienced diarrhoea and those haven't experience diarrhoea. Furthermore data analyzed with few samples can be unclear on association of Norovirus infection with diarrhoea. One of the gel run after PCR had multiple bands and the negative control didn't show any contamination, therefore the band were excised from the gel, at the respective NoV GI and GII band size. These gels were treated to extract DNA and the procedure for NoV PCR was carried out with the DNA from the gel, the same as the PCR from prepared samples. PCR is a very sensitive method for amplification, and sensitive to contamination. When PCR did not show bands visible enough for results, a Nested PCR was performed with the same specific primers as the first run.

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5.3 CONCLUSION

The present study is the first study that identifies the presence of NoV inflectional through seasonal changes, and aimed at identifying the prevalent strain of NoV in the Vhembe district. The present study shows in details, how NoV affect the children's health. It also shows how rapid the virus can be detected with reverse-transcriptase RT-PCR, in order to use the results outcome as a source of information to give light on NoV infection in a given area, to prevent the spread of a virus in a community. The study shows, and also agrees with other research studies, that NoV GII is the most predominant genogroup of NoV infections in different areas, though strains can be different from place to the other. Although it is deduced that a target gene for NoV elimination is the RdRp, to inhibit growth, genotyping of the strains found in the present study showed a significant difference in nucleotide sequence found in Vhembe and strains found in other countries, supporting the difficulty for producing a target vaccine for NoV. In the present study NoV infections were far from convincing to have caused or to be associated with most of the diarrhoea experienced. Regardless of the key developments in the area of NoV research, a deeper understanding of the structure and dynamics of NoV binding to different HBGAs is necessary for the design of potential inhibitors to block the binding of HBGAs to NoVs (Nasir, 2009). As the nucleotide sequences of the primers for the capsid N/S domain are highly conserved among the NoV, the amplification of the unknown genotype can be easily performed.

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5.4 RECOMMENDATIONS

Understanding how NoVs evolve is important for finding more effective ways to control this disease and mostly its impact in healthcare settings. Also, the use of this genetic clustering together with serological differentiation may provide useful information to determine the relationship between genetic and antigenic differences among NoV. These analyses would provide insights into the epidemiology of NoV infection. Regardless of the recent successes of detecting NoV, a number of challenges still remain for NoV research. Efforts towards a vaccine, a suitable human cell culture system and an effective antiviral agent remain a high priority for many NoV research teams. Further investigations are necessary to assess the significance of NoV and related viruses to diarrheal illness in Vhembe and other regions of South Africa. The RT-PCR will make such studies possible and in addition sequence analysis will be necessary to determine the differences between local strains and recognized international strains of NoVs.

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REFERENCES

Allen DJ, Gray JJ, Gallimore CI, Xerry J, Iturriza GM (2008). Analysis of Amino Acid Variation in the P2 Domain of Norovirus VP1 Protein Reveals Putative Variant-Specific Epitopes. *PLoS One*. **3**: 1-9.

Almanza H, Cubillos C, Angulo I, Mateos F, Castón JR, van der Poel WHM, Vinje J, Bárcena J, Mena I (2008). Self-Assembly of the Recombinant Capsid Protein of a Swine Norovirus into Virus-Like Particles and Evaluation of Monoclonal Antibodies Cross-Reactive with a Human Strain from Genogroup II. *Journal of Clinical Microbiology*. **46** (12): 3971-3979.

Aminu M, Esona MD, Geyer A, Steele AD (2008). Epidemiology of rotavirus and astrovirus infections in children in North-western Nigeria. *Annual African Medical Journal*; **7**: 168-174.

Appleton H (1987). Small round viruses: classification and role in food-borne infections. *Ciba Foundation Symposium*. **128**: 108-125.

Arias A, Emmtt E, Vashist S, Goodfellow L (2013). Progress towards the prevention and treatment of Norovirus infections. *Future Microbiology*. **8** (11): 1475-1487.

Atmar RL, Bernstein DI, Harro CD M.D, Al-Ibrahim MS, Chen WH, Ferreira J, Estes MK, Graham DY, Opekun AR, Richardson C, Mendelman PM (2011). Norovirus Vaccine against Experimental Human Norwalk Virus Illness. *New England Journal of Medicine*. **365**: 2178-2187.

Ayolabi CI, Ojo DA, Armah GE, Akpan I, Mafiana CF (2010). Detection and partial characterization of Norovirus among children with acute gastroenteritis in Lagos, Nigeria. *International Journal of Medicine and Medical Sciences*. **2** (7): 216-221.

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Belliot G, Sosnovtsev SV, Chang KO, Babu V, Uche U, Arnold JJ, Cameron CE, Green KY (2005).Norovirus proteinase-polymerase and polymerase are both active forms of RNA-dependent RNA polymerase. *Journal of Virology*. **79**: 2393-2403.

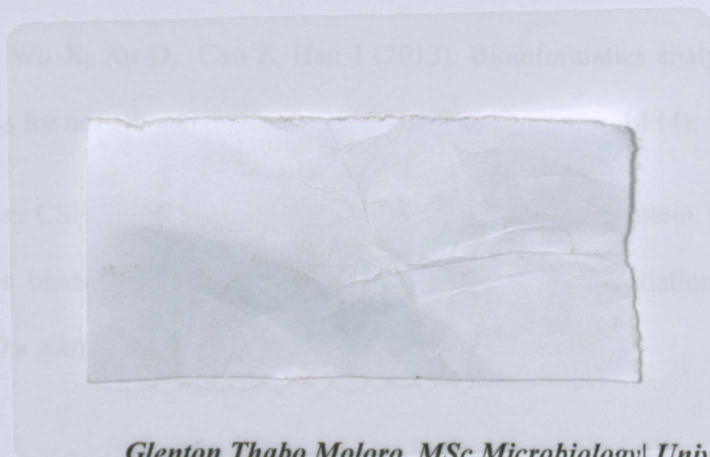
Bok K, Abente EJ, Realpe-Quintero M, Mitra T, Sosnovtsev SV, Kapikian AZ, Green KY (2009). Evolutionary Dynamics of GII.4 Noroviruses over a Thirty-four Year Period. *Journal of Virology*. **83**: 11890-11901.

Bok K, Parraa GI, Mitraa T, Abentea E, Shaverb CK, Boona D, EngleaR,Yua C, Kapikiana AZ, Sosnovtseva SV, Purcella RH, Greena KY (2011). Chimpanzees as an animal model for human Norovirus infection and vaccine development. *Proceedings of the National Academy of Sciences of the United States of America.* **108** (1): 325-330.

Bonfield JK, Smith KF, Staden R (1995). A new DNA sequence assembly program. *Nucleic Acids Research*. **23**: 4992-4999.

Bull RA, Tu ETV, McIver CJ, Rawlinson WD, White PA (2006). Emergence of a New Norovirus Genotype II.4 Variant Associated with Global Outbreaks of Gastroenteritis. *Journal of Clinical Microbiology*. **44** (2): 327-333.

Cao S, Lou ZY, Tan M, Chen YT, Liu YJ, Zhang ZS, Zhang XJC (2007). Structural Basis for the Recognition of Blood Group Trisaccharides by Norovirus. *Journal of Virology.* **81** (11): 5949-57.



© University of Venda

Carlsson B, Kindberg E, Buesa J, Rydell GE, Lidón MF, Montava R, Abu Mallouh R, Grahn A, Rodríguez-Díaz J, Bellido J, Arnedo A, Larson G, Svensson L.; Kindberg; Buesa; Rydell; Lidón; Montava; Mallouh; Grahn; Rodríguez-Díaz; Bellido; Arnedo; Larson; Svensson (May 2009). The G428A Nonsense Mutation in FUT2 Provides Strong but Not Absolute Protection against Symptomatic GII.4 Norovirus Infection. *Plos One*. **4** (5): 1-10.

Centre for disease Control and Prevention (2011). Updated Norovirus Outbreak Management and Disease Prevention Guidelines. *Recommendations and Reports*. **60** (3): 1-12.

Cheetham SM (2006). Pathogenesis of Human Norovirus in Gnotobiotic pigs. A dissertation Presented in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University.1-300. *Clinical and Diagnostic Research*. **6** (2): 188-191.

Chen R, Neill JD, Noel JS, Hutson AM, Glass RI, Estes MK, Prasad BVV (2004). Inter- and intragenus structural variations in caliciviruses and their functional implications. *Journal of Virology*. **78** (12): 6469-6479.

Chen CJ, Lartey B, Agbemabiese C, Mahmoud A, Armah G (2013). The Epidemiology of Noroviruses in Ghana: A Case Study of Norovirus Detection. *The Journal of Global Health*. **54** (6): 2166-3602.

Chen L, Wu D, Ji L, Wu X, Xu D, Cao Z, Han J (2013). Bioinformatics analysis of the epitope regions for norovirus capsid protein. *BMC Bioinformatics*. **14** (4): 1-6.

Daughenbaugh K, Fraser CSHJW, Hardy ME (2003). The genome linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. *The EMBO Journal*. **22**: 2852-2859.

© University of Venda

Dolin R (2007). Noroviruses-Challenges to Control. *New England Journal of Medicine*. **357**: 1072-1073.

Donaldson EF, Lindesmith LC, LoBue AD, Baric RS (2010).Viral shape-shifting: norovirus evasion of the human immune system. *Nature Reviews/ Microbiology*. **8**: 231-241.

Furman LM, Maaty WS, Petersen LK, Ettayebi K, Hardy ME, Bothner B (2009). Cysteine protease activation and apoptosis in Murine Norovirus infection. *Virology Journal*. **6**: 139.

Gallimore CI, Iturriza-Gomara M, Xerry J, Adigwe J, Gray JJ (2007). Inter-seasonal diversity of Norovirus genotypes: Emergence and selection of virus variants. *Archives of Virology*. **152**: 1295-1303.

Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK (2000). Norwalk virus open reading frame 3 encodes a minor structural protein. *Journal of Virology*. **74**: 6581-6591.

Glass RI (2013). Beyond discovering the Viral Agents of Acute Gastroenteritis. *Emerging Infectious Diseases*. **19** (8): 1180-1191.

Goetz G (2012). Pet Dogs Can Carry Human Norovirus, Study Shows. *Food Safety News.*

Gray J (2009). Enteric viruses: Mechanisms for generating diversity. *Enteric Virus Unit CfI, HPA*. A presentation.

Green KY (2007). Caliciviridae: The Noroviruses. *Fields Virology 5th ed*. **28** (28): 949-979.

Hall (1999). Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. **41**: 95-98.

© University of Venda

Hall AJ, Vinjé J, Lopman B, Park GW, Yen C, Gregoricus N, Parashar U (2011).Updated Norovirus Outbreak Management and Disease Prevention Guidelines. *Recommendations and Reports*. **60** (3): 1-15.

Hansman GS, Christian Biertumpfel, Georgiev I, McLellan JS, Chen L, Zhou T, Katayama K, Kwong PD (2011). Crystal Structures of GII.10 and GII.12 Norovirus Protruding Domains in Complex with Histo-Blood Group Antigens Reveal Details for a Potential Site of Vulnerability. *Journal of Virology*. **85** (13): 6687-6701.

Hansman GS, Natori K, Shirato-Horikoshi H, Ogawa S, Oka T, Katayama K, Tanaka T, Miyoshi T, Sakae K, Kobayashi S, Shinohara M, Uchida K, Sakurai N, Shinozaki K, Okada M, Seto Y, Kamata K, Nagata N, Tanaka K, Miyamura T, Takeda N. (2006). Genetic and antigenic diversity among Noroviruses. *Journal of General Virology*. **87**: 909-919.

Hardy ME (2005). Mini Review: Norovirus protein structure and function. *FEMS Microbiology Letters*. **253**: 1-8.

Hardy ME, Crone TJ, Brower JE, Ettayebi K (2002). Substrate specificity of the Norwalk virus 3C-like proteinase. *Virus Research*. **89**: 29-39.

Herbert TP, Brierley I, Brown TD (1997). Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. *Journal of General Virology*. **78** (5): 1033-1040.

Huang PT, Farkas S, Marionneau W, Zhong N, Ruvoën-Clouet AL, Morrow M, Altaye LK, Pickering DS, Newburg J, Pendu L, Jiang X (2003). Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *Journal of Infectious Diseases*. **188**: 19-31.

© University of Venda

Huang PT, Farkas W, Zhong M, Tan S, Thornton AL, Morrow M, Jiang X (2005). Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. *Journal of Virology.* **79**: 6714-6722.

Hughes PJ and Stanway G (2000). The 2A proteins of three diverse picornaviruses are related to each other and to the H-rev107 family of proteins involved in the control of cell proliferation. *Journal of General Virology*. **81**: 201-207.

Hutson, AM; Atmar RL, Graham DY, Estes MK (2003). Norwalk virus infection and disease is associated with ABO histo-blood group type. *Journal of Infectious Diseases.* **188** (1): 176–177.

Huynen P, Mauroy A, Martin C, Savadogo LGB, Boreux R, Thiry E, Melin P, De Mol P (2013). Molecular epidemiology of norovirus infections in symptomatic and asymptomatic children from Bobo Dioulasso, Burkina Faso. *Journal of Virology.* **58**: 515-521.

Hyde JL and Mackenzie JM (2012). Pathogenesis and replication of Norovirus: following the mouse tail? *Microbiology Australia.* **1** (1): 74-76.

International Committee on Taxonomy of Viruses data base (ICTVdB) Management (2006). Norovirus. In: ICTVdB — The Universal Virus Database, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA

Iritani N, Kaida A, Kubo H, Abe N, Murakami T, Vennema H, Koopmans M, Takeda N, Ogura H, Seto Y (2008). Epidemic of Genotype GII.2 Noroviruses during Spring 2004 in Osaka City, Japan. *Journal of Clinical Microbiology.* **46** (7): 2406-2409.

© University of Venda

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Creating Future Leaders

Karst SM (2010). Pathogenesis of Noroviruses, Emerging RNA Viruses. *Viruses Review*. **2**: 748-781.

Karst SM, Wobus CE, Lay M, Davidson J, Virgin HW 4th (2003).STAT1-dependent innate immunity to a Norwalk-like virus. *Science*. **299** (5612): 1575-1578.

Katayama K, Horikoshi HS, Kojima S, Kageyama T, Oka T, Hoshino FB, Fukushi S, Shinohara M, Uchida K, Suzuki Y, Gojobori T, Takeda N (2002). Phylogenetic Analysis of the Complete Genome of 18 Norwalk-like Viruses. *Virology*. **299**: 225-239.

Kawada JI, Arai N, Nishimura N, Suzuki M, Ohta R, Ozaki T, Ito Y (2012). Clinical characteristics of Norovirus gastroenteritis among hospitalized children in Japan. *Microbiology and Immunology*. **56** (11): 756-759.

Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, Natoric K, Takeda N, Katayama K (2002). Genogroup-specific PCR primers for detection of Norwalk-like viruses. *Journal of Virological Methods*. **100**: 107-114.

LoBue AD, Thompson JM, Lindesmith L, Johnston RE, Baric RS (2009). Alphavirus-adjuvanted norovirus-like particle vaccines: heterologous, humoral, and mucosal immune responses protect against murine norovirus challenge. *Journal of Virology*. **83**: 3212-3227.

Mans J, de Villiers JC, du Plessis NM, Avenant T, Taylor MB (2010). Emerging Norovirus GII.4 2008 variant detected in hospitalised paediatric patients in South Africa. *Journal of Clinical Virology*. **8** (11): 1-7.

© University of Venda

University of Venda
Creating Future Leaders

Mans J, Netshikweta R, Magwalivha M, Van Zyl WB, Taylor MB (2013). Diverse Norovirus genotypes identified in sewage-polluted river water in South Africa. *Epidemiology of Infection*. **141**: 303-313.

Matsuyama K (2013). Vomiting –Bug vaccine seen as shot in the arm for cruises. *Bloomberg*. Article.

Matthews JE, Dickey BW, Miller RD, Felzer JR, Dawson BP, Lee AS, Rocks JJ, Kiel J, Montes JS, Moe CL, Eisenberg JNS, Leon JS (2012). The epidemiology of published Norovirus outbreaks: a review of risk factors associated with attack rate and genogroup. *Epidemiology of Infection*. **140**: 1161-1172.

Mattison K, Grudeski E, Auk B, Charest H, Drews SJ, Fritzinger A, Gregoricus N, Hayward S, Houde A, Lee BE, Pang XL, Wong J, Booth TF, Vinjé J (2009). Multicenter comparison of two norovirus ORF2-based genotyping protocols. *Journal of Clinical Microbiology*. **47** (12): 3927-3932.

Mattison K, Sebunya TK, Shukla A, Noliwe LN, Bidawid S (2010). Molecular detection and characterization of noroviruses from children in Botswana. *Journal of Medical Virology*. **82** (2): 321-324.

Mesquita JR, Barclay L, Nascimento MSJ, Vinjé J (2010). Novel Norovirus in Dogs with Diarrhea. *Emerging Infectious Diseases*. **16** (6): 980-982.

Monroe SS (2011).Control and Prevention of Viral Gastroenteritis. *Emerging Infectious Diseases*. **17** (8): 1347 – 1579.

© University of Venda

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Creating Future Leaders

Moyo SJ , Gro N, Kirsti V, Matee MI, Kitundu J, Maselle SY, Langeland N, Myrmel N (2007). Prevalence of enteropathogenic viruses and molecular characterization of group A rotavirus among children with diarrhoea in Dares Salaam Tanzania. BCM: 7; doi: 10.1186/1471-2458-7-359.

Nakata S, Honma S, Numata K, Kogawa K, Ukae S, Adachi N, Jiang X, Estes MK, Gatheru Z, Tukei PM, Chiba C (1998). Prevalence of Human Calicivirus Infections in Kenya as Determined by Enzyme Immunoassays for Three Genogroups of the Virus. *Journal of Clinical Microbiology*. **36** (11): 3160-3163.

Nasir W (2009). Computational studies on the interaction of norovirus surface protein with ABO- blood group active saccharides: A study of norovirus-HBGA interactions. *Chalmers.* **44**: 1-8.

Netshikweta R, van Zyl WB, Wolfaardt M, Taylor MB (2011). Comparison of conventional nested RT-PCR and reverse-transcriptase RT-PCR for the detection of enteroviruses in water samples. Poster presentation. Faculty of Health Sciences, University of Pretoria. 1-185.

Noda M, Fukuda S, Nishio O (2007). Statistical analysis of attack rate in Norovirus foodborne outbreaks. *International Journal of Food Microbiology.* **122** (2): 216–20.

Nordgren J , Nitiema LW, Ouermi D, Simpore J, Svensson L (2013). Host Genetic Factors Affect Susceptibility to Norovirus Infections in Burkina Faso. *Plos One*. **8** (7): 1-10.

Nordgren J, Kindberg E, Lindgren PE, Matussek A, Svensson L (2010). Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden". *Emerging Infectious Diseases.* **16** (1): 81-87.

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Creating Future Leaders

Ntafis V, Xylouri E, Radogna A, Buonavoglia C, Martella V (2010). Outbreak of Canine Norovirus Infection in young dogs. *Journal of Clinical Microbiology*. **48** (7): 2605-2608.

Obara M, Hasegawa S, Iwai M, Horimoto E, Nakamura K, Kurata T, Saito N, Oe H, Takizawa T (2008). *Journal of Clinical Microbiology*. **46** (10): 3397-3403.

Oluwatoyin JM, Adeyemi AO, Famurewa O, Svensson L, Nordgren J (2012).Molecular epidemiology of rotavirus and Norovirus in Ile-Ife, Nigeria: high prevalence of G12P[8] rotavirus strains and detection of a rare Norovirus genotype. *Journal of Medical Virology*. **84** (9): 1489-1496.

Otto PH, Clarke IA, Lambden PR, Salim O, Reetz J, Liebler-Tenorio EM (2011). Infection of Calves with Bovine Norovirus GIII.1 Strain Jena Virus: an Experimental Model to Study the Pathogenesis of Norovirus Infection. *Journal of Virology*. **85** (22): 12013-12021.

Parashar UD and Monroe SS (2001).Norwalk-like viruses as a cause of foodborne disease outbreaks. *Revised Medical Virology*. **11** (4): 243-252.

Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinjé J, Parashar UD (2008). Systematic Literature Review of Role of Noroviruses in Sporadic Gastroenteritis. *Emerging Infectious Diseases*. **14** (8): 1224-1231.

Prasad BV, Crawford S, Lawton JA, Pesavento J, Hardy M, Estes MK (2001).Structural studies on gastroenteritis viruses. *Novartis Foundation Symposium*. **238** (26-37): discussion 37-46.

© University of Venda

Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK (1999). X-ray crystallographic structure of the Norwalk virus capsid. *Science*. **286** (5438): 287-290.

Puustinen L, Blazevic V, Huhti L, Szakal ED, Halkosalo A, Salminen M, Vesikari T (2012). Norovirus genotypes in endemic acute gastroenteritis of infants and children in Finland between 1994 and 2007. *Epidemiology and Infection.* **140**: 268-275.

Ramirez S, Giammanco GM, De Grazia S, Colomba C, Martella V, Arista S (2008). Genotyping of GII.4 and GIIb norovirus RT-PCR amplicons by RFLP analysis. *Journal of Virological Methods* **147** (2): 250-256.

Ramudingana P (2009). Detection of rotavirus and Noroviruses in children under 5 years of age in Pretoria, South Africa and molecular characterization of rotavirus strains. *University of Limpopo Institutional Repository.* **268**.

Ren Z, Kong Y, Wang J, Wang Q, Huang A, Xu H (2013). Etiological study of enteric viruses and the genetic diversity of norovirus, Sapovirus, adenovirus, and astrovirus in children with diarrhoea in Chongqing, China. *BMC Infectious diseases*. **13** (412): 1-7.

Rockx B, de Wit M, Vennema H, Vinjé J, de Bruin E, van Duynhoven Y, Koopmans M (2002). Natural History of Human Calicivirus Infection: A Prospective Cohort Study. *Clinical Infectious Diseases*. **35**: 246-253.

Ruvoen-Clouet N, Ganiere JP, Andre-Fontaine G, Blanchard D, Le Pendu J (2000). Binding of Rabbit Hemorrhagic Disease Virus to Antigens of the ABH Histo-Blood Group Family. *Journal of Virology.* **74**: 11950-11954.

© University of Venda

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Creating Future Leaders

Rydell GE, Kindberg E, Larson G, Svensson L (2011). Susceptibility to winter vomiting disease: a sweet matter. *Reviews in Medical Virology*. **21** (6): 370-382.

Said MA, Perl TM, Sears CL (2008). Gastrointestinal Flu: Norovirus in Health Care and Long-Term Care Facilities. *Healthcare Epidemiology*. **47**: 1202-1208.

Shieh Y, Monroe SS, Fankhauser RL, Langlois GW, Burkhardt W, Baric RS (2000).Detection of Norwalk-like virus in shellfish implicated in illness. *Journal of Infectious Diseases*. **181** (2): 360-366.

Shirato H (2011). Norovirus and histo-blood group antigens. *Japan Journal of Infectious Diseases*. **64** (2): 95-103.

Shirato H, Ogawa S, Ito H, Sato T, Kameyama A, Narimatsu H, Xiaofan Z MiyamuraT, Wakita T, Ishii K, Takeda N (2008). Noroviruses Distinguish between Type 1 and Type 2 Histo-Blood Group Antigens for Binding. *Journal of Virology*. **82**: 10756-10767.

Siebenga JJ, Vennema H, Zheng D, Vinje´ J, Lee BE, Pang X, Ho ECM, Lim W, Choudekar A, Broor S, Halperin T, Rasool NBG, Hewitt J, Greening GE, Jin M, Duan Z, Lucero Y, O'Ryan M, Hoehne M, Schreier E, Ratcliff RM, White PA, Iritani N, Reuter G, Koopmans M (2009). Norovirus Illness Is a Global Problem: Emergence and Spread of Norovirus GII.4 Variants, 2001–2007. *The Journal of Infectious Diseases*. **200**: 802-812.

Smit TK, Bos P, Peenze I, Jiang X, Estes MK, Steele AD (1999).Seroepidemiological study of genogroup I and II calicivirus infections in South and southern Africa. *Journal of Medical Virology*. **59** (2): 227-231.

© University of Venda

Sosnovtsev SV and Green KY (2000).Identification and genomic mapping of the ORF3 and VPg proteins in feline calicivirus virions. *Virology*. **277**: 193-203.

Tan M, Huang PW, Meller J, Zhong WM, Farkas T, Jiang X (2006). Mutations within the P2 domain of Norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. *Journal of Virology*. **78** (6): 3201.

Tan M, Xia M, Chen Y, Bu W, Hegde RS, Meller J, Li X, Jiang X (2009). Conservation of Carbohydrate Binding Interfaces — Evidence of Human HBGA Selection in Norovirus Evolution. *Plos One*. **4**: e5058.

The Division of Medical Virology, University of Cape Town (2012). *National Health Laboratory*.

Trainor E, Lopman B, Iturriza-Gomara M, Dove W, Ngwira B, Nakagomi O, Nakagomi T, Parashar U, Cunliffe N (2013).Detection and molecular characterization of noroviruses in hospitalized children in Malawi, 1997-2007. *Journal of Medical Virology*. **85** (7): 1299-1306.

Tu ET, Bull RA, Greening GE, Hewitt J, Lyon MJ, Marshall JA, McIver CJ, Rawlinson WD, White PA (2008). Epidemics of gastroenteritis during 2006 were associated with the spread of norovirus GII.4 variants 2006a and 2006b. *Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America*. **46** (3): 413-420.

Vega E, Barclay L, Gregoricus N, Williams K, Lee D, Vinjé J (2011). Novel Surveillance Network for Norovirus Gastroenteritis Outbreaks, United States. *Emerging Infectious Diseases*. **17** (8): 1389-1395.

© University of Venda

Victoria M, Miagostovich MP, Ferreira MS, Vieira CB, Fioretti JM, Leite JP, Colina R, Cristina J (2009). Bayesian coalescent inference reveals high evolutionary rates and expansion of Norovirus populations. *Infection, Genetics and Evolution: journal of molecular epidemiology and evolutionary genetics in infectious dieases.* **9** (5): 927-932.

Vinjé J, Vennema H, Maunula L, von Bonsdorff CH, Hoehne M, Schreier E, Richards A, Green J, Brown D, Beard SS, Monroe SS, de Bruin E, Svensson L, Koopmans MPG (2003). International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. *Journal of Clinical Microbiology*. **41** (4): 1423-1433.

Widdowson M, Monroe SS, Glass RI (2005). Are Noroviruses Emerging? *Emerging Infectious Diseases*. **11**(5): 735-737.

Wu TC, Liu HH, Chen YJ, Tang RB, Hwang BT, Yuan HC (2008). Comparison of Clinical Features of Childhood Norovirus and Rotavirus Gastroenteritis in Taiwan. *Journal of Chinese Medical Association.* **71** (11): 566-570.

Yoon JS, Lee SG, Hong SK, Lee SA, Jheong WH, Oh SS, Oh MH, Ko GP, Lee CH, Paik SY (2008). Molecular Epidemiology of Norovirus Infections in Children with Acute Gastroenteritis in South Korea in November 2005 through November 2006. *Journal of Clinical Microbiology*. **46** (4): 1474-1477.

Zaafrane MH, Kaplon J, Loulizi KS, Aouni Z, Pothier P, Aouni M, Balay KA (2012). Molecular prevalence of bovine noroviruses and neboviruses detected in central-eastern Tunisia. *Archives of Virology*. **157**: 1599-1660.

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Zakikhany K, Allen DJ, Brown D, Iturriza-Gomara M (2012). Molecular Evolution of GII-4 Norovirus Strains. *PLoS ONE*. **7** (7): 1-8.

Zheng DP, Ando T, Fankhauser RL, RS Beard, Glass RI, Monroe SS (2006). Norovirus classification and proposed strain nomenclature. *Virology*. **346**: 312-323.

Zhou X, Li H, Sun L, Mo Y, Chen S, Wu X, Liang J, Zheng H, Ke C, Varma JK, Klena JD, Chen Q, Zou L, Yang X (2012). Epidemiology and molecular analysis of waterborne outbreak of Norovirus GII.4.*Epidemiology and Infection.* **140** (12): 2282 - 2289.

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