HUMAN NOROVIRUS IN RURAL COMMUNITIES OF VHEMBE DISTRICT, LIMPOPO PROVINCE- SOUTH AFRICA

by

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DECLARATION

I, Mulondo Goodman (student number: 11620995), declare that this dissertation for the award of MSc in Microbiology (MSc MBY) at the University of Venda, has not previously been submitted for a degree at this or any other institution and that all reference materials contained herein have been duly acknowledged.

……. ……..

Signature Date

DEDICATION

I dedicate this research to my siblings (Avhasei, Mukhethwa, Dakalo, Mulisa, Murendeni, Hlekani, Miyelani) and my parents, Mulondo Tshilidzi Walter and Mukhese Vhonani Violet.

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ABSTRACT

BACKGROUND: Human norovirus (NoV) is the etiological agent associated with acute gastroenteritis (AGE) in both children and adults worldwide. Children of <5 years of age, the elderly and individuals suffering from chronic diseases are potentially at high risk of NoV-associated illness. High morbidity and mortality rate associated with NoV have been reported worldwide. In children under the age of 5 years about 1.8 million death cases have been reported in developing countries alone. Despite the fact that the virus is affecting people of all age groups, there is lack of data to elucidate the importance and the role of NoV in children of the age above 5 years and adults.

OBJECTIVE: To characterize human norovirus in patients with diarrhoea in rural communities of Vhembe district, Limpopo province.

MATERIALS AND METHODS : From August 2017 to October 2018, outpatient between 5 and 68 years of age from rural communities of Vhembe district, Limpopo province were recruited for this study. A total of n=80 stool samples were collected from patients with diarrhoea and were kept at 4˚C throughout the transportation to the laboratory and refrigerated at - 20˚C prior to RNA extraction. Stool samples were tested for norovirus using the RIDA©GENE NOROVIRUS I & II real-time RT-PCR. The RNA extracts tested positive for norovirus were subjected to RT-PCR amplification. The RT-PCR products of the amplified fragments were sequenced, and phylogenetic trees were constructed by the neighbor-joining method using MEGA 7 software.

RESULTS: NoV was detected in 13(16%) out of 80 stool samples collected, of which 6 (46%) strains belonged to norovirus GII and 7 (54%) strains to norovirus GI. A total of 5 genotypes were detected (GII.Pg, GII.1, GII.2, GII.4 Sydney 2012). The phylogenetic analysis revealed circulation of NoV genotypes with considerable diversity.

CONCLUSION: This study illustrates NoV prevalence and substantial genetic diversity in patients above 5 years of age living in rural communities of Vhembe district, Limpopo province. Continued systematic surveillance to evaluate norovirus association with diarrhoea is needed to have a full picture on the epidemiology and disease burden in people of all the age groups.

KEY WORDS: Norovirus (NoV), Diarrhoea, Adults, Acute gastroenteritis (AGE).

LIST OF ABBREVIATIONS

˚C	-	Degree Celsius
%	-	Percentage
g	-	gram
µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
mmol	-	Millimole
3', 5'	-	3 Prime, 5 Prime
ABO	-	Blood types A, B and O
AGE	-	Acute Gastroenteritis
APC	-	Abdominal pain cramp
bp	-	Base pair
CDC	-	Centers for Disease Control and Prevention
cDNA	-	Complementary deoxyribonucleic acid
CT	-	Cycle threshold
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediamine Tetraacetic Acid
EIA	-	Enzyme Immuno Assays
EM	-	Electron microscopic
et al	-	(et aliii) and others
F	-	Forward
FUT2	-	Fucosyl transferase two
GI	-	Genogroup one
GII	-	Genogroup two
GIV	-	Genogroup Four
HBGA	-	Histo Blood Group Antigens
ICR	-	Internal control RNA
Ig (A/G)	-	Immunoglobulin (A/G)
Kb(p)	-	Kilobase (pair)
kDa	-	Kilodaltons
Le type	-	Lewis type

M	-	Molar
min(s)	-	Minutes
nm	-	Nanometer
NoV	-	Norovirus
ORF(s)	-	Open Reading Frame(s)
ORF1,	-	Open reading frame 1
ORF2	-	Open reading frame 2
ORS	-	Oral rehydration therapy
P Domain	-	Protruding (Domain of the capsid)
PBS	-	Phosphate buffered saline
PCR	-	Polymerase Chain Reaction
pH	-	Potential of Hydrogen
PHC	-	Primary Health Care (PHC)
R	-	Reverse
RdRp	-	RNA-dependent RNA polymerase
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcription PCR
RT-qPCR	-	Real-time reverse transcriptase polymerase
SA	-	South Africa
S Domain	-	Shell (domain of the capsid)
S type	-	secretor type
S	-	Second(s)
ssRNA	-	Single Stranded-Ribonucleic acid
TAE buffer	-	Tris EDTA buffer
Tris	-	Tris (hydroxymethyl)-methylamine
USA	-	United States of America
VLPs	-	Virus-like particles
VP1	-	Viral protein 1
VP2	-	Viral protein 2
VPg	-	Viral protein genome-linked
w/v	-	Weight per unit volume
WHO	-	World Health Organisation

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Chapter 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Africa is the second largest continent where approximately 70% of poor people are located in rural villages (UNICEF, 2012; WHO, 2010), with most people living in poor environmental conditions, that lack good quality water and poor hygiene practices, resulting in wide variety of diseases which include diarrhoea (Vantarakis et al., 2011). Diarrhoeal diseases pose significant human threat leading to high morbidity and economic loss in both developing and developed countries (Lopman et al., 2004). Diarrhoeal disease can be caused by different enteropathogens such as parasites, bacteria and viruses (Cardemil et al., 2017b; Vantarakis et al., 2011; Patel et al., 2009).

In countries where vaccination against rotavirus has been implemented, there is a large decline in diarrheal disease cases. Consequently, noroviruses (NoV) are now becoming a major role player as the cause of acute gastroenteritis with severe complications in young children, elderly and people suffering from chronic diseases (Vantarakis et al., 2011).

There are seven classified NoV genogroups with majority of the human infections caused by strains which belong predominantly to Genogroup II, followed by Genogroup I and rarely from Genogroup IV (Shamkhali and Deng, 2017; Howard et al., 2017b; Sisay et al., 2016; Vega et al., 2014). Amoung the GII genotypes, the GII.4 strain is the most predominant strain causing NoV gastroenteritis globally, resulting in more hospitalization and death in both the young and elderly (Cardemil et al., 2017; Morioka et al., 2006; Drinka, 2005; Khanna et al., 2003; Fankhauser et al., 1998). In Africa, NoV cause about 200,000 deaths in children under 5 years of age (Lopman et al., 2016; Zeng et al., 2012) and 1 million hospitalizations (Rupprom et al., 2017). Between January 1990 and February 2008, diarrheal cases in children younger than 5 years, older children and the elderly was 12%, indicating that NoV infection is causing an increase in diarrheal cases reported each year (Mans et al., 2016).

Noroviruses have the ability to remain stable outside the host (Ramani et al., 2014; Park et al., 2012). NoV are resistant to common disinfectants in use (Smith, 2013; Park et al., 2010; Duizer et al., 2004). Generally, NoV can be transmitted through food-borne, air-borne routes, water-borne and direct contact with the infected person (Ramani et al., 2014; Park et al., 2012; Vantarakis et al., 2011). Primary outbreaks are usually related to exposure with contaminated food (Vantarakis et al., 2011; Koopmans et al., 2006), while secondary transmission often results directly from person-to-person contact (Mathijs et al., 2012; Kroneman et al., 2008; Hedberg and Osterholm, 1993). In rural communities, NoV disease can be easily accumulated due to a wide variety of factors including lack of standard hygiene practices, poor quality water and poor living conditions (Kabue et al., 2016a) and high number of people living in a household (Utsumi et al., 2017).

The climatic change affects the occurrence of NoV outbreaks by influencing the host susceptibility, transmissibility and the resistance of NoV to different environmental conditions (Rohayem, 2009). Previous studies have reported that NoV infections peak during the winter season (Ahmed et al., 2013; Dey et al., 2010; Rohayem, 2009; Greer and Fisman, 2008). Mans and colleagues (2016) found that the seasonality of NoV in the Africa region is less obvious. In South Africa, NoV infection was observed during spring and early summer season (Mans et al., 2016)

1.2 STUDY RATIONALE

Very little data on the prevalence of human NoV in children older than 5 years and adults in Africa is not well studied (Romero et al., 2017; Kabue et al., 2016a; Mans et al., 2016; Mans et al., 2015; Matussek et al., 2015; Kabayiza et al., 2014; Mans et al., 2014). In fact, no study could be found on adolescents and older people with diarrhoea in rural communities of the Vhembe district, Limpopo Province. This will, therefore, to our knowledge be the first study to investigate the prevalence of NoV strains in young children older than 5 years and adult people from rural communities suffering from diarrhoea. Additionally, this study will provide more information on the role of Human NoV as the causative agent of gastroenteritis in people above 5 years of age living in rural communities of Vhembe district, South Africa. This contribute to the NoV vaccine development in order to target possibly all age groups.

1.3 RESEARCH QUESTION

What are the circulating NoV strains in patients older than 5 years of age living in rural communities of Vhembe District?

1.4 OBJECTIVES OF THE STUDY

1.4.1 PRIMARY OBJECTIVE

- To characterize human NoV in patients with diarrhoea in rural communities of Vhembe district, Limpopo province.

1.4.2 SECONDARY OBJECTIVES

- To determine the prevalence of human norovirus (NoV) in patients older than 5 years of age using real-time one-step RT-PCR.
- To determine the genetic characteristics of human Norovirus in patients older than 5 years of age based on partial capsid sequences.
- To determine the relationship between the strains using Phylogenetic analyses of the capsid NoV genotypes identified.

Chapter 2

LITERATURE REVIEW

2.1 BACKGROUND

In rural villages of Limpopo province (South Africa), most people are living in extremely poor environmental conditions with poor sanitation, lack of safe drinking water and poor hygiene practices. These poor living conditions may enhance the exposure to enteric pathogens such as bacteria, parasites, and viruses (UNICEF 2009; 2006). Amongst the viruses, NoV is now becoming a major role player as the cause of diarrhoea after the introducing of two licensed vaccines against Rotavirus (Smith, 2013). Several studies have confirmed high morbidity and mortality rates by Nov worldwide (Ahmed et al., 2014; Aragão et al., 2013; Okoh et al., 2010).

2.2 HISTORY OF HUMAN NOROVIRUS

Hyperemesis hemis or winter vomiting disease was first described by Zahorsky in 1929. The illness was characterised by the sudden onset of self-limiting vomiting and diarrhoea that typically peaked during the winter season (Zahorsky et al., 1929). Kapikian et al (1972) first discover the causative agent of this syndrome by immune electron microscope examination of stool from volunteering students and teachers in Norwalk elementary school who were affected by outbreak of gastroenteritis in 1968 (Figure 2.1) (Kapikian et al., 1972).

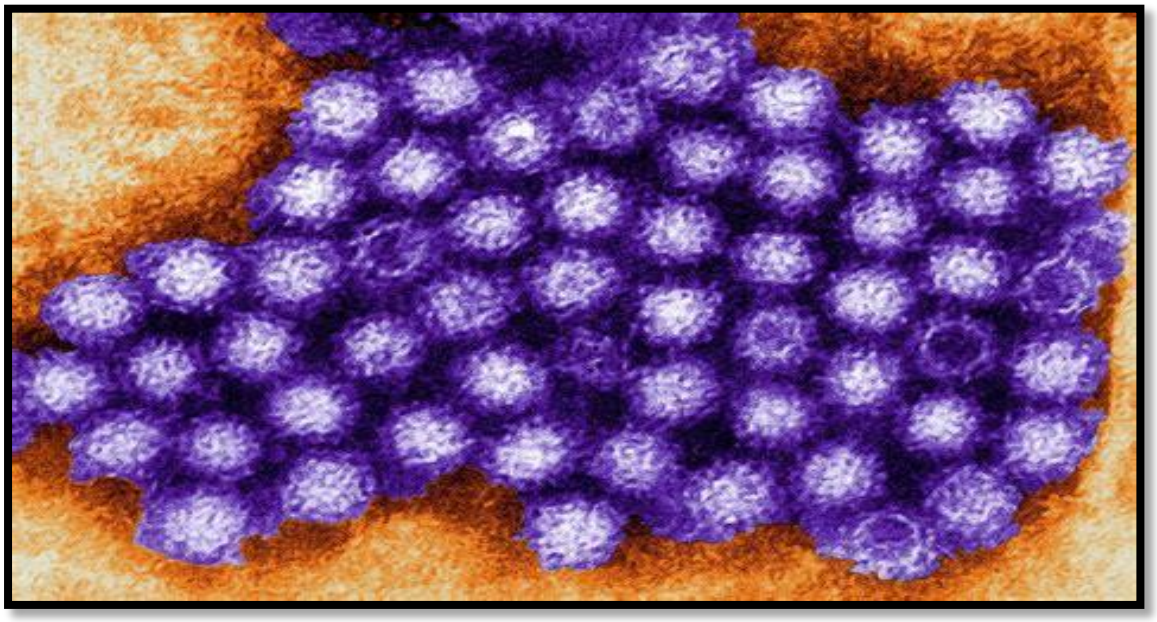

Figure 2.1: NoV particles visualised by electron microscopy (CDC/Charles D. Humphrey)

2.3 NOROVIRUS CLASSIFICATION AND GENOME STRUCTURE

Noroviruses belong to the family *Caliciviridae* with four other genera including: *Sapovirus*, *Nebovirus, Lagovirus* and *Vesivirus* (Green, 2013). They are small (approximately 38nm in diameter), non-enveloped, positive sense, single-stranded RNA (ssRNA+) genome of approximately 7.5 Kbs in length (Green, 2013; Park et al., 2012; Jiang et al., 1993). Norovirus genome contains three open reading frames (ORFs) which are covalently linked to VPg at the 5'end and polyadenylated at the 3'end (Green, 2013; Jiang et al., 1993) (Figure 2.2). ORF1 encodes a polyprotein comprising of all non-structural protein including the RNA-dependent RNA polymerase (RdRp) (Belliot et al., 2003; Green, 2013). ORF2 and ORF3 encodes for the major capsid protein of ~60 kDa known as VP1 and minor (VP2) structural protein of ~20 kDa, respectively (Pletneva et al., 2001).

The major capsid protein (VP1) forms two domains which are the (protruding, P1 and P2) and the (S) shell (Figure 2.2; Tan et al., 2004; Nilsson et al., 2003). The P domains are then divided into subdomains (Tan et al., 2004). Most of cellular interaction and immune recognition take place in P2 subdomains (Chakravarty et al., 2005; Tan et al., 2004). Though the capsid protein provides shell structure for the virus, it also contains the viral phenotype and cellular receptor binding site (Tan et al., 2004).

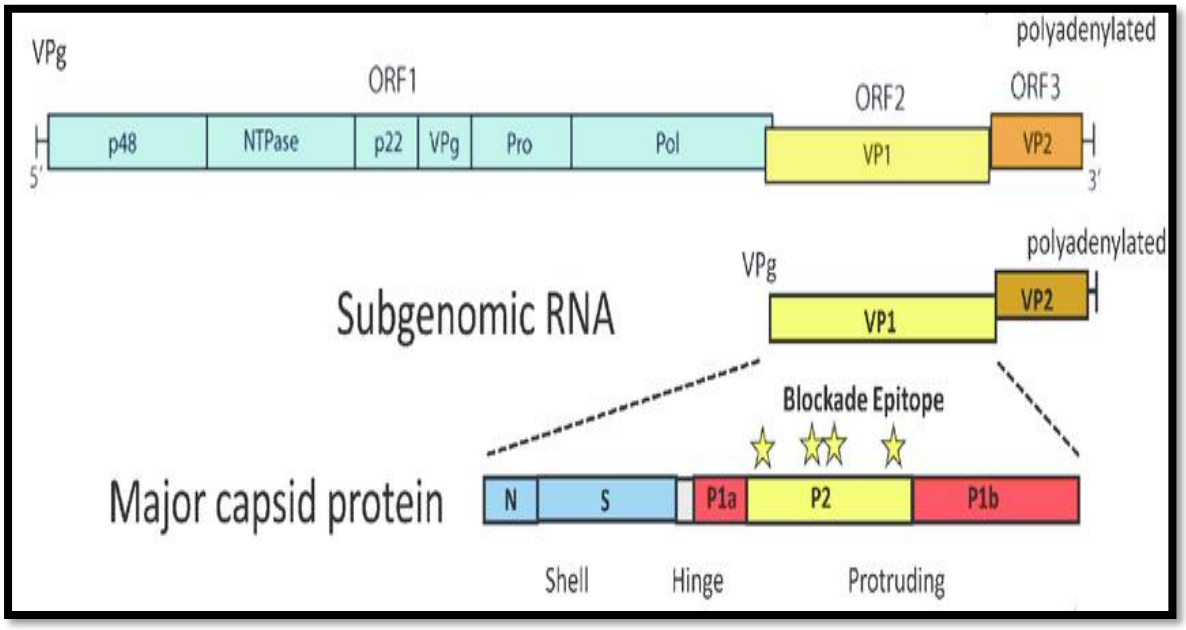

Figure 2.2: Norovirus genome Organisation

(https://microbenotes.com/wp-content/uploads/2018/02/Structure-and-Genome-of-NoV.png)

2.4 NOROVIRUS GENETIC DIVERSITY AND RECOMBINATION

Noroviruses are classified into at least seven genogroups (GI - GVII) which are then further divided into 41 genotypes based on the amino acid sequence of the capsid protein VP1 (Figure 2.3). However, virus from genogroup I, II and IV are known to infect human (Shah and Hall, 2017; Vinjé, 2015; Kroneman et al., 2013; Zheng et al., 2006), and some of porcine strains (GII.11, GII.18 and GII.19) within the Genogroup II (Figure 2.3; Cortes-Penfield et al., 2017; Vinjé, 2015). Genogroup III strains are associated with bovine and sheep, while genogroup IV strains are associated with mice (Pinto et al., 2012; Martella et al., 2008; Batten et al., 2006).

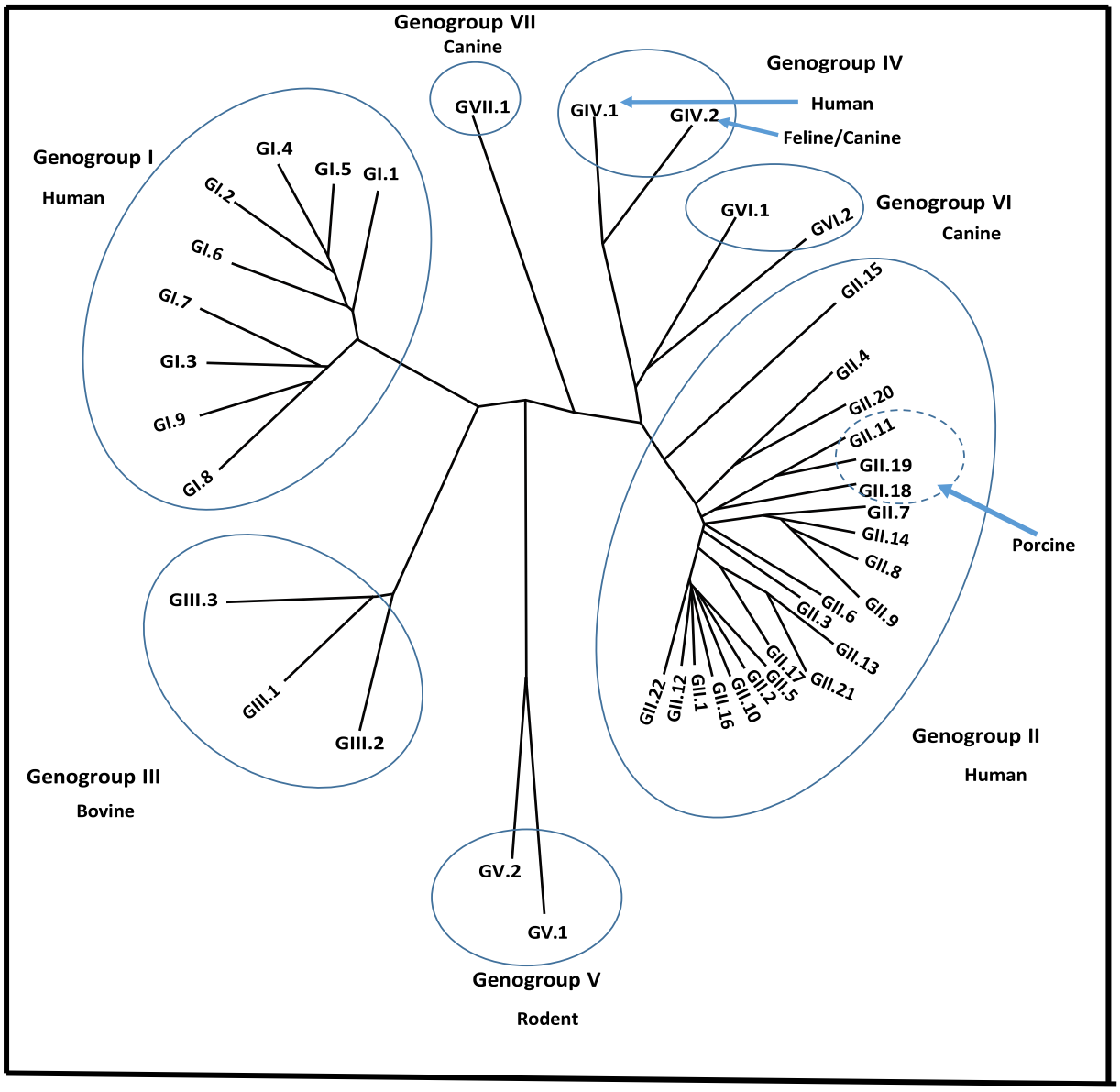

Figure 2.3: Phylogenetics of noroviruses based on the capsid protein (Cortes-Penfield et al., 2017).

Norovirus RNA recombination is regarded as one of major driving forces of viral evolution (White, 2014; Bull et al., 2007; Worobey and Holmes, 1999) which has been linked with the emergence of new NoV strains circulating worldwide (Siqueira et al., 2017; Arana et al., 2014; Eden et al., 2013). NoV recombination confuses molecular epidemiologic studies and greatly affect phylogenetic groupings leading major implications and complications in viral vaccine design (Bull et al., 2005). A recombinant NoV can be defined as the clusters of two different groups of NoV strains when two different regions from polymerase and capsid region of the genome are genotyped (Figure 2.4) (Bull et al., 2007; Vinjé et al., 2004). However, genotyping based on one region (capsid or polymerase) of the NoV genome does not give true representation of the epidemiology of the virus due to frequent recombination within NoVs (Bull et al., 2007).

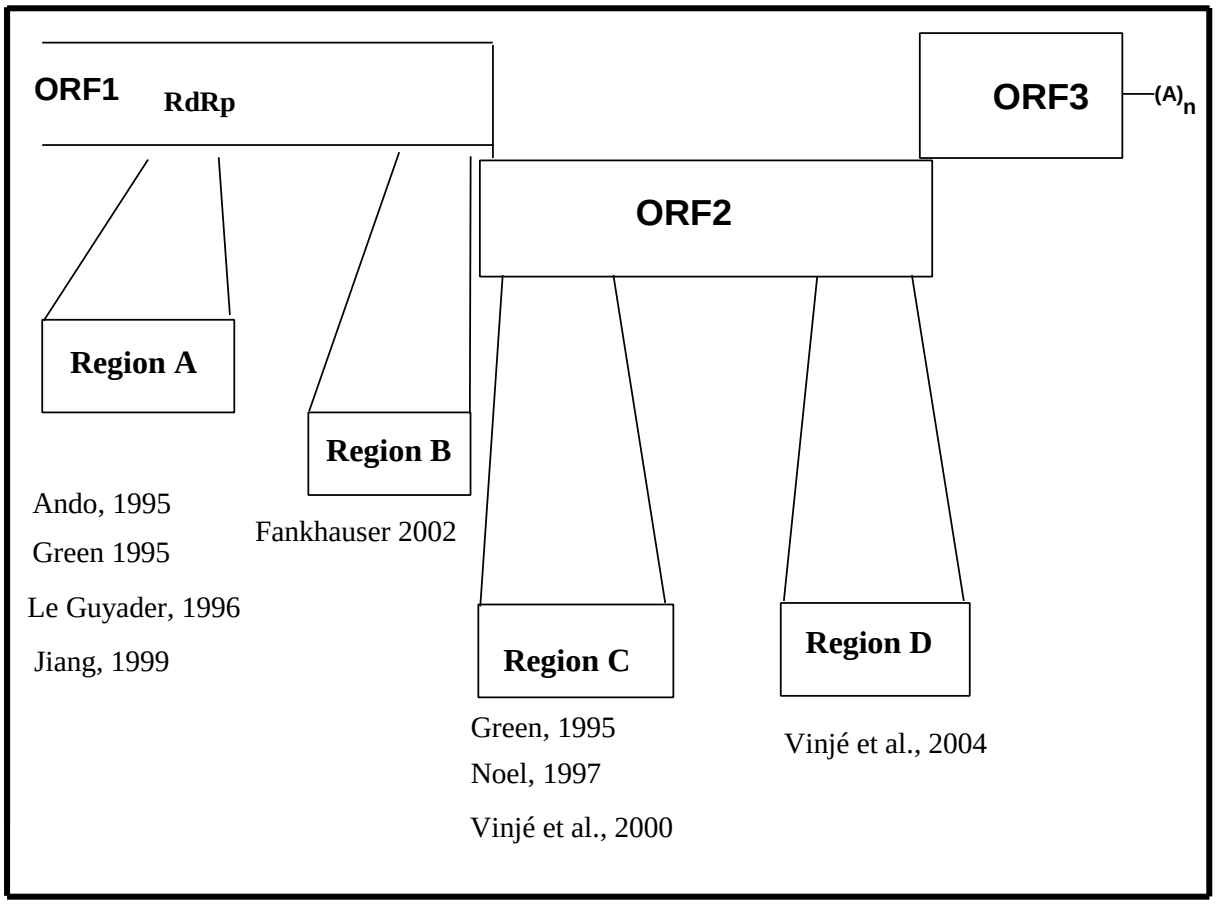

Figure 2.4: Location of the genomic region (A-D) on NoV used for detection and genotyping (Vinjé et al., 2004).

Norovirus genogroup II (GII) is the most prevalent genogroup in patients with acute gastroenteritis which account for 50-80% when strains are genotyped based on partial RNA-dependent RNA polymerase (RdRp) and capsid nucleotide sequences. NoV (GII.4) variant causes NoV outbreaks worldwide (Howard et al., 2017; Mans et al., 2015), in particular GII.4 undergo genetic evolution resulting in a new variant of this strain emerging every two to three years (de Rougemont et al., 2011; Shanker et al., 2011; Bok et al., 2009; Allen et al., 2008) rapidly replacing the circulating variant and then becomes dominant globally (Mans et al., 2016). Over the last four decades, there has been only limited evolution of GI variant (Lindesmith et al., 2011).

Although GII.4 was first documented as a major epidemic NoV strain in the middle of the last decade of the 20th century, it has been predominantly circulating ever since 1974 (Bok et al., 2009). Since the 1990's, there have been 7 different GII.4 variants associated with global epidemics worldwide (Ramani et al., 2014), which occurred in late 1990's (US 96) (White et al., 2002; Noel et al., 1999), Farmington Hills 2002 (Lopman et al., 2004; Widdowson et al., 2004), Hunter 2004 (Bull et al., 2006), De gaan 2006b (Eden et al., 2010; Tu et al., 2008), New Orleans 2009 (Yen et al., 2011), and most recently Sydney 2012 (Eden et al., 2014; 2013). 67% of these GII.4 variants evolved from previous GII virus through antigenic expression and mutation in the p2 domain of major capsid protein (Debbink et al., 2012b; Bull et al., 2010; Donaldson et al., 2010). Interestingly, the most recently dominant variant (New Orleans 2009 and Sydney 2012) evolved from the processes that involves both antigenic shift and antigenic drift (Robilotti et al., 2015; Eden et al. 2013).

High mutation rate in GII.4 variant leads to high evolution rate (Bull et al., 2010). However, it is not clear whether other NoV variant RNA polymerase has the same fidelity rate as compared to GII.4 strains (Lindesmith et al., 2017). In Africa, there are few studies which have been carried out on combined RdRp/capsid genotypes. Kabue et al (2016a) detected 8 GII.Pe/GII.4 (Sydney 2012) recombinants strains among the RdRp/Capsid genotypes in paediatric stool samples collected from rural communities of Vhembe district (SA). There is a paucity of data regarding the NoV recombination and its prevalence in SA (Mans et al., 2016). Mans et al (2016) reveals that data on the emergence of new NoV recombinants in a short period of time confirms the need to genotype both the RdRp and capsid region.

2.5 HOST SUSCEPTIBILITY, IMMUNITY AND PATHOGENESIS

Knowledge and understanding of NoV-induced responses are of outmost important for vaccine development (Kocher et al., 2018). Historically, both innate and acquired immunity host factors are thought to plays an important role in susceptibility to infection (Cardemil et al., 2017). Data from volunteer challenge studies showed a short-term acquired immunity with protection against same strain of NoV lasting for a week up to 2 years in human (Kocher et al., 2018; Cortes-Penfield et al., 2017; Parrino et al., 1977; Wyatt et al., 1974). Immunity to NoV is thought to be self-limiting with most people experiencing re-infection through their lifetime (Cardemil et al., 2017).

Antibodies from natural infection have been studied as possible markers of immunity and its prevalence correlates with an increase in age. However, Nurminen et al (2011) demonstrated the lowest prevalence of GII.4-speciffic IgA and IgG in hospitalized children compared to adults. Although the antibody seroprevalence to norovirus is high in adults, that does not necessarily guarantee the protection against the norovirus disease (Johnson et al., 1990; Parrino et al., 1977).

Norovirus protective response is genotypic-specific, whereas re-infection with other genotypes from the same genogroup is highly common (Cardemil et al., 2017; Blazevic et al., 2015; Parra and Green, 2014; Saito et al., 2013; Wyatt et al., 1974). Therefore, this might be the reason why high seroprevalence to norovirus does not necessarily equate with protection from disease (Cardemil et al., 2017). Different studies have shown protection against homologous genotypes but lack cross protection to heterogeneous strains of the same genogroups (Cardemil et al., 2017; Wyatt et al., 1974).

In addition to acquired immunity, innate host factors are of outmost important role in immunologic protection against the NoV disease. Susceptibility to norovirus infection depends on the presence of Histo-blood group antigens (HBGAs), which are the large family of complex carbohydrates classified by terminal glycosylation moieties which include: A or B antigen, secretor (S) and Lewis (Le) type (Kocher et al., 2018; Karst, 2010).

The HBGAs are docking site or receptor for Nov infection (Caddy et al., 2014). Histo-blood group antigens are believed to play an important role for entry of the virus to the gut mucosal epithelial cells (Harrington et al., 2002). The expression of HBGAs is regulated in part by the *FUT2* (fucosyltransferase 2) gene, which encodes an alpha (1,2) fucosyltransferase to generate H-antigens, which are catalyzed to produce A or B blood group antigens (Currier et al., 2015). However, for individuals who have functional gene *(FUT2)* which results in ABH glycan secreted into body fluids are regarded as secretor positive and are found to be at risk of being infected by certain NoV genotypes (Currier et al., 2015), more specially with GII.4, which is the dominant genotype causing high morbidity and mortality worldwide (Kocher et al., 2018; Chen and Chiu, 2012) with new strains emerging every 2 to 4 years (Lindesmith et al., 2008; Siebenga et al., 2007).

Pathogenesis of NoV infection is not well understood (Karst, 2015). Even though the intestinal epithelial remains undamaged during the infection, there are histo-pathological changes in the small intestine causing the broadening and blunting the villi, (Dolin et al ., 1975; Schreiber et al., 1973) as well as decreasing the activities of specific brush border enzyme on enterocytes including sucrase, alkaline phosphate and trehalase (Blacklow et al., 1972). Moreover, mal-absorption of lactose, fats and D-xylose which decrease the brush border enzyme activity and abridge the microvilli have also been reported following NoV infection (Schreiber et al., 1973; Agus et al., 1973). Acute gastroenteritis caused by NoV can results in severe intestinal pathologies including the death of enterocytes in the small and large intestine of infants (Pelizzo et al., 2013; Stuart et al., 2010; Turcios-Ruiz et al., 2008) . Currently, literature from different studies suggests that NoV infection norovirus-induced diarrhoea is not caused by structural damage of the intestinal wall but instead by alterations of secretory and processes.

2.6 CLINICAL PRESENTATION OF NOROVIRUS INFECTION

Immediately after an incubation period, the classic norovirus symptoms include non-bloody watery diarrhoea, sudden onset of vomiting, abdominal pain/cramps (APC) (Lee et al., 2013; Atmar and Estes, 2006), followed by constitutional symptoms such as generalized myalgias, low grade fever, headache and chills and malaise (Cardemil et al., 2017b). Several studies have shown that most patients experience a brief self-limiting infection which usually disappears after 2-3 days (Cardemil et al., 2017b; Kabue et al., 2016a). Asymptomatic infections are common, leading to spread of infection although their viral load is lower compared to symptomatic infections (Utsumi et al., 2017; Barreira et al., 2010; Kindberg et al., 2007). These are due to high NoV shedding in faeces (Lindsay et al., 2015) and a low infectious doses (Teunis et al., 2008). Hossain et al (2018) and Kabue et al (2016a) reported no significant difference between symptomatic and asymptomatic NoV infections. Several studies have shown that asymptomatic NoV infection seems to contribute more to the spread of NoV infection due to subclinical symptoms, which makes clinical diagnoses more difficult (Utsumi et al., 2017; Ayukekbong et al., 2015)

Depending on the variation of clinical spectrum of NoV infection the young, elderly and immunocompromised patients are the most vulnerable group of people who are at high risk of severe symptoms and complications (Chen et al., 2017; Bok and Green, 2012; Schwartz et al., 2011) such as hemodialysis due to acute renal failure, cardiac problem which includes arrhythmia, acute graft organ rejection in transplant recipient resulting in death (Harris et al., 2008; Mattner et al., 2006).

Elderly people are at high risk of severe NoV symptoms and clinical outcomes (Mattner et al., 2006). Prolonged diarrhoea from about 3 to 9 days in older adults has been reported in NoV outbreaks (Mattner et al., 2006; Goller et al., 2004). However, other clinical symptoms excluding diarrhoea may also be prolonged in adult people and such symptoms include: headache, dehydration (thirst), and sensation condition of whirling and loss of balance even though vomiting and diarrhoea had resolved (Cardemil et al., 2017; Lopman et al., 2004). In addition, these symptoms can lead to the high mortality rate in adult patients with NoV illness (CDC, 2007; Lopman et al., 2004; Lopman et al., 2003).

2.7 TRANSMISSION OF NOROVIRUS INFECTION

Norovirus spread through a variety of transmission route (Figure 2.5) (Lopman et al., 2011). Both the characteristics and behaviours of the infected host with potential susceptibles may also play a role in mitigating the risk of transmission. Lopman et al (2011) demonstrated that all the pathway requires shedding of the virus from infectious host (Lopman et al., 2011). NoVs are transmitted directly through person-to-person contact (faecal-oral or vomit-oral) or indirectly through ingestion of faecally contaminated food or water (Shankhali Chenar and Deng, 2017; Barclay et al., 2014). Environmental transmission is other route of NoV transmission from infected people to uninfected people (Lopman et al., 2012).

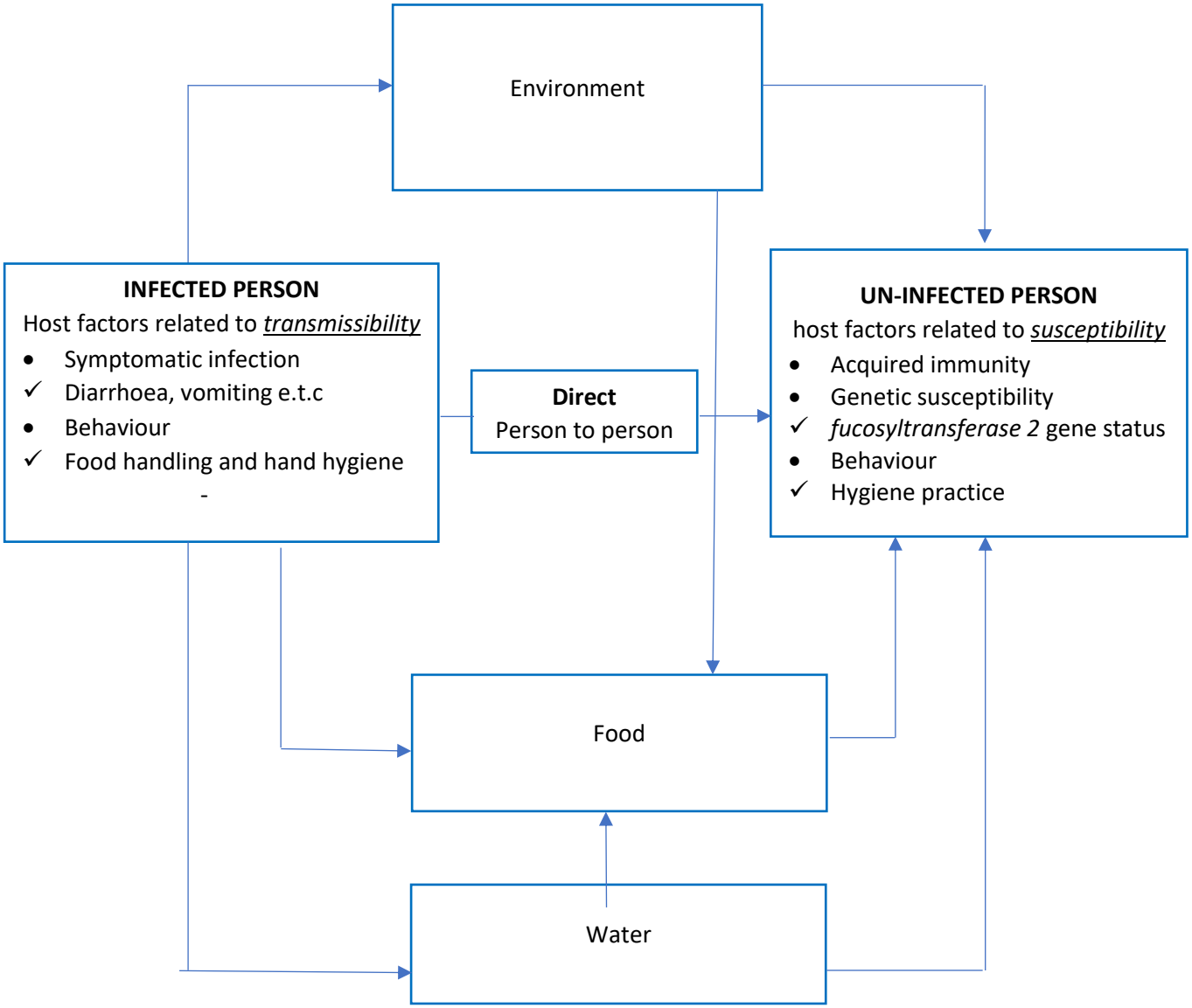

Figure 2.5: Route of transmission of NoV infection (Adapted from Lopman et al., 2011)

2.7.1 PERSON TO PERSON TRANSMISSION

Although Norovirus can be transmitted through various routes, direct transmission via person to person is the common transmission route of NoV (Figure 2.5) (Barclay et al., 2014; Kroneman et al., 2008). Outbreaks due to person to person (faecal-oral and vomit-oral) transmission occur in the diverse range of settings (hospitals, cruise ships, day-care centers, and military settings) where humans congregate and can affect a few to a lot of people (Barclay et al., 2014; Wick, 2012; Takkinen, 2006; Grotto et al., 2004). Norovirus GII.4 strain is mostly associated with gastroenteritis outbreaks caused by person-person transmission (Verhoef et al., 2010; Kroneman et al., 2008). From 348 NoV outbreaks which were reported between 1996-2000, foodborne transmission was the most common source of NoV outbreak with 39%, followed by person to person transmission with 12% (Lopman et al., 2003). The existence of a zoonotic reservoir for human infective NoV has been investigated, but zoonotic transmission of human infective NoV has thus far never been proven (Bank-Wolf et al., 2010).

2.7.2 WATERBORNE TRANSMISSION

Municipal waste is a mixture of human excreta (sewage), debris and suspended solids generated by households and commercial establishment (Argaw, 2004). Raw sewage is the major carrier of human enteric viruses because they are shed in large quantities in the faecal waste of infected individual (Zhang and Farahbakhsh, 2007). Due to their stability and persistence, most enteric virus outbreaks havargawe been previously reported on humans resulting from exposure to contaminated drinking water sources and recreational waters (Jurzik et al., 2010; Lodder et al., 2010; Okoh et al., 2010; Svraka et al., 2007; Westrell et al., 2006). Outbreaks of NoV have been reported to be associated with swimming in lakes and swimming pools during the summer season. Mans et al (2013) data on diverse Norovirus genotypes identified in sewage-polluted river water in South Africa demonstrated that 66% (70/106) Klip river samples were detected with NoV. Recently in KwaZulu Natal, a total of 11 samples were positive with 8 NoV genotypes from which the recreational water served as a vehicle in transmitting NoV among people swimming in Lagoons (Sekwadi et al., 2018).

2.7.3 ENVIRONMENTAL TRANSMISSION

Noroviruses are highly persistent and also influenced by different factors for their transmission, and as such include: large reservoir, copious shedding in faeces, widespread and rapid dissemination by vomit and many different fomites become contaminated. The viral particle can be easily transmitted from one object to another during the time of outbreaks through surfaces such as computer keyboards or mouse, door handles, switches, televisions, cellular phones, public phones, water taps, toilet light switches, (Gallimore et al., 2008, 2006). Contamination of mouse and keyboards have been reported correlating with the outbreaks in schools. Observation together with the epidemiological data suggests that projectile vomiting accelerates the spread of norovirus in large area (O'Neill and Marks, 2005).

2.7.4 FOODBORNE TRANSMISSION

Foodborne transmission is regarded as one of the common pathways that contribute to foodborne disease outbreaks worldwide (Widdowson et al., 2005; Mead et al., 1999). Foodborne outbreaks may be due to contamination during production, processing and preparation (Barclay et al., 2014; Hall et al., 2012). However, ready to eat food which includes salads, sandwiches and those that requires handling after cooking remains the most common transmission for NoV infection (Hall et al., 2014). As such, food with high content of water can easily transmit NoV because small inoculum of infectious viral particle can be mixed in a large volume of liquid served to many people which could lead to large outbreaks (Parashar and Monroe 2001).

Shellfish which includes molluscs such as oysters, mussels, cockles and clams harvested directly from sewage-contaminated water are the important source of NoV food-borne transmission (Acheson et al., 2002; Parashar et al., 2001). Shellfish filtrate clean water after harvesting for their nutrients and such tends to concentrate the NoV because NoV particles can retain and survive for several months in shellfish tissue by either specific binding or ionic binding (Le Guyader et al., 2006). The filtration process reduces the level of faecal coliform contamination, but do not reliably purify the NoV from the shellfish, which is either consumed as raw or with inadequate cooking (Patel et al., 2009), this increase the risk of illness by NoV infectious particles which could have been inactivated by high temperature during cooking process.

2.8 EPIDEMIOLOGY OF NOROVIRUS

Norovirus has been documented for high mortality with serious and long-term conditions (Siebenga et al., 2009). According to data from Centers for Disease Control and Prevention (CDC) (2015), norovirus impact over 250 million individuals annually and is known to contribute about 95% of viral gastroenteritis cases worldwide. Globally (between 2008 - 2014), NoV prevalence was 18% with lower rates in the inpatient (17%) compared to outpatient individuals (20%) and community setting (24%) (Shah and Hall, 2017; Ahmed et al., 2014). NoV prevalence is found to be higher in developing countries with low mortality rate (19%) and developed countries (20%) compared with high mortality in developing countries (14%) (Shah and Hall, 2017).

Globally, GII.4 viruses have been shown to be the predominant circulating pathogen causing 80% of NoV outbreaks, leading to greater complications in children and the elderly (Cardemil et al., 2017). Several studies have demonstrated that norovirus accounted for 6%-27% of AGE cases in adults of all ages, and 8%-41% of acute gastroenteritis in elderly of 65 years old and above (Leblanc et al., 2017; Costa et al., 2015; Grytdal et al., 2015; Verhoef et al., 2013; Fernández et al., 2011).

Recently in USA, Griffea-Hollis and Melton-Riddle (2018) reported that during 2016 and 2017 NoV cases have continued to be reported by public health agencies from universities, primary schools, day care centers, cruise ships and nursing homes. From September 2016 to April 2017 number of outbreaks have been reported among the general population of United States, with most predominant strain being Sydney (GII.P16-GII4) accounting for 60% of outbreak followed by GII.2 (14% of outbreaks), GI.3 (7%), GII.6 (4%) and GII.Pe-GII.4 with (3%). However, the authors also revealed that other NoV cases are undetected because sick people mistaken the illness as flu.

Mans et al (2016a) reported a prevalence of 13.5% of diarrhoeal disease from 14 African studies in children. However, the authors identified lack of data in older children and adults as a critical gap in Africa and the same gap exists worldwide (Lopman et al., 2016; Ahmed et al., 2014). Ahmed et al (2014) reported the lower prevalence in this setting compared to 20% which was reported in developed countries. In addition, NoVs are often detected in asymptomatic individuals (Phillips et al., 2009).

Kabue and co-workers (2016b) reported that out of 55 studies in their analysis, 65.4% and 20% of norovirus related studies reported in Africa between 1990-2013 were conducted from outpatients and hospitalised patients, respectively. Data of the study clearly show that GII strain was predominant variant in human samples. In addition, 42 studies contained only human samples and 12 only environmental samples and one combined. The authors reported a NoV prevalence rate of 11%. Moreover, in these studies, 18% pooled per percentage prevalence of GI and 81% of GII was reported (Kabue et al., 2016b).

In SA, Rouhani et al (2016) reported an overall prevalence of 30.4% from the asymptomatic carriage in children <2 years from the Malnutrition and the Consequences for Child Health and Development (MAL-ED) study. In addition, NoV incidence was 9.52 (95%, CI) for GII and 5.09 (95%, CI) for GI strain. Additionally, Moyo et al (2014) detected 13% of NoV (with 88.7% of GII and 13.2% of GI strain) from 408 diarrhoeal stool samples collected during the study in Tanzania infants.

Recently, Kabue et al (2017) reported NoV prevalence of 41% from both the symptomatic and asymptomatic children under 5 years of age. Additionally, high NoV diversity of circulation strains with GII.4 Sydney 2012 variant predominantly have also been reported. These findings are not in agreement with the findings from other previous studies (Moyo et al., 2014; McAtee, et al., 2014). High NoV detection rate was because their study was conducted in rural communities which is different compared to studies conducted from semi-urban settings. This finding was however, in agreement with several studies conducted in outpatients' children from other developing countries (Shioda et al., 2015; Zou et al., 2015; Jia et al., 2014), because young children are more susceptible to NoV infection as they are more exposed to the contaminated environment (Kabue et al., 2016a). There is paucity of data regarding the disease burden in older children and elderly in Limpopo province and globally.

2.9 PREVENTION OF NOROVIRUS OUTBREAKS

The prevention of NoV outbreaks by initiating the investigation through collection of clinical and epidemiological data can help in identifying the predominant mode of transmission and the reliable sources (Centers for Disease Control and Prevention (CDC), 2011). The solution for prevention of NoV can be done by using appropriate measures focusing on maintenance of strict hygiene by food handlers, control of contamination of water and food, environmental disinfection and reduction of outbreaks caused by direct contact through person to person spread (Robilotti et al., 2015; Kosa et al., 2014; Patel et al., 2009).

To prevent transmission of NoV, appropriate personal hand hygiene by sick individuals is the most important method for prevention of NoV infection and control of transmission (CDC, 2011; Patel et al., 2009). Reducing norovirus present in hands is accomplished by frequent handwashing with soap for a minimum of 20 seconds with running water (CDC, 2011), this reduces the number of pathogens in hands through mechanical removal of adherent microorganisms (Girard et al., 2010; Harris et al., 2010; Macinga et al., 2008; Sickbert-Benett et al., 2005). CDC suggests that alcohol-based hand sanitizer (e.g. ≥ 70% Ethanol) can be used as an adjunct in between proper handwashing but should not replace soap and water handwashing.

Food handlers should be excluded from the work during the time of infection and must be given 48 - 72 hours after recovery from gastroenteritis to prevent the transmission of viral particles to other individuals (Parashar et al., 2001). Since the virus can shed for a longer duration after illness and during the absence of clinical disease, the need to exclude these individuals for long period of time is of outmost important to avoid transmission (Rockx et al., 2002). Even though, the exclusion of food handlers from work may not be practical for economic purpose, other options such as temporal workers or other jobs that do not require food handling should be considered. Chemical disinfection of environmental contaminated surfaces is one of the key approaches to avoid the transmission of norovirus. Particular approaches should be given to areas of greatest environmental contamination such as high touch surfaces (hand rails and door knobs).

2.10 TREATMENT AND UPDATES ON NOV VACCINE DEVELOPMENT

In recent years, the increase in high burden of norovirus disease have given the impetus to the development of antiviral treatments and vaccines as well as the effective methods to prevent infections and illness of NoV disease (Bartsch et al., 2016; Kambhampati et al., 2015; Ramani et al., 2014). Moreover, current studies have shown that Norovirus (NoV) vaccines will be available in a few years to come (Kocher et al., 2018; Cortes-Penfield et al., 2017).

The major complication of norovirus disease is dehydration that results. Presently, there is no specific treatment implemented for human norovirus disease, but cases are being managed by the use of supportive first-line therapy such as Oral Rehydration Therapy (ORS) (Kambhampati et al., 2015) which is often not frequently used in the management of diarrhoea in children of under the age of 5 years (Van der Westhuizen, 2011). However, effective treatment is of utmost important especially to children, adults and immunocompromised individual since they will be experiencing high loss of fluids. Favipiravir and ribavirin drugs which work by inhibiting viral entry and replication, have demonstrated activity against other RNA viruses as well as norovirus replicons (Kambhampati et al., 2015; Kaufman et al., 2014; Arias et al., 2013). However, these treatment options have not been implemented in humans (Kambhampati et al., 2015).

To date, norovirus vaccine development has been proposed through the development of virus-like particles (VLPs) (Cortes-Penfield et al., 2017; Debbink et al., 2014; Richardson et al., 2013; Atmar and Estes, 2012). Although the VLPs lack a viral genome, the expression of recombinant norovirus protein results in the assembly of virus-like particles that are antigenically and morphologically identical to the infectious agents thereby mimicking the native virus (Ramani et al., 2014; Jiang et al., 1993). Preclinical studies done in mice showed that VLPs candidate vaccines are more immunogenic when delivered through the following route: intranasal, oral and parenteral route, inducing serum and mucosal immunity (Ramani et al., 2014; Atmar and Ester, 2012; Estes et al., 2000).

The Initial phase I clinical studies assessed the safety and immunogenicity of oral immunization with increasing dosages of GI.1 Norwalk virus VLPs in adults (Tacket et al., 2003; Ball et al., 1999), afterwards the intranasal route for administration was assessed. The safety and immunogenicity of two doses of dry powder formulation adjuvanted with monophosphoryl lipid A and mucoadherent chistosan was tested in adults (El-Kamary et al., 2010). The vaccine was tolerated with no side effects and was immunogenic showing-independent increase in serum antibody titers thereby inducing the functional antibodies which shows the homing potential to the gut mucosa and peripheral lymphoid tissues (Ramani et al., 2014). The intranasal administration route for Norwalk vaccine VLPs was tested in proof-of-concept efficiency in healthy, secretor-positive adults who receive two doses of vaccine or placebo 3 (Debbink et al., 2012a). The vaccinated individual shows less possibility to develop gastroenteritis infection compared to the placebo patients (Ramani et al., 2014)

Proof of concept studies are of utmost importance to reveal that NoV vaccine can induce protective immunity. From these studies, only GI.1 strains are tested though the circulating genotype is GII.4. Therefore, challenge studies using GI and GII strain failed to demonstrate the heterotypic protection, suggesting that vaccines should include VLPs from two major strain from GI and GII genogroup (Ramani et al., 2014). Studies on new VLPs showed that intramuscular immunization of a bivalent formulation including GI.1 and consensus VLPs induced higher antibody levels compared with intranasal route of immunization. The first studies on intramuscular immunization with the bivalent vaccines in adult volunteers are accomplished, with no side effects and the vaccine was immunogenic, with IgA and IgG responses to both VLPs (Frey et al., 2011).

Following different studies which were conducted in past years, there was a little promise on the development of successful NoV vaccine together with establishing recommendations for them. However, recent studies on the treatment and update of NoV vaccine development indicated that successful licensed NoV vaccine will be available in few years to come (Lucero and Vidal, 2018; Cortes-Penfield et al., 2017). But several important challenges remain for development of inexpensive, safe and efficacious NoV vaccines (Kocher et al., 2018; Cortes-Penfield et al., 2017).

Firstly, Preclinical studies of therapeutics for NoV have long been limited by the lack of cheap and appropriate small animal model systems as well as by a lack of cell culture systems for NoV infection (Cortes-Penfield et al., 2017). However, a recent breakthrough on the development of affordable NoV culture techniques (Kolawole et al., 2016; Qu et al., 2016; Jones et al., 2015), could lead to successful culturing of NoV in future giving opportunity for live vaccine candidates (Lucero and Vidal, 2018). In addition, these developments are still in early stages and further discussion for their capabilities require further advances and such will be available in a few years to come.

Secondly, NoV are genetically diverse, and they undergo antigenic drift giving rise to new strains (Ramani et al., 2014). As such, the diversity between and within genogroups will require the multivalent vaccine (LoBue et al., 2006), because previously studies have shown that NoV protective response is genotypic specific (Blazevic et al., 2015; Vesikari and Blazevik 2014). This raise a serious question as to how many strains of NoV should be included in the vaccine (Atmar and Estes, 2012).

Thirdly, because of self-limiting duration of protection following the NoV infection, Natural infection does not confer long-term immunity as seen in data from volunteer challenge studies (Cardemil et al., 2017; Ramani et al., 2014), even though modelling studies are suggesting a memory response that lasts for 4-9 years (Lopman et al., 2014; Ramani et al., 2014; Lindesmith et al., 2013). Therefore, vaccine candidates will need closer attention to the duration of protection following exposure to the infection, need for the booster doses and reformulation (Cardemil et al., 2017).

Lastly, another difficulty in the development of vaccine for enteric pathogens in general, is the inability to correlate the specific antibody titers with protection. From previously adult volunteering study, patients with high serum and duodenal titers who were challenged with the same NoV variant were highly susceptible to NoV infection compared to individual who were seronegative (Johnson et al., 1990). Nowadays, it is known that the susceptibility to NoV infection depends upon the HBGAs (Currier et al., 2015; O'ryan et al., 2009; Hutson et al., 2005; Lindesmith et al., 2003). One strategy to overcome these limitations is rational targeting of NoV strains through conducting systematic surveillance studies annually similar to the annual targeting for seasonal influenza vaccines (Cortes-Penfield., et al., 2017).

2.11 DIAGNOSIS OF NOROVIRUS INFECTION

It is difficult to diagnose norovirus infection based on clinical features alone (Chen et al., 2017). As such, diagnosis of Nov infection requires both the clinical presentation as well as the laboratory testing (Stone et al., 2012). Saliva, rectal swabs and vomitus can be used for detection of NoV but whole-stool samples are more preferred clinical sample for detection of NoV because stool sample contains high quantity of virus since the primary source of transmission is faecal-oral route (Vinjé et al., 2015). Laboratory testing of NoV has undergone significant development from traditional electron microscopic (EM) examination to nucleic acid amplification testing (Vinjé, 2015; Kapikian et al., 1972).

During the past decades EM was the only method for detection of NoV. Although EM can also visualize other enteric viruses, this method is now not widely used in many diagnostic microbiology laboratories because is costly and insensitive for detection purpose (Vinjé, 2015). Since 1990's the detection methods have evolved from electron microscopy to conventional end-point reverse transcription polymerase chain reaction (RT-PCR) (Vennema et al., 2002). But, real-time RT-quantitative PCR assays (RT-qPCR) are being widely used in public health and research laboratories because of increased specificity, sensitivity and its rapidness (Vinjé et al., 2015; Patel et al., 2009; Trujillo et al., 2006). One-step RT-qPCR assays have been recently developed for rapid detection of NoV in large number of stool specimen during the outbreaks (Patel., 2009; Trujillo et al., 2006).

Recently, several diagnostic platforms have been developed and receive the FDA-clearance for simultaneous detection of gastroenteric pathogens (Hawash et al., 2017). The FilmArray GI Panel, the xTAG GPP and Verigene Enteric Pathogens Test (EP) platforms provide the multiplex molecular diagnostic tests for relevant GE pathogens with good specificity and sensitivity. The Luminex® xTAG® platform detects up to 15 diarrheal causing pathogens including, NoV GII and GI, rotavirus group A, 7 bacterial species, and 2 parasite species (Gosert et al., 2018; Hawash et al., 2017). The FilmArray GI Panel detects 23 enteric pathogens, including NoV GII and GI, rotavirus group A, adenovirus group F, astrovirus, sapovirus, 14 bacterial species and 4 parasite species. The Verigene EP assay detects NoV, rotavirus, 5 bacterial species and 2 Shiga toxins (Kroneman et al., 2013).

2.12 NEW CELL LINES DISCOVERED

While attempts for culturing human noroviruses have been unsuccessful, Ettayebi et al (2016) reported replication of several human norovirus strains in a culture system derived from human intestinal stem cell-derived enteroids (HIEs) isolated from intestinal crypts in human intestinal tissues (Saxena et al., 2016; Sato et al., 2011). These HIEs offer advantages over existing cell lines because they contain multiple intestinal epithelial cell types (enteroendocrine, enterocytes, goblet and Paneth cells), are non-transformed, and demonstrate many of the biological and physiological properties that responds to agonist (Foulke-Abel et al., 2014). However, these findings provide hope for the development of live attenuated or inactivated NoV vaccines.

2.13 SUMMARY OF LITERATURE REVIEW

Acute gastroenteritis (AGE) cause high morbidity, mortality and economic costs across the globe each year particularly in less developed countries of the world. Every year, acute gastroenteritis cause approximately 1.4 million deaths in low and middle countries. Although different pathogens are involved in causing diarrhoea, enteric viruses play a major role as disease causing agents. Among these viruses, Norovirus (NoV) is recognised as the second leading cause of AGE in people of all age groups. Norovirus is classified into 7 genogroups (GI – GVII) of which GII, GI and rarely GIV infect humans. These genogroups are further devided into 38 genotypes. However, GII.4 genotype is responsible for approximately 55-85% of clinical cases worldwide.

Studies on the molecular epidemiology of NoV infections in outpatients children of less than 5 years of age in rural communities of Vhembe district, Limpopo Province (South Africa) and worldwide have already been conducted. Nevertheless, in rural communities of Vhembe district Limpopo province, South Africa, studies on the molecular epidemiology of NoV infection in outpatient children older than 5 years of age and adults are lacking. Therefore, this is the first study that aimed to characterize human NoV in patients with diarrhoea in rural communities of Vhembe district. After the completion of this study, the role of NoVs as causative agents of gastroenteritis and their diversity in rural villages of Vhembe district will be known. Data from this study will help target clinical development of vaccines that will cover people of all the age group and inform future vaccination policy decisions.

Chapter 3

MATERIALS AND METHODS

3.1. INFORMED AND ETHICAL CLEARENCE

Approvals were obtained from the Ethics committees of the Department of Health in the Limpopo province (Ref. 4/2/2) and University of Venda (Ref.SMNS/18/MBY/03) (Appendices 1, 2 and 3). Written informed consent was given to each participant before stool collection (Appendices 4,5 and 6).

3.2. DEMOGRAPHICS OF PATIENTS

In this study, patients were between 5 years and 68 years of age. After obtaining written informed consent from the participants, an interview by health professional nurses (using a questionnaire: Appendix 7) was conducted with the adult patients to gather the information on personal details regarding the date of birth, gender, starting date of diarrhoea and symptoms associated with patient illness such as abdominal pain cramps (APC), fever and dehydration. The consistency of the stool according to the Bristol stool chart was documented (Appendix 8) (Lewis and Heaton, 1997). Living conditions of the patients based on the type of water used, the presence of livestock, type of toilet being used, and employment status was also included in the questionnaire.

3.3. SAMPLE COLLECTION

The study design for this research was cross-sectional. Stool samples (n=80) were randomly collected from symptomatic cases of diarrhoea in patients above 5 years attending Primary Health Care (PHC) centres. Diarrhoea in this study was referred to as the passage of three or more loose or watery liquid stools within the preceding 24 hours (WHO, 2005). Sterile specimen containers were used for collection of stool sample and kept at 4˚C during the transportation to the University of Venda molecular laboratory and immediately refrigerated at - 20˚C prior to RNA extraction. Between August 2017 to October 2018, a total of 80 stool specimens was randomly collected at 6 different clinics (Rabali, Thengwe, Marseilles, Vhufuli, Thohoyandou, Phiphidi) situated in the rural communities of Vhembe district in Limpopo Province, South Africa (Figure 3.1).

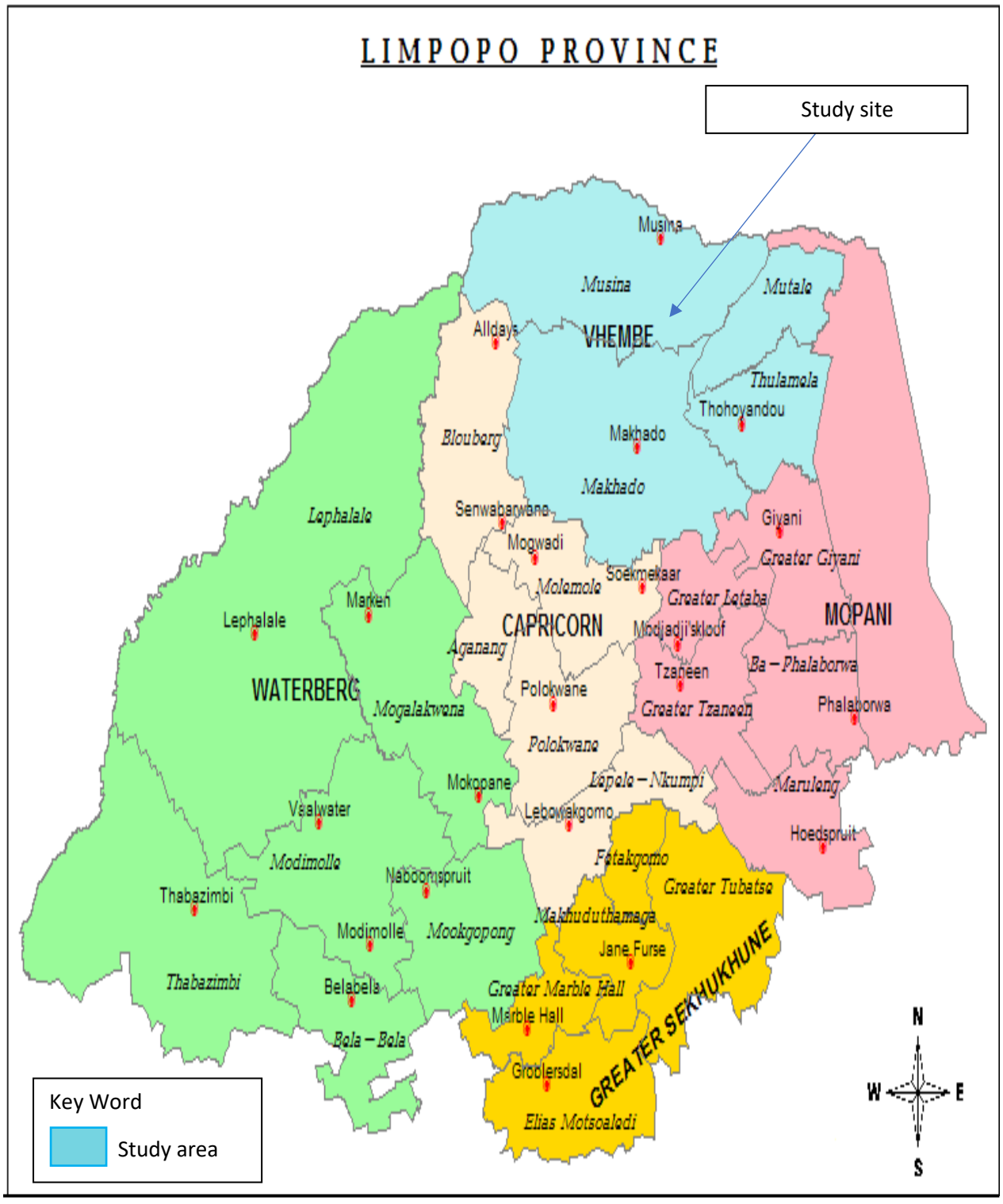

Figure 3.1: Map of Vhembe district, Limpopo Province

http://www.capeinfo.com/blogs/wp-ntent/blogs.dir/akela/files/2009/05/limpopo_dlghgovza.gif

3.4 RNA EXTRACTION (BOOM METHOD)

The published Boom extraction method was used (Boom et al., 1990). Before RNA extraction, sample processing was done on each raw specimen by diluting the stool 1:10 in phosphate buffer saline (PBS, 0.01 M, pH 7.2) (Thermo-Fisher Scientific, Waltham, Massachusetts, United States) and thoroughly vortexed to allow proper mixing. RNA was extracted from the faecal suspension using an adapted version of the guanidium thiocyanate/silica method reported by Boom et al (1990) (Appendix 9). The method is based on the lysis and nuclease inactivating properties of chaotropic agents guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent.

3.5 Real-time PCR (NOROVIRUS DETECTION METHOD)

Different molecular-based techniques are used for detection of viruses such as Human Norovirus (Atmar and Estes, 2001). In this study, the RIDA©GENE Norovirus I & II real time RT-PCR (r-BiopharmAG, Darmstadt, Germany) was used for detection of Human Norovirus (Figure 3.2).

Figure 3.2: RIDA©GENE Norovirus I & II real-time RT-PCR Kit (http://www.r-biopharm.com)

The RIDA©GENE NOROVIRUS I & II real-time RT-PCR (r-BiopharmAG, Darmstadt, Germany) kits was used because: (1) The multiplex RT rtPCR is a qualitative detection method and differentiate Norovirus GI and GII in human stool samples targeting the ORF1/ORF2 junction region according to the manufacturer validation; (2) It is not thought to cross-react with other common enteric pathogens; (3) The assay contains an internal control RNA (ICR) to monitor the extraction efficiency and also determine PCR inhibition; and (4) Has high (98%) sensitivity and specificity. [The one step real-time PCR was performed on the Corbett Research Rotor Gene 6000 (Corbett Life Sciences, Concorde, Australia) according to manufacturer's instructions with the following temperature profile: Reverse transcription for 10 min at 58°C; initial denaturation step for 1 min at 95°C followed by 45 cycles of 95°C for 15 seconds and 55°C for 30 seconds with continuous fluorescence reading].

3.6 NOROVIRUS RT-PCR AMPLIFICATION

Extracts tested positive for norovirus by one step Real-time PCR was then subjected to RT-PCR amplification for the purpose of nucleotides sequencing. Primers used for RT-PCR are given in Table 3.1. The specific oligonucleotide primer pair G1SKF/G1SKR to amplify 330 bp of the capsid region of NoV genogroup I and G2SKF/G2SKR to amplify 344 bp of the capsid region of NoV genogroups II was used to perform One - step Ahead RT-PCR (QIAGEN, GmbH, Germany) as previously described by Kojima et al (2002). Designed primers (WGS 9F/WGS 9R) were used to amplify a 751 bp product of GII capsid that were not detected by GIISK primers.To amplify the genome of NoV strains, the cDNA was synthesized from 10% of diluted 5µl of RNA extracts for 10 mins at 50°C followed by heating at 95°C for 5 min to inactivate the enzyme and then amplified using the supplied 0.5 µM of each oligonucleotide primer in 25µl reaction mixture. Thermocycling condition for PCR was performed for 40 cycles as follows: denaturation at 95°C for 10 seconds, annealing at 50°C for 10 seconds (GISK primers or WG9 primers) or 56°C (GII SK primers), extension at 72°C for 10 seconds and final extension at 72°C for 10 minutes. In addition, 326 bp of RdRp fragment was amplified using primers set JV12/JV13(Vinjé et al., 2003) with the same PCR conditions as of GISK primers. PCR products were then analysed using a 2% (w/v) agarose gel in TAE buffer (40mmol l21 Tris acetate; 2 mmol l21 EDTA, pH 8.3) stained with ethidium bromine (Appendix 10).

Table 3.1: Primers used for Norovirus genotyping in this study

PRIMERS	SEQUENCES (5′ - 3′)	POLARITY	GENOTYPE	TARGET SIZE (BP)	NUCLEOTIDE	POSITION	REFERENCES
G1SK(F)	CTGCCCGAATTYGTAAATGA	F	GI NoV	Capsid	330 bp	5342	Kojima et al., 2002
G1SK(R)	CCAACCCARCCATTRTACA	R	GI NoV			5671	
G2SK(F)	CNTGGGAGGGCGATCGCAA	F	GII NoV	Capsid	344 bp	5058	Kojima et al., 2002
G2SK(R)	CCRCCNGCATRHCCRTTRTACAT	R	GII NoV			5401	
JV12	ATACCACTATGATGCAGATTA	F	GI and GII NoV	RdRp	326 bp	4552	Vinjé et al., 2003
JV13	TCATCATCACCATAGAAAGAG	R	GI and GII NoV			4878	
WGS 9F	CACCCACAGTTGAGTCAAGAAC	F	GII NoV	Capsid	751 bp	5734[a]	
WGS9R	GGAGCTGCCTCTTGGTAGA	R	GII NoV			6484[a]	

[a] Accession number of reference strain from Genbank used to design the primer WGS9F/9R: JN595867.1.

3.7 GENOTYPING AND PHYLOGENETIC ANALYSIS OF NOV STRAINS

The RT-PCR products of the amplified fragments were directly purified with Zymoclean™ Gel DNA recovery Kit following manufacturer's instructions. The Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer POP7™ (Thermo-Scientific) using the same specific primers. The raw sequence reads were edited with Finch TV v1.4 (Geospiza, Seattle, USA). The nucleotide sequences obtained from the selected NoV strains were used to search similar sequences in the NCBI genetic database using the BLAST tool (available at http://www.ncbi.nlm.nih.gov/) and then aligned using Noronet typing tools (Kroneman et al., 2011) (available at http://www.rivm.nlm/norovirus/typingtool). The reference strains from Genbank were randomly selected among the Blast hits with >80% similarities on the query sequence of the NoV strains identified from this study.

Phylogenetic trees were constructed by the neighbor-joining method (Saitou et al., 1987) using MEGA 7 software, with 1,000 bootstrap replicates for each gene (Felsenstein, 1985). The evolutionary distances were computed using the *P*-distance method (Nei, 2000).

3.8 STATISTICAL ANALYSES

Data were recorded in Microsoft excel. The t-test comparing cycle threshold (CT) values in diarrhoeal cases was performed. Non-parametric receiver operating characteristic analyses to assess the association between CT values and illness was also performed A *P*-value of < 0.05 was considered to be statistically significant.

Chapter 4

RESULTS AND DISCUSSION

4.1 NOROVIRUS PREVALENCE

During the study period, a total of 80 stool samples were collected from participants suffering from Acute gastroenteritis (AGE) and screened for Norovirus (NoV). Bloody stool diarrhoeal samples were excluded from this study. All of the participants were residents from rural communities of the Vhembe district, Limpopo Province. The age distribution of the study participants was ranging from 5 years to 68 years (Table 4.1). The proportion of the participants by age and sex are summarized in Table 4.1.

Out of 80 diarrhoeal faecal samples, 13 samples were tested positive by one step real-time RT-PCR for NoV (16%), of these positive samples 6 [46%] were GII , 7 [54%] were GI. The internal control (IC) was detected in 100% of all positive stool samples (n=13), and IC recovery rate was 33.25 ± 0.97 [mean ± standard deviation (sd)]. However, there was a significant difference (Student's t-test, unpaired, t = -2.60, $p < 0.001$) in the CT values of GII genotypes and GI genotypes with their mean of 22.19 (n = 6, sd = 5.59) and 32.23 (n = 7, sd = 7.78) respectively. NoV GII is the predominant genogroup worldwide associated with AGE as described ealier on the literature. However, the inconsistency of this study with previous observations may be probably due to small number of samples collected.

Among diarrhoeal patients, forty-nine (61%) were female and 31 (39%) were males. From those detected positive for NoV, 10/13 (77%) were females, with only 2/13 (15%) of them being female of less than 35 years of age and only 3 (23%) were adult male children (Table 4.1). The age distribution of the study participants was ranging from 5-68 years, with 39 (49%) of participants between the age of 21-59 years followed by 36 (45%) of 5-20 years of age and lastly by elderly with 5 (6%) (Table 4.1).

Table 4.1: Demographic profile (age and sex distribution) of Norovirus tested positive in adult patients living in rural communities of the Vhembe district, Limpopo Province.

	No of cases collected (n= 80)	No (%) of cases tested positive by Real-time PCR (n=13)
Age (years)		
5 - 20	36	5 (38%)
21 - 59	39	7[a] (54%)
≥ 60	5	1 (8%)
Total	80	13 (16%)
Gender		
Female	49	10 (77%)
Male	31	3 (23%)
Total	80	13 (100%)

[a] individual of 35 years of age and above; NoV, norovurs.

The findings of this study are consistent with previous studies done, where NoV was mostly detected in female patients between 30-59 years of age than in age groups between 5-29 and >60 years (Gong et al., 2018; Lindsay et al., 2015). High NoV infection in adult women may be due to the fact that younger people such as infants and children are more susceptible to NoV infections because they are more exposed to the contaminated environment and they have not acquired sufficient immunity. Thus, it is likely that NoV infection spread among young people to adults and elderly more especially females who are childminders. In addition, poor living environmental and hygiene conditions of people living in rural communities of Vhembe district could also have been responsible for spread of NoV in patients from local communities of Vhembe district. However, in different countries across the world, most children were found to develop antibodies against NoV infection between 5 to 15 years of age (Sakon et al., 2014). Therefore, this could be the reason for low NoV detection rate within those age group.

NoV was frequently detected in diarrhoea with mixed symptoms 6 (46%) followed by diarrhoea with abdominal cramp pain 5 (39%) and lastly from diarrhoea alone 2 (15%) (Table 4.2). Mixed symptoms include, vomiting, fever and dehydration. These findings are inconsistent to the study done by Gong et al (2018) where the author reported a low detection rate of NoV positive cases from adult patients suffering from diarrhoea and abdominal cramp than in diarrhoea and other clinical symptoms. In addition, these findings are also inconsistent with findings from other studies conducted in children of less than 5 years of age were NoV infection was mostly associated with diarrhoea with vomiting only (Matsuyama et al., 2018; Kabue et al., 2016a; Gotz et al., 2001). However, with regard to clinical symptoms, NoV infection is more associated with fever and vomiting (Cui et al., 2017; Zhou et al., 2017), but its frequency is low in this study.

Similarly, to other studies, most NoV cases were detected in watery stool (46%). In this study, fresh stool sample 9 (69%) of less than three days were more associated with NoV infection as also described in previous studies (Table 4.2) (Matsuyama et al., 2018; Kabue et al., 2016a). There are different stages during viral infection, there are possibilities that the fresh stool samples were collected when the virus replication cycle is at the exponential stage.

The majority of diarrheal cases (67/80, 84%) were collected from patients who depends on tap water for consumption than other water sources. Out of 13 NoV positive samples detected, 10 (77%) of cases were from people who depend on tap water in their daily basis followed by borehole with 23% (Table 4.2). The findings of this study are compatible with speculation from a study by Ayukekbong et al (2014) in Cameroon who found that tap water are more contaminated with NoV than borehole water. In rural communities of the Vhembe district, municipalities supply tap water from municipal water treatment plants. Unlike other enteric viruses, NoV are highly resistant to common chlorine disinfectants which are being used in municipality water treatment plants (Ayukekbong et al., 2014; Smith, 2013). It is possible that supplied water from treatment plants are being contaminated by NoV from streams which are likely to be contaminated with human sewage. However, hygiene practices of people collecting water and storage containers being used could also have played a major role in causing NoV infection in people living in rural community of the Vhembe district.

Out of the 13 cases of NoV detected, 11 (85%) cases were obtained from patients who used pit latrine toilets for sanitation (Table 4.2). Pit latrines with slab bases are the most common toilet being used in rural communities of the Vhembe district (Banda, 2018; unpublished). Faecal-oral contamination is a common route of transmission for NoV (Ayukekbong et al., 2014). Therefore, toilet facilities that allow disposable of faeces plays a major role in transmission of NoV and other enteric viruses. To the best of our knowledge, pit latrines may be associated with high risk of NoV infection compared to flush toilets. Improved toilets such as those which are being connected to sewer lines are needed to reduce the transmission of enteric viruses in rural communities of the Vhembe district and in developing countries as a whole. Though the zoonotic transmission of human infective NoV has thus far never been proven, only 1 (8%) NoV positive case was detected from patient with livestock at home (Table 4.2).

Though there was inconsistency on stool sample collection in this study, most of diarrhoeal cases were collected between November - March. Among samples tested positive, NoV were most detected from specimens collected during the rainy season (November to March). In term of seasonal distribution, the findings of this study agree with findings from other studies in the literature. Similarly, NoV peak during the rainy season (Gong et al., 2018).

Table 4.2: Clinical features and family conditions of study participant above 5 years of age from rural communities of the Vhembe district, South Africa.

Parameters	NoV cases (n=80)	No of NoV positive (%) by real-time PCR (n=13)
Clinical symptoms		
Diarrhoea alone	17	2 (15%)
Diarrhoea and vomiting only	2	0 (0%)
Diarrhoea and APC	36	5 (39%)
Diarrhoea with other symptoms	25	6 (46%)
Types of stool		
Watery	23	6 (46%)
Formed	9	2 (15%)
Soft	48	5 (39%)
Interval[a]		
≤3 days	70	9 (69%)
>3 days	10	4 (31%)
Family conditions		
Water source		
Tap	67	10 (77%)
Spring/Wells	1	0 (0%)
Boreholes	12	3 (23%)
River	0	0 (0%)
Sanitation		
VIP/Pit latrine	71	11 (85%)
Flush toilets	9	2 (15%)
Livestock		
Yes	7	1 (8%)
No	73	12 (92%)
Total	80	13 (100%)

[a] between stool sample collection and onset of diarrhoea; APC, Abdominal pain cramp; PCR, polymerase chain reaction. other symptoms include (vomiting , fever, dehydration)

4.2 SEQUENCE ANALYSIS OF CAPSID AND POLYMERASE REGION FRAGMENTS

4.2.1 VALIDATION OF PRIMERS SPECIFICITY

The positive samples from previous published study done by Kabue et al (2016a) in children of less than 5 years of age were used to test the primer set GISKF/G1SKR and GIISKF/G2SKR which is used to amplify the capsid gene fragment of NoV GI and GII, respectively (Figure 4.1 and 4.2; Kojima et al., 2002). The primers sets JV12/JV13 were also used to detect a fragment of the NoV RNA polymerase gene (Figure 4.3). The primers for a long sequence (WGS 9F and WGS 9R) were used to detect the fragments of GII capsid which were not detected by the short sequence GIISKF and GIISKR (Figure 4.4). The primer set tested generated the PCR product of expected size indicating an amplification of NoV strains (Figure 4.1, 4.2, 4.3 and 4.4). Different studies have shown that the detection of viruses by RT-PCR is difficult and inconsistence due to genetic diversity which makes it difficult to select the primers to use (Vinjé et al., 2004).

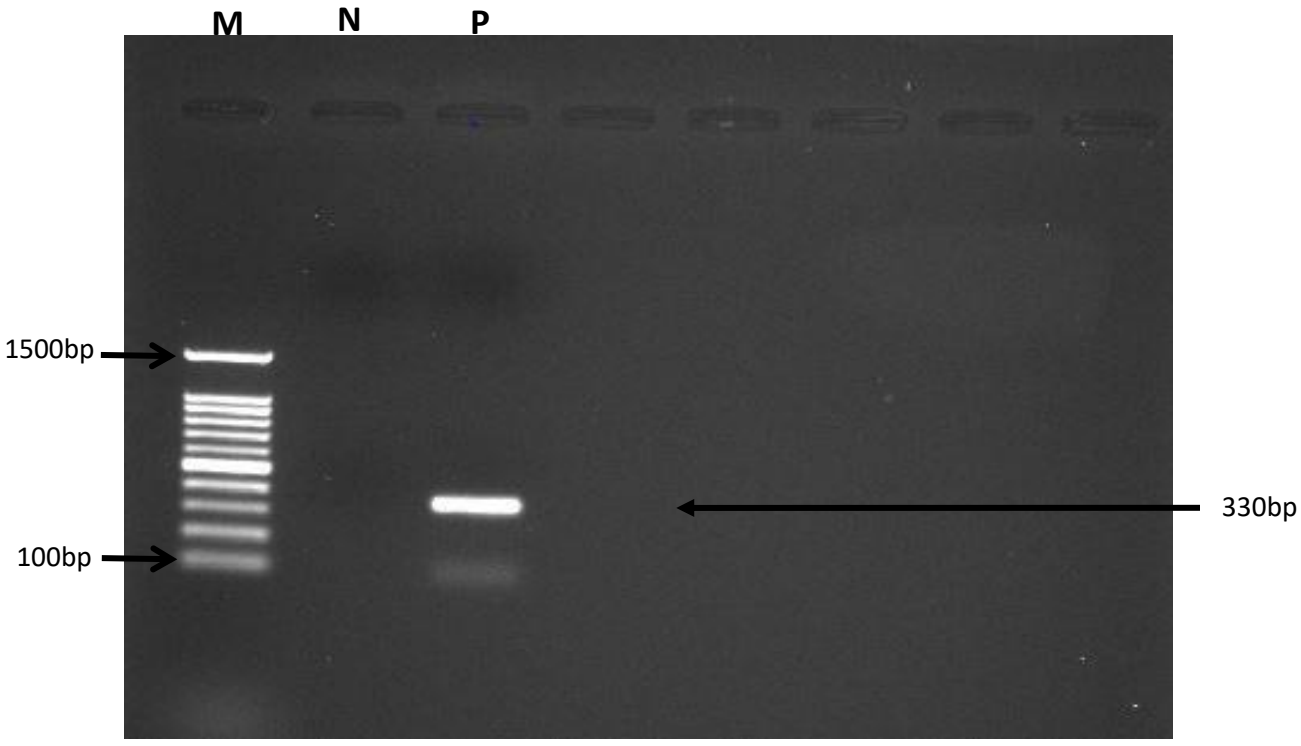

Figure 4.1: Gel electrophoresis results of NoV GI capsid gene PCR product of 330 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study.

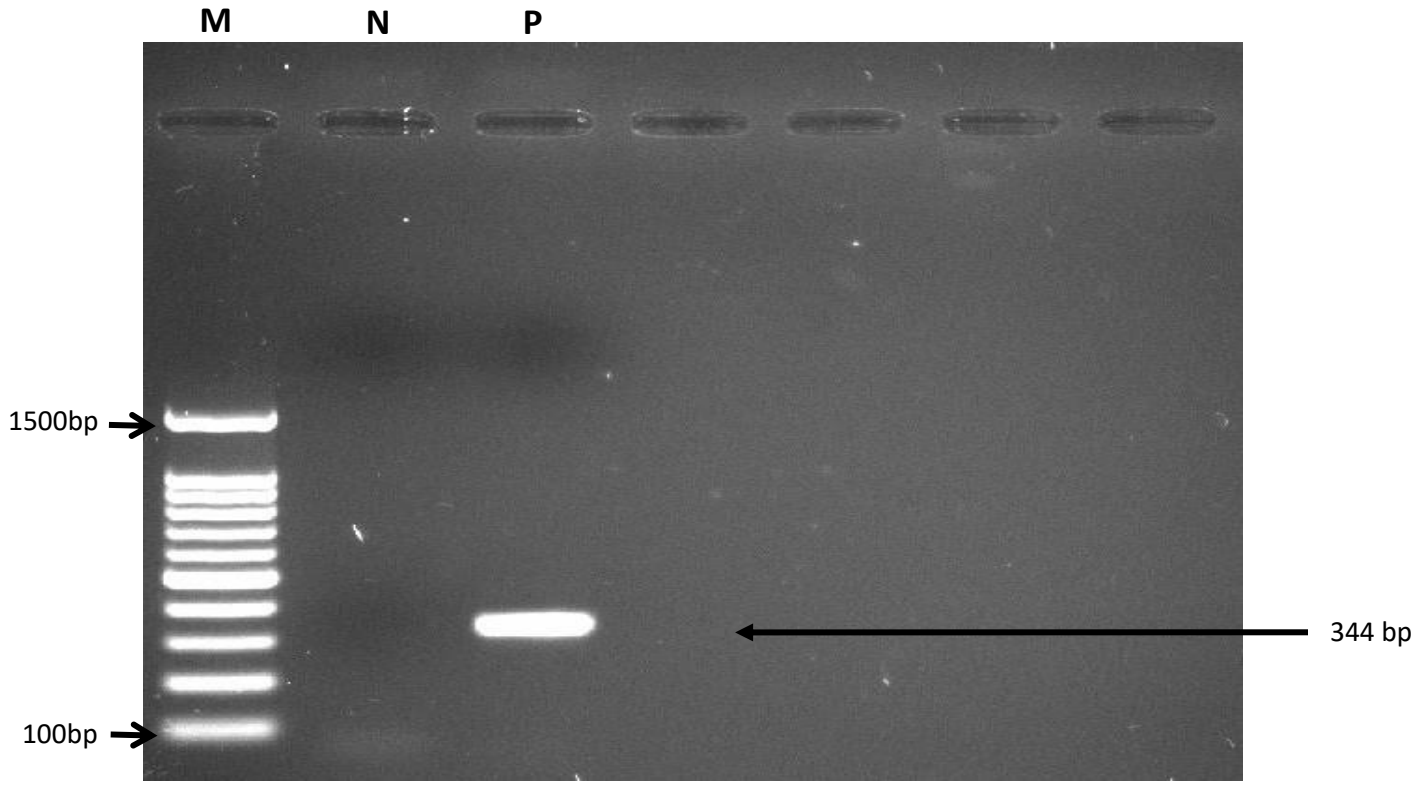

Figure 4.2: Gel electrophoresis results of NoV GII capsid gene PCR product of 344 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study.

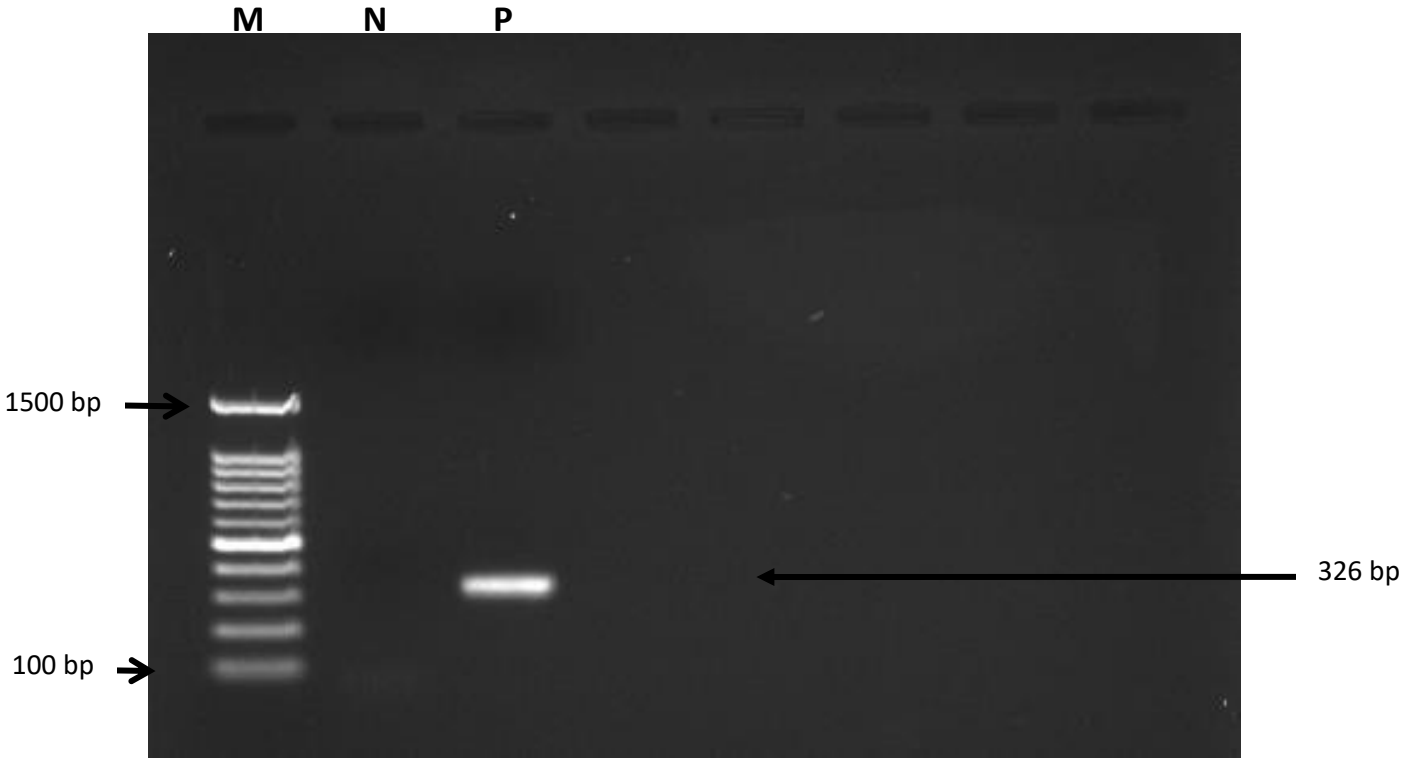

Figure 4.3: Gel electrophoresis results of NoV GII polymerase gene PCR product of 326 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study.

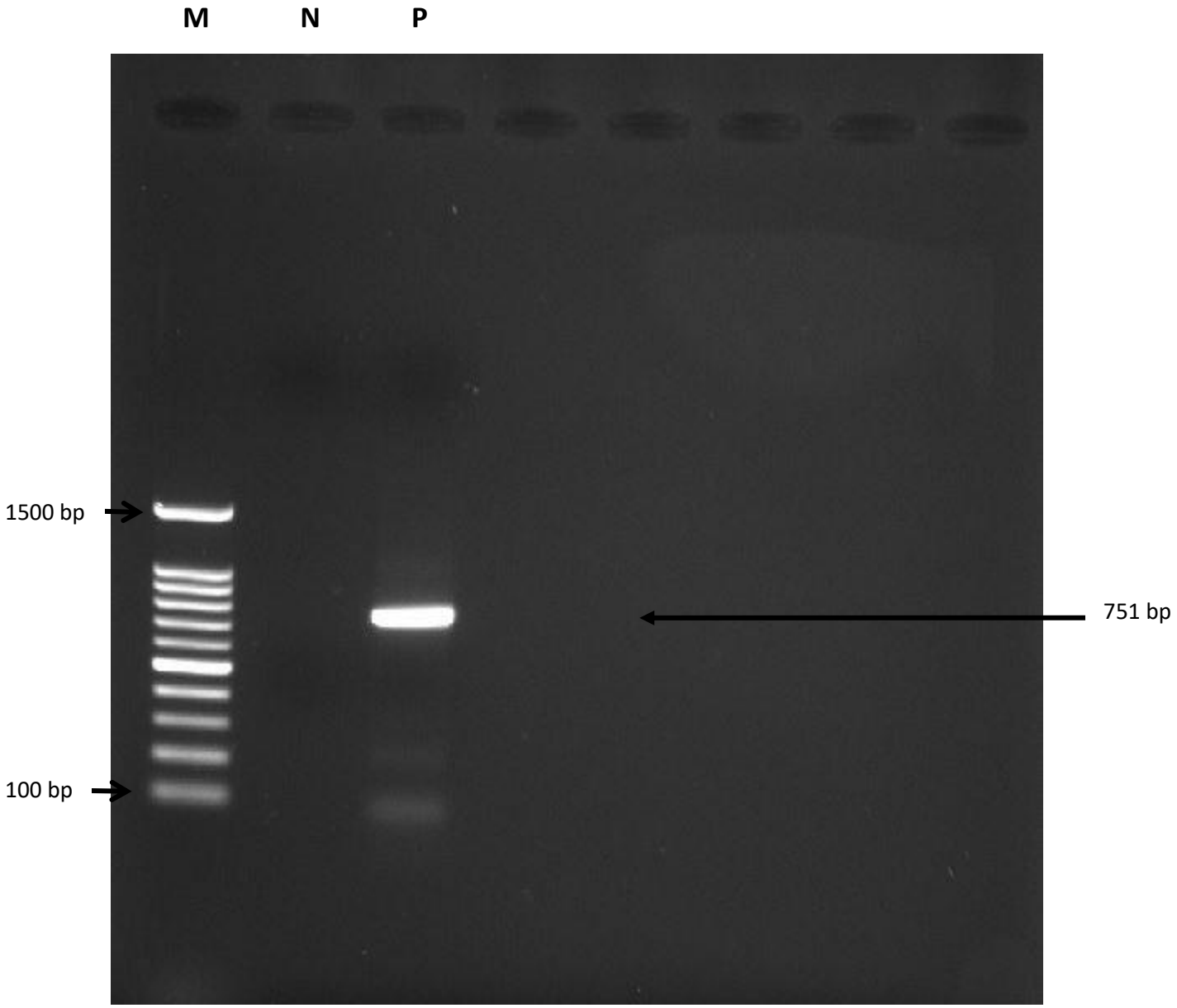

Figure 4.4: Gel electrophoresis results of NoV GII capsid gene PCR product of 751 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study.

4.2.2 AMPLIFICATION RESULTS

From the total of 13 samples confirmed positive by real-time PCR, 5 samples (38%, 5/13) were successfully amplified (Figure 4.5, 4.6 and 4.7). However, none of GI samples could successfully be amplified (Figure 4.8). Overall, 8 (62%, 8/13) samples could not be successfully amplified by conventional PCR, which might be due to various reasons including:

- RNA degradation in the sample because of load-shedding of electricity which makes the storage of the refrigerators unstable. Load-shedding might have reduced the viral load of RNA sample (Vinjé et al., 2004).

- Time interval between the storage of RNA extracts and amplification could also have influenced the amplification results (Vinjé et al., 2004).

- Freezing and thawing of samples during amplification might have also contributed to the degradation of RNA template (Duizer et al., 2004).

- Mismatch between the primers and RNA template (Kanwar et al., 2018; Lochridge and Hardy, 2003). However, this was not considered in this study because preliminary testing for positive controls were done before. In addition, amplification for GI was retested using different concentration of RNA templates (1:10, 1:5 and 1:2 dilution) so to verify if the poor GI results are maybe affected by the low concentration of RNA. However, all the samples were still negative for GI.

- Previous studies have reported that unlike GII strain, GI strains are not stable (As such, there is high possibility of detection using the real-time PCR due to its greater sensitivity compared to conventional PCR (Kabue et al., 2016a; Kumar et al., 2016; Duizer et al., 2004).

To avoid contamination in this study, RNA extraction, NoV detection and Amplification were performed in a separate rooms . In addition, the usage of internal control (IC) during RNA extraction helped to monitor PCR inhibitors during real-time PCR as this was also previously described (Hata et al., 2017; Rodriguez et al., 2012; Schrader et al., 2012; Le Guyader et al., 2009).

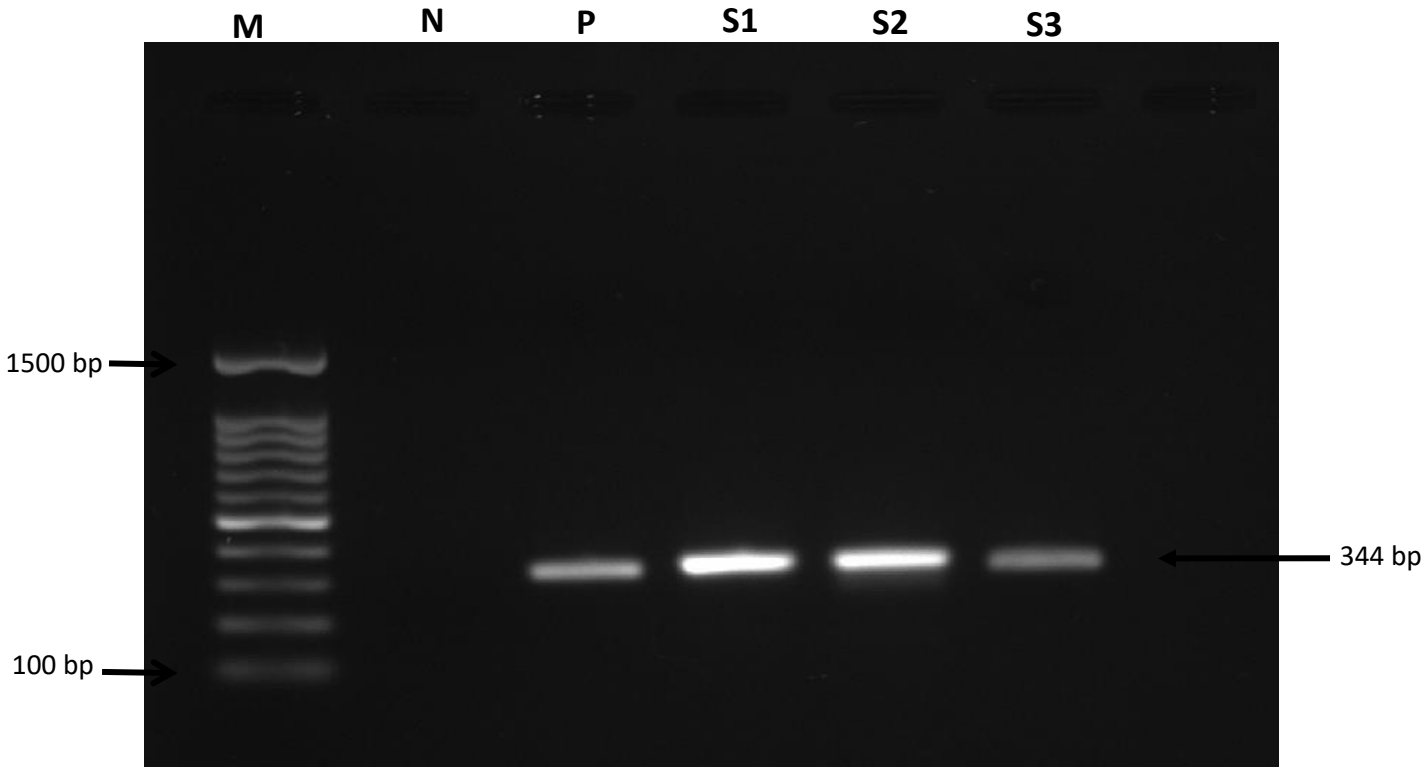

Figure 4.5: Gel electrophoresis results of NoV GII Capsid gene purified PCR product of 344 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study; lane S1: sample 22; lane S2: sample 24; lane S3: sample 27.

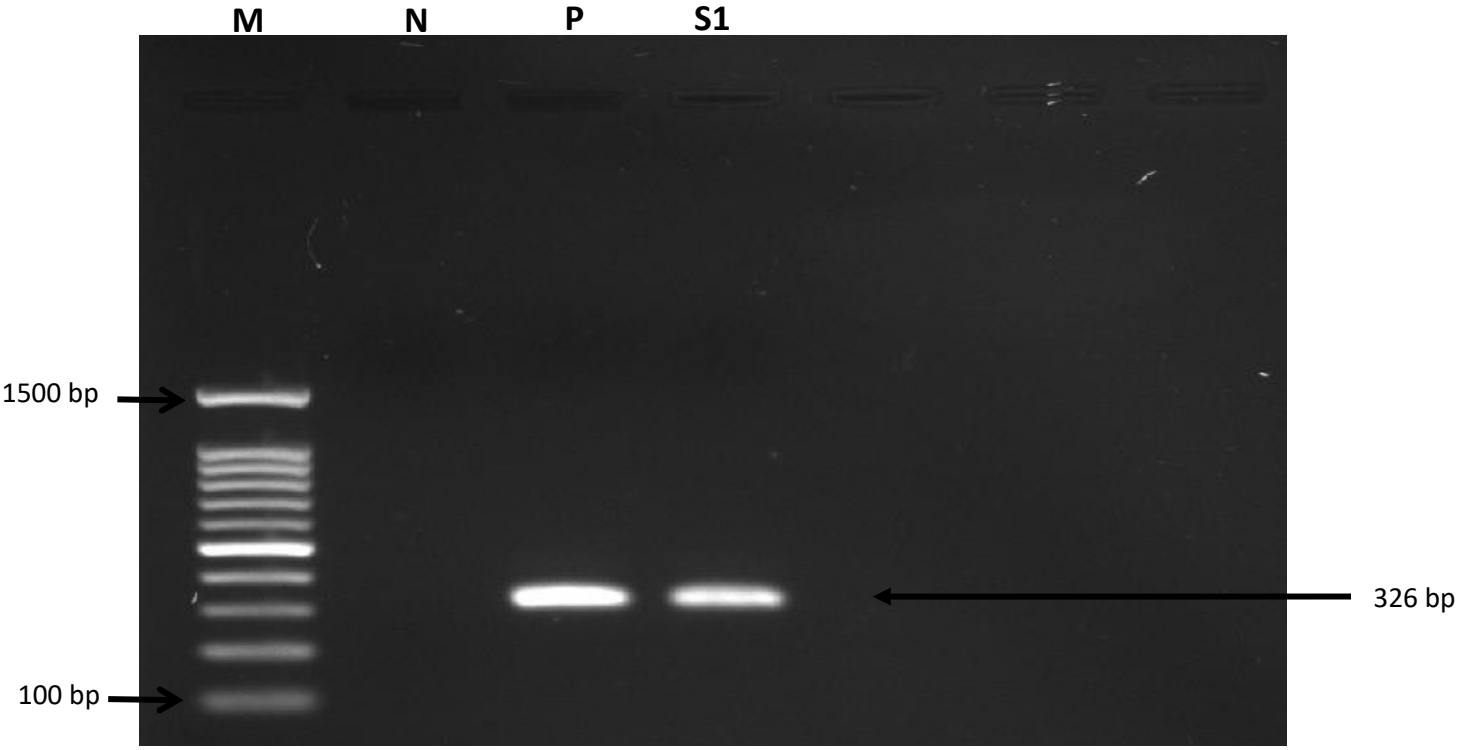

Figure 4.6: Gel electrophoresis results of NoV GII polymerase gene purified PCR product of 326 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control; lane S1: sample 22.

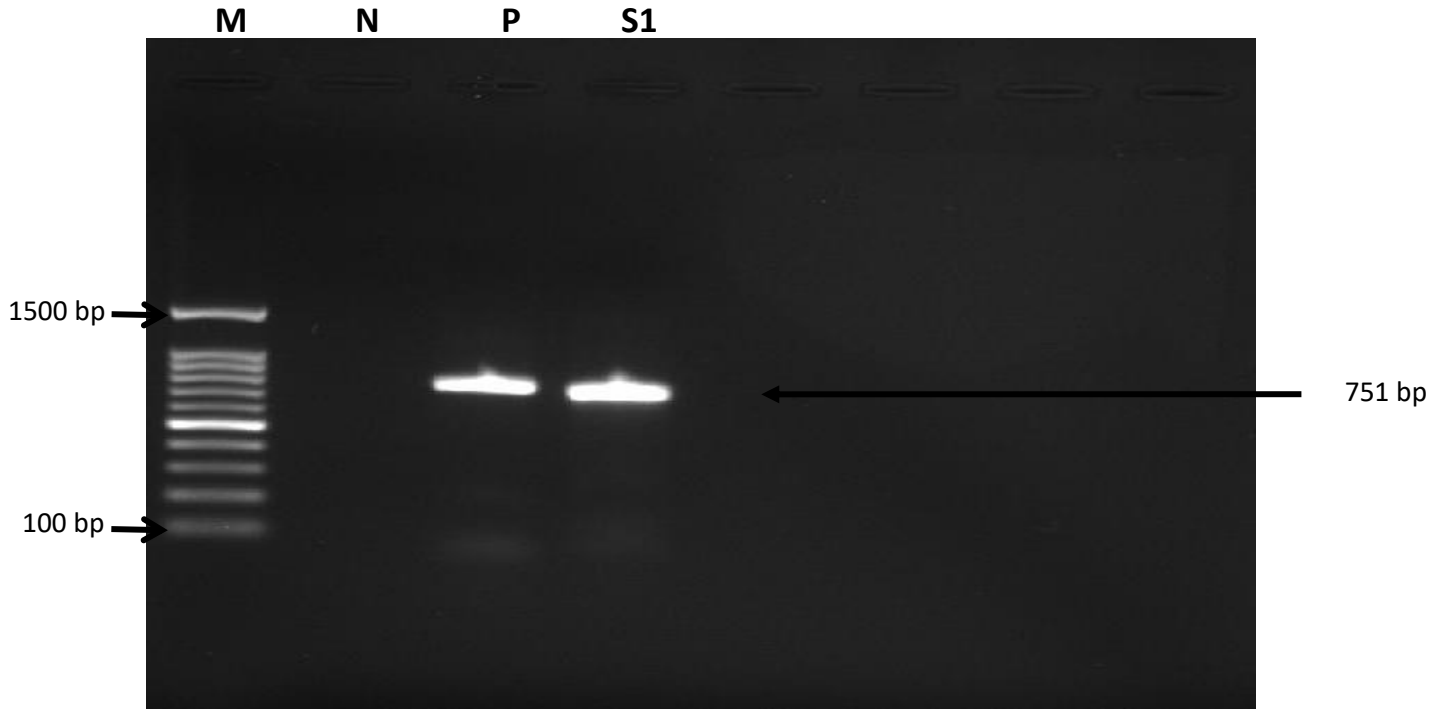

Figure 4.7: Gel electrophoresis results of NoV GII capsid gene purified PCR product of 751 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study; lane S1: sample 42.

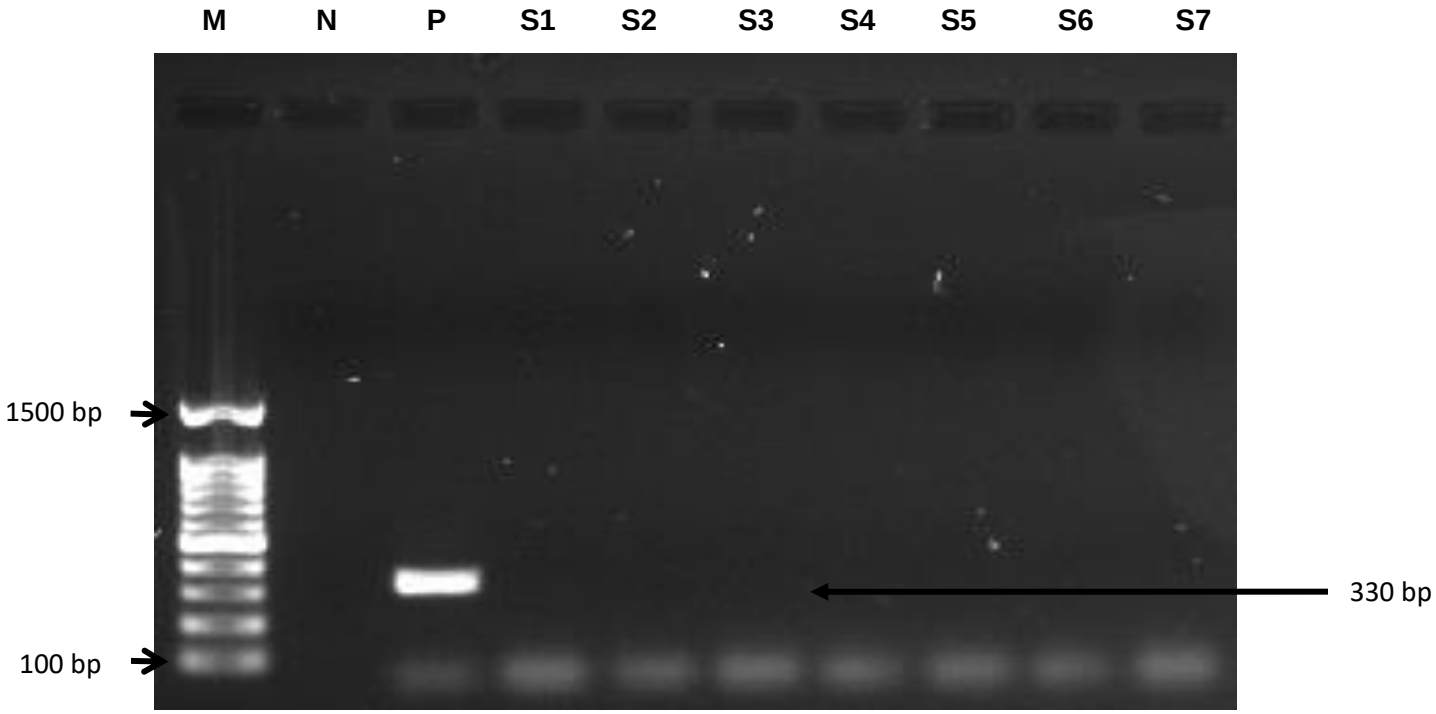

Figure 4.8: Gel electrophoresis results of NoV GI capsid gene PCR product of 330 bp analyzed by electrophoresis in 2% agarose gel. Lane M: 100-bp DNA ladder; lane N: Negative control; lane P: positive control from the previous study; lane S1: sample 10; lane S2: sample 22; lane S3: sample 24; lane S4: sample 26; lane S5: sample 26; lane S6: sample 38; lane S7: sample 63.

4.2.3 GENOTYPING RESULTS

Four (4/5, 80%) samples were successfully sequenced. Based on the capsid sequences and polymerase region of NoV GII, at least 4 genotypes were identified for GII (Table 4.3), consisting of:

- GII.1 (sample 22), GII.2 (sample 27) and GII.4 Sydney 2012 viriant (sample 24) for the sequence with only the capsid fragment available.

- GII.Pg (sample 22) for the sequences with only the RdRp fragment gene available.

- GII.Pg/GII.1 (sample 22) variant for the sequence with both the polymerase and capsid fragment. This is the putative recombinant form of the main findings of this study. However, further sequencing of the full junction region of ORF1/ORF2 is required to confirm the recombination.

Table 4.3: Genotype distribution of identified NoV strains in stool specimens between September 2017 and October 2018 in Rural communities of Vhembe district/South Africa.

Genogroup	Genotypes		Genotypes		Genotypes	
	RdRp	n (%)	Capsid	n (%)	RdRp/capsid	N (%)
GI		0(0)		0(0)		0(0)
GII	GII.Pg	1 (100)	GII.1 GII.2 GII.4[a]	1 (33.3) 1 (33.3) 1 (33.3)	GII.Pg/GII.1	1(100)
Total		1		3		1

[a] Predominant GII.4 Sydney 2012 variant

4.3 PHYLOGENETIC ANALYSES OF NOV GII CAPSID AND GII POLYMERASE GENOTYPES

To evaluate the genetic relationships among NoV-positive samples, representative phylogenetic trees based on partial nucleotide sequences of RdRp and capsid genes were generated by MEGA 7 software (Kumar et al., 2016) (Figure 4.9 for 326 bp of the RdRp fragment and Figure 4.10 for the 344 bp of a GII capsid fragment).

Besides the detection of capsid fragment for GI and GII, this study also went further to assess the combined characterization of both the capsid and polymerase regions of NoVs so to investigate the occurrence of recombination in NoV variants. The similarity of all the reference strains used in this study were as follow: (89-99%) for GI, (99%) for GII.Sydney 2012, (95-99%) of GII.1 and (99-100%) for GII.Pg genotype (Figure 4.9 and Figure 4.10). Though with the limited number of sequences from our sequencing set, the results of this study reveal a genetic diversity of NoV in diarrheal stool samples collected from patients living in rural communities of Vhembe district.

The GII.Pg NoV has been circulating before 2008 following an outbreak which occurred in Victoria in 1983 (Bruggink et al., 2016). GII.Pg genotype is associated with outbreaks in both healthcare and non-healthcare settings worldwide (Bruggink et al., 2016). Reports of the detection of GII.Pg norovirus in humans include studies in Australia (Dunbar et al., 2014; Lodo et al., 2014), Belgium (Mathijs et al., 2011), Spain (Arana et al., 2014), Taiwan (Tsai et al., 2014), Germany (Hoffmann et al., 2013), Italy (Medici et al., 2014), China (Sang et al., 2014), France (Loury et al., 2015), Tanzania (Moyo et al., 2014) and South Africa (Mans et al., 2014).

Results of this study have demonstrated that GII.Pg strain shares common ancestors with other common strains circulating around the world. In contrast, this strain (GII.Pg) is not clustered with other established genotypes as shown in Figure 4.9. The variation of GII.Pg strain with other circulating strains may be due to different field of setting (clinics located in different areas), patients enrolled and time interval for sample collection from this study. This indicates genetic variation and necessitate further analysis of the full genome of this strain.

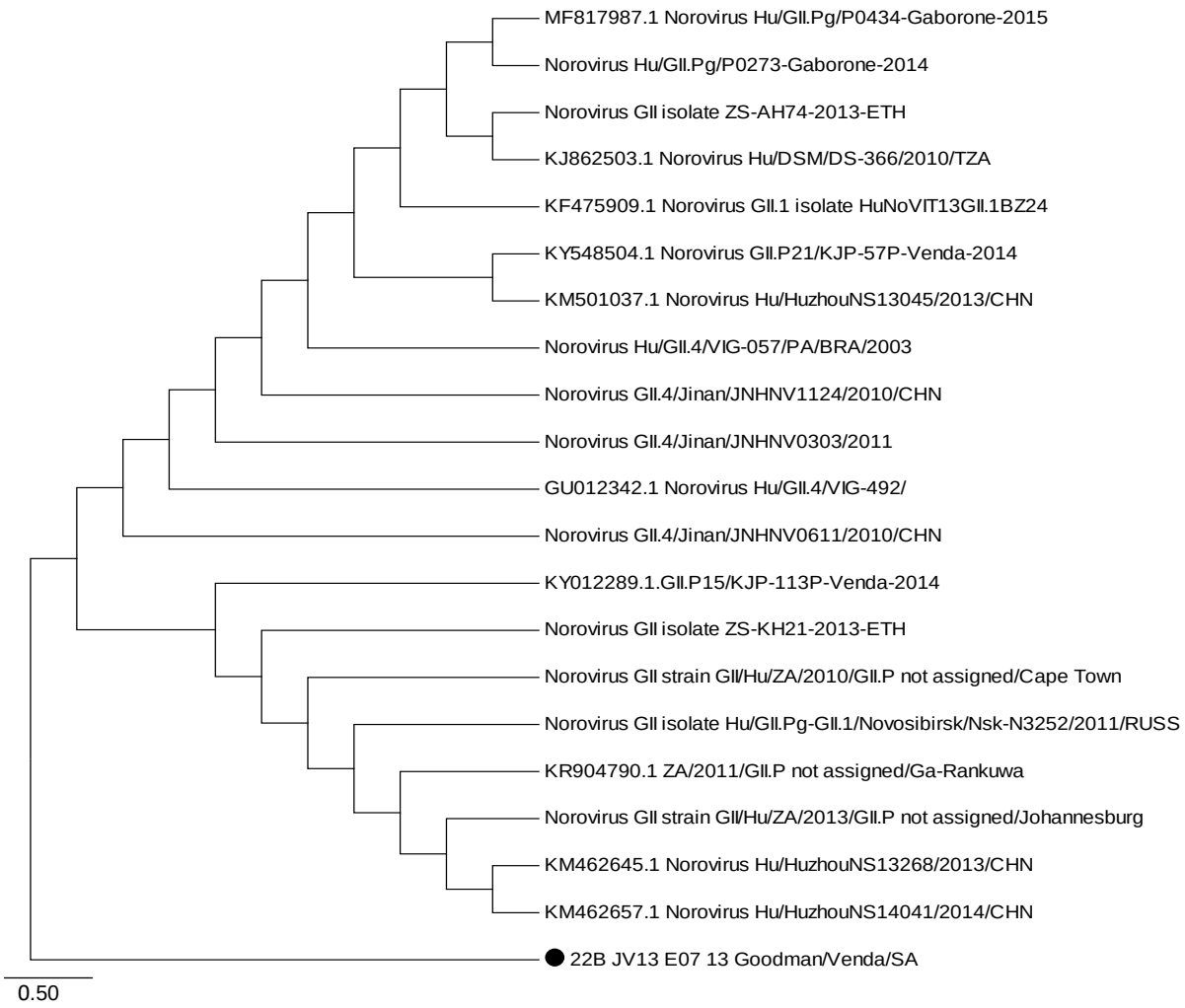

Figure 4.9. Phylogenetic tree based on 326-nucleotide sequence of the NoV GII polymerase gene fragment. The Neighbor-Joining tree of the GII capsid strains circulating between August 2017 and October 2018 in the rural communities of Vhembe district, Limpopo province/South Africa. Round Black dots indicated the GII.Pg polymerase genotypes from this study. All the bootstrap values were 100%. Twenty references strains of NoV were randomly selected from Genbank with their respectively accession numbers. Evolutionary analyses were conducted in MEGA7 and bootstrap tests (1000 replicates) based on the Kimura two-parameter model.

In the current study, NoV recombinant genotype (GII.Pg/GII.1) was identified in 1 out of 4 (25%) of the successfully sequenced amplicons (Table 4.3). The recombinant form of (GII.Pg/GII.1) have been previously reported in South Africa (Mans et al., 2016) and other countries such as France (Loury et al., 2015), Germany (Hoffmann et al., 2013) and Belgium (Mathijs et al., 2011). Combined characterization of both the capsid and polymerase regions is substantial to monitor the new recombinant strains and also new emerging NoV genotype worldwide.

GII.1 capsid genotype found in this study has been recently reported in Brazil (Fumian et al., 2019; Siqueira et al., 2017), Australia (Lun et al., 2018), India (Gupta et al., 2018) and elsewhere (Santos et al., 2017; Tao et al., 2015). This genotype is frequently reported from clinical cases and environmental samples (Fumian et al., 2019). However, the GII.1 variant obtained from this study shows a lower nucleotides sequence similarity with other GII.1 genotypes previously published elsewhere. In the phylogenetic tree, GII.1 clustered together with the recombinant form (GII.4 Sydney 2012) previously reported by Kabue et al (2016a) (Figure 4.10).

GII.2 variant reported in this study have been previously reported more common in hospitalised older children than in younger children (Bruggink et al., 2017; Zhirakovskaia et al., 2015; Sakon et al., 2014). Similarly, between 2016 to 2017, the GII.2 genotype was predominant in China (Liu et al., 2018) and Cameroon (Mugyia et al., 2018). This finding is in agreement with previous study done by Bruggink et al (2017) who reported the GII.2 as the common variant in children suffering from AGE. In addition, this study detected the GII.2 strain even in different age groups with close relationship with other GII.2 genotypes reported elsewhere (Figure 4.10).

During the last decades, GII.4 NoV has been the predominant genotype circulating in humans worldwide and was mainly associated with Acute gastroenteritis (AGE) outbreaks in people of all the age groups. Interestingly, our study also detected one of the major emerging variant (GII.4 Sydney 2012). This variant has a common ancestor with the NoV GII.4 variants Apeldoorn 2008 and New Orleans 2009 (Fonager et al., 2013; Van Beek et al., 2013). GII.4 Sydney 2012 variant was first identified in March 2012 in Australia. Since 2012, NoV GII.4 variant Sydney 2012 was reported to lead to an increase in NoV outbreaks in countries such as Australia, China, France, South Africa and the United States. However, based on the phylogenetic position of the GII.4 Sydney 2012 strain obtained in this study, this variant is not clustered with GII.4 variants previously reported in a study conducted by Kabue et al (2016a) in children <5 years in rural communities of Vhembe district (Figure 4.10). The recombinant form reported in this study might be due to viral evolution or mutation from previously reported recombinants in the Vhembe district (Kabue et al., 2016a).

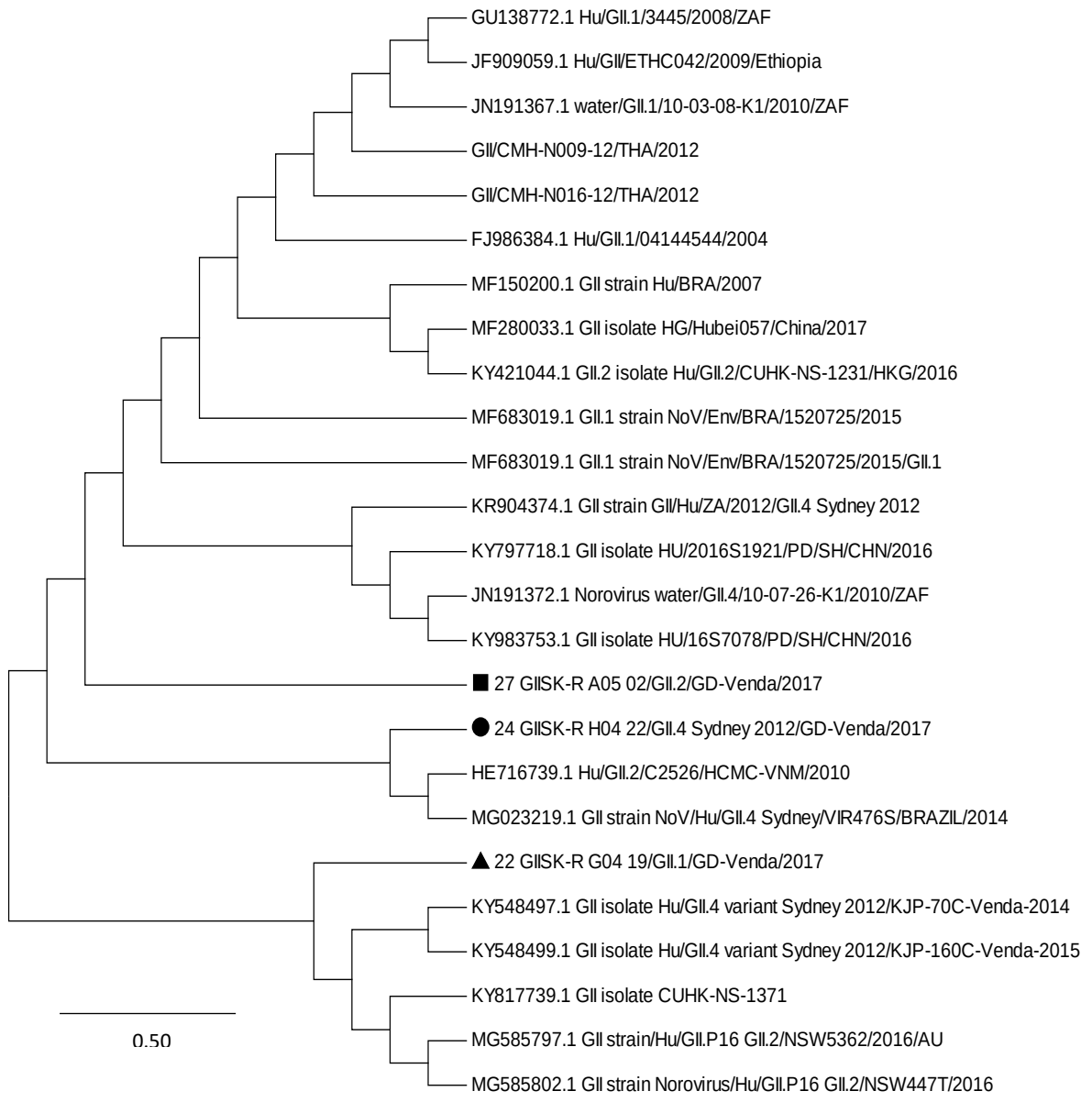

Figure 4.10: Phylogenetic tree based on 344-nucleotide sequence of NoV GII capsid gene fragment. The Neighbor-Joining tree of the GII capsid strains circulating between August 2017 and October 2018 in the rural communities of Vhembe district, Limpopo province/South Africa. Squared black dot indicated the GII.2 capsid genotype, round Black dot for GII.4 Sydney 2012 genotype and triangle black dot for GII.1 capsid genotype. All the bootstrap values were 100%. Twenty-two reference strains of NoV were selected from Genbank with their respectively accession numbers. Evolutionary analyses were conducted in MEGA7 and bootstrap tests (1000 replicates) based on the Kimura two-parameter model.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Though NoV is recognised worldwide affecting people of all the age groups, most of norovirus studies have been intensively reported in young children under 5 years of age leaving a gap knowledge about the burden of NoV in older children and adults. The aim of this study was to characterize human norovirus in patients with diarrhoea in rural communities of Vhembe district, Limpopo province. The results showed that NoV is circulating in patients above 5 years of age living in rural communities of Limpopo province. The findings revealed considerable multiple genotypes of norovirus circulating in rural communities of Vhembe district. Phylogenetic analysis showed that the GII NoV genotypes sequenced in this study shares common ancestors with other strains previously reported elsewhere associated with NoV outbreaks. The study findings suggest that norovirus vaccine should be targeting also individual above 5 years of age

Study limitations includes the following:

- **First,** Small sample sizes and sampling strategy in the present study was bias. Although sampling took place throughout a period of 12 months, the small number of diarrhoeal cases collected have limited our full ability to understand the seasonality of circulating strain and the role of NoV as a causative agent for Acute gastroenteritis (AGE) in our study population. This mostly due to the reluctance of adults to provide stool specimens for analysis.

- **Second,** diarrhoea can be caused by a variety of Gastrointestinal pathogens with similar clinical symptoms as those of the norovirus infection. However, it is possible that our enrolled patients experienced diarrhoea because of small number of other pathogens such as *Salmonella*, *Escherichia coli*, rotavirus, adenovirus, and sapovirus. Our study did not look at other enteric pathogens associated with diarrhoea because the focus was on norovirus prevalence.

- **Third,** only stool samples from patients with AGE was evaluated. Therefore, we do not know the occurrence of AGE pathogens in healthy or asymptomatic individuals.

- **Fourth:** only stool samples were collected. Environmental samples (toilets swabs and water samples) are needed to confirm the association with NoV.

- **Fifth,** norovirus infection is self-limiting. Therefore, Infants and young children became weak immediately after being infected, unlike in older children and adult patients were one can present with diarrhoea which then disappear after 1 or 2 days without individual going for a consultation. So, it is difficult to frequently encounter reported norovirus gastroenteritis in young adults in primary health care centers unless the infection is getting worse.

- **Sixth,** in this study, only partial genomic analysis was done. Full genomic sequencing enables more data on the viral recombination, evolutionary and relationships of NoV.

5.2 RECOMMENDATIONS

✓ This study provides, for the first time, the prevalence and genotypes of NoV strains circulating in adults living in rural communities of Vhembe district. However, it is of outmost importance that continuous surveillance and constant monitoring of circulating strain of NoV should be conducted to better understand the prevalence and genetic diversity of NoV in people living in rural communities of the Vhembe district. Further investigation to analyse the full genomes of NoV circulating strains are needed.

✓ Poor living conditions with lack of standard hygiene practices and lack of good/safe quality water which could play a major role as potential sources of introducing NoV infection into rural communities must be considered in future to control enteric pathogens.

✓ Practicing good parental care should be done to protect infants and young children from getting exposed to a contaminated environment and a wide variety of activities (e.g. swimming) to prevent NoV transmission.

✓ Since there is no antiviral therapy for NoV infection, the development of licensed vaccines may play an important role in preventing NoV infection among individuals who are vulnerable.

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Appendix 4

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

(DEPARTMENT OF MICROBIOLOGY)

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Tittle of the research project:

HUMAN NOROVIRUS IN RURAL COMMUNITIES OF VHEMBE DISTRICT, LIMPOPO PROVINCE.

Investigator: Mr G Mulondo, Hons MBY, Department of Microbiology (UNIVEN)

Study leader: Prof N Potgieter, PHD Virology, Department of Microbiology (UNIVEN)

Co-study leader : Prof AN Traore, PHD BCM, Department of Microbiology (UNIVEN)

Co-study leader: Dr JP Kabue,PHD MBY, Department of Microbiology (UNIVEN)

ADDRESS

Department of Microbiology

Life science Building

School of Mathematics and Natural Sciences

University of Venda

Contact Number: 015 962 8107

You are being invited to take part in this research project. Please take some time to read the information presented here, which will explain briefly the project. Please ask the study staff any question about any part you do not fully understand. **Your participation is entirely voluntary, and you are free to decline to participate.**

This study has been approved by the committee for human Research at the University of Venda. And will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for good practice and the Medical Research Council (MRC) ethical Guidelines for Research.

What is this research project study all about?

- ✓ The study will include stools sample from adult patients with diarrhoea
- ✓ The project aimed to characterize the circulating strains of Norovirus in rural communities of Vhembe district Limpopo province.
- ✓ This information will help decisions making in public prevention strategies against diarrhoea disease. The findings of this study will also assist in the development of vaccine.
- ✓ General information will be given to you, including contact details, gender, use of toilets, date of diarrhoea, HIV status and other illness e.t.c. Stool sample will be transported to the laboratory for the analysis.

Why have you been invited to participate?

You were selected for this study because you are suffering from diarrhoea

What will your responsibilities be?

Participation in this study is completed voluntary.

Will you benefit from taking part of this research?

No monetary compensation is offered for your participation. But you will be receiving the results of bacteriological and virological analysis if possible

Are there any risk involved in your taking part in this research?

there is no risk involved in participating. Collection of stools will be done after or when the participant is eliminating waste during diarrhoea episodes

who will access your medical records?

Only the medical doctor/nurse and the research team will have access to your medical information. The participants identity will not be made public and if the results are published or presented, a participant will only be referred to by code number. The participants identity will be strictly kept confidential.

Is there anything else that you should know?

You may contact Prof N Potgieter (University of Venda/Life Science Offices) at tel. 015 962 8256 if you have any further queries or encounter any problem.

DECLARATION BY PARTICIPANTS

By signing below**, I**…… Agree to take part in research studies entitled ‘’Characterization of human norovirus in adults with diarrhoea in rural communities of Vhembe District, Limpopo province**.**

I declare that:

- ✓ I have read or was read to me this information and consent form and it is written in a language with which I am fluent and comfortable
- ✓ I have had a chance to ask Questions and all my questions have been adequately answered
- ✓ I understand that taking part in this study is voluntary and I have not been pressurised to take part.

Signed at (place)…… on (Date)……..…….

…….. **……**

Signature of Participant **signature of Witness**

DECLARATION BY INVESRIGATOR(S):

I ……. Declare that:

- ✓ I explained the information in this document to the participant
- ✓ I encouraged the participant to ask questions and take adequate time to answer them
- ✓ I am satisfied that the participant adequately understands all aspects of the research, as discussed above.
- ✓ I did/did not use an interpreter (if an interpreter is used then the interpreter must sign the declaration below).

Signed at (place)…… on (date) ……..

…… **……..**

Signature of investigator **signature of witness**

DECLARATION BY INTERPRETER:

I …… Declare that:

- ✓ I assisted the investigator (name) ……..to explain the information in this document to (name of participant)…….. using the language medium of Venda/Tsonga
- ✓ We encouraged the participant to ask question and took adequate time to answer them
- ✓ I conveyed a factually correct version of what was related to me
- ✓ I am satisfied that the participant fully understands the content of this informed consent document and has all the question satisfactorily answered.

Signed at (place) ……..on (date) ……

……..Signature of interpreter

APPENDIX 5

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES (DEPARTMENT OF MICROBIOLOGY)

Thalutshedzo ya thandela ya thoduluso na fomo ya thendelo vharangaphanda vha thoduluso edzi.

Thoho ya thandela iyi:

HUMAN NOROVIRUS IN RURAL COMMUNITIES OF VHEMBE DISTRICT, LIMPOPO PROVINCE.

Investigator: Mr G Mulondo, Hons MBY, department of microbiology (UNIVEN)
Study leader: Prof N Potgieter, PHD Virology, Department of Microbiology (UNIVEN)
Co-study leader: Prof AN Traore, PHD BCM, Department of Microbiology (UNIVEN)
Co-study leader: Dr JP Kabue, PHD MBY, Department of Microbiology (UNIVEN)

Thoduluso ya vhulwadze hau tshuluwa na mbadelo dzi vhangwaho nga vhulwadze uvhu zwi tshi vhambedzwa na madi ane vhathu vha a shumisa mahayani na dzidoroboni.

Ri a vha ramba na u vha humbela u dzhenelela thoduluso dza thandela ya vhulwadze ha u tshuluwa ha vhathu vhahulwane. Tshipikwa tsha thandela iyi ndi u todulusa uri zwi durela hani muvhuso u thogomela vhathu vhano khou tshuluwa, na u todulusa nyimele ya vhulwadze uvhu zwibadela. Tsedzuluso idzi dzi dzhia tshifhinga tshisa padi 30 minutes. **Rido humbela zwitevhelaho:**

- ✓ U fhinduleliwa mbudziso dzi yelanaho na u vhu vhulwadze. Radovha hafhu ra humbela dzi tshika dza musi vhatshi khou tshuluwa hu u itela u todulusa zwithu zwovhangaho uho u tshuluwa.

Zwine zwa nga itisa uri vhasi dzhenelele:

- ✓ Vhatea uvha vhasa dzuli Vhembe District, Arali vhodi thusa lusa padiho luvhili kha duvha na musi Arali vho tshuluwa luno pada vhege.

Zwi vha thusa mini kha u dzhenelela:

- ✓ Vha do thusa muvhuso kha u kona u divha uri u shumisa vhugai khau lafha vhathu vha no khou tshuluwa.
- ✓ Muvhuso udodivha nyimele yavho khauvhu vhulwadze nahone tshumelo ya khwine ine ya fanela uya kha tshitshavha ido divhea.
- ✓ Vhado vhidziwa vha divhadzwa nga mvelele idzi

Ndugelo dzavho ndi dzifhio?

- ✓ A vha kombetshedziwi u dzhenelela thoduluso idzi
- ✓ U sa dzhenelela a zwi khakhisi tshumelo ine vha tea u iwana. U ya nga mulayo, u dzhenelela kha ngudo idzi azwi thithisi ndugelo dzavho
- ✓ Rivha thembisa uri mawana a thoduluso edzi a do vha tshiphiri kha rine na vhone nahone musi ritshi nwala dzi report dzashu dzina lavho alinga buliwi**.**

Thendelo:

Ndi khou tenda u dzhenelela tsedzuluso idzi nahone ndo talutshedzwa nda pfesesa uri thoduluso idzi ndi dza mini. Ndo vhala zwo nwalwaho afho ntha. Ndi a tenda uri a thongo kombetshedzwa u dzhenelela kha thoduluso idzi, ndo tou zwifuna nne mune. Ndo tendelwa u litsha tshifhinga tshinwe na tshinwe arali ndisa tsha funa u isa phanda. Ndo fhiwa tshifhinga tsho edanaho tsha u vhudzisa mbudziso. Ndi a tenda uri musi hutshi nwalwa dziripoto madzina anga ha nga buliwi.

…… …… …… ……

Dzina la Mudzheneleli **U saina** **Duvha**

…… …….. ……

Dzina la Mutodulusi **U saina** **Duvha**

Thendelo:

Ndi khou tenda u dzhenelela tsedzuluso idzi nahone ndo talutshedzwa nda pfesesa uri thoduluso idzi ndi dza mini. Ndo vhala zwo nwalwaho afho ntha. Ndi a tenda uri a thongo kombetshedzwa u dzhenelela kha thoduluso idzi, ndo tou zwifuna nne mune. Ndo tendelwa u litsha tshifhinga tshinwe na tshinwe arali ndisa tsha funa u isa phanda. Ndo fhiwa tshifhinga tsho edanaho tsha u vhudzisa mbudziso. Ndi a tenda uri musi hutshi nwalwa dziripoto madzina anga ha nga buliwi. A thi koni u nwala na u vhala, ndi humbela uri mutodolusi a ntsainele

…… …… …… ……

Dzina la Mudzheneleli **U saina** **Duvha**

…… …….. ……

Dzina la Mutodulusi **U saina** **Duvha**

Vhasa farea zwavhudi vhanga kwama muhulwane wa sibadela kana vhara ngaphanda vha thoduluso idzi vhane vha vha **Vho-Professor Natasha Potgieter kha 015 962 8000**

N.B: Rivha thembisa uri mawanwa a thoduluso idzi a do vha a tshiphiri kha rine nav hone. musi ri tshi nwala dzi repoto dzashu dzina lavho a li nga bulwi na luthihi.

APPENDIX 6

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

(DEPARTMENT OF MICROBIOLOGY)

Nhlamuselo ya projeke ya ndzavisiso na fomo ya mpfumelelano xikan'we na vurhangeri bya vulavisisi lebyi.

Thoho ya thandela iyi:

HUMAN NOROVIRUS IN RURAL COMMUNITIES OF VHEMBE DISTRICT, LIMPOPO PROVINCE.

Investigator: Mr G Mulondo, Hons MBY, Department of microbiology (UNIVEN)

Study leader: Prof N Potgieter, PHD Virology, Department of Microbiology (UNIVEN)

Co-study leader: Prof AN Traore, PHD BCM, Department of Microbiology (UNIVEN)

Co-study leader: Dr JP Kabue, PHD MBY, Department of Microbiology (UNIVEN)

Ha mi rhamba xikan'we na Ku mi kombela Ku nghenelela eka vulavisisi bya projeke ya vuvabyi bya Ku khoma hile ndzeni eka vanhu lavakulu. Xikongomelo xa projeke leyi I ku lavisisa leswaku mfumo wu durheliwa hi ndlela yihi Ku tlhogomela vanhu lava nga khomiwa hi vuvabyi bya le ndzeni na Ku lavisisa xiyimo xa vuvabwi lebyi eswibedlhele. Vulavisisi lebyi byi teka nkarhi lowu nga hundzeki makume-nharhu wa timinete. **Hita kombela leswi landzelaka:**

- ✓ Ku hlamuriwa eka swivutiso leswi swi yelanaka na vuvabyi lebyi. Ntlhadlakambirhi, hi kombela na thyaka leri humaka loko munhu a khomi hile ndzeni. Leswi i Ku endlela Ku hi kota Ku lavisisa xivangelo xa vuvabyi lebyi.

Leswi swi nga mi sivelaka ku teka xiave eka ndzavisiso lowu:

- ✓ A wu fanelanga kuva mutshami wa le Vhembe District. Loko munhu a ti pfunile Ku tlula kambirhi eka Siku rin'we xikan'we na Ku khomeka endzeni Ku hundza vhiki.

Mbuyelo wa ku va na xiave eka ndzavisiso lowu:

- ✓ Mi ta pfuna mfumo leswaku wu kota Ku tiva leswaku Ku nga tirhisiwa ntsengo wo tani hi kwihi Ku kota Ku tshungula vanhu lava nga hlaseriwa hi vuvabyi byale ndzeni.
- ✓ Mfumo wu ta tiva xiyimo xa wona eka vuvabyi lebyi naswona vukorhokeri byo antswa lebyi fanelaka vanhu byi ta tiveka.
- ✓ Mi ta vitaniwa mi tivisiwa hi mbuyelo wa ndzavisiso lowu.

Timfanelo ta nwina hi tihi?

- ✓ Ami sindzisiwi Ku nghenelela eka vulavisisi lebyi
- ✓ Loko mi nga ngheneleli aswi kavanyeti vukorhokeri lebyi mi fanelaka Ku byi kuma, hikuya hi nawu, Ku nghenelela ka dyondzo leyi a swi kavanyeti timfanelo ta nwina.
- ✓ Hi mi tshembhisa leswaku mbuyelo wa vulavisisi lebyi, wu ta va xihundla eka hina na nwina naswona loko hi tsala swiviko swa hina mavito ya nwana a ya nga paluxiwi.

Mpfumelelano:

Ndza pfumela Ku nghenelela eka ndzavisiso lowu naswona ndzi hlamuseriwile ndzi tlhela ndzi swi twisisa leswaku vulavisisi lebyi i bya mayelana na yini. Ndzi hlayile leswi nga tsariwa Laha henhla. Ndza pfumela leswaku a ndzi sindzisiwanga Ku nghenelela eka vulavisisi lebyi. Ndzi lo swi tsakela mina hi ndzexe. Ndza pfumeleriwa Ku tshika eka nkarhi wun'wana na wun'wana loko ndzi nga ha swi tsakeli Ku yisa emahlweni. Ndzi nyikiwile nkarhi wo ringanela Ku vutisa swivutiso. Ndza pfumela leswaku loko Ku tsariwa swiviko mavito ya nga a ya nga paluxiwi.

.....	…….	.. …….....
Vito ra mungheneleri	**Nsayino**	**Siku**
.....	…….....	…….....
Vito ra mulavisisi	**Nsayino**	**Siku**

Mpfumelelano:

Ndza pfumela Ku nghenelela eka ndzavisiso lowu naswona ndzi hlamuseriwile ndzi tlhela ndzi swi twisisa leswaku vulavisisi lebyi i bya mayelana na yini. Ndzi hlayile leswi nga tsariwa Laha henhla. Ndza pfumela leswaku a ndzi sindzisiwanga Ku nghenelela eka vulavisisi lebyi. Ndzi lo swi tsakela mina hi ndzexe. Ndza pfumeleriwa Ku tshika eka nkarhi wun'wana na wun'wana loko ndzi nga ha swi tsakeli Ku yisa emahlweni. Ndzi nyikiwile nkarhi wo ringanela Ku vutisa swivutiso. Ndza pfumela leswaku loko Ku tsariwa swiviko mavito ya nga a ya nga paluxiwi.

.....	…….	…….....
Vito ra mungheneleri	**Nsayino**	**Siku**
.....	…….....	…….....
Vito ra mulavisisi	**Nsayino**	**Siku**

Mi nga va mi nga khomekangi kahle, mi nga ti hlanganisa na Nkulukumbha wa xibedlhele kumbe vurhangeri bya ndzavisiso lowu lava va nga **Prof. Potgieter eka 015 9628000**.

APPENDIX 7

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES, UNIVERSITY OF VENDA

Research project data capture form: **Symptomatic patient**

Subject Number……

Consultation details			
Date:	Visit Number:	Hospital/Clinic name:	
Patient information			
Name _____ _	Date of birth _____ _	Gender M ☐ F ☐	Contact details: _____ _
Parental status:	Unemployed ☐	Employed ☐	Self-employed ☐
Family condition			
Water source:	Tap ☐	Spring/wells ☐	Boreholes ☐
Sanitation:	VIP/Pit latrine ☐	Flush toilet ☐	
Other :	Livestock ☐		
Medical History			
Clinical symptoms:	Diarrhea ☐	Fever ☐	Vomiting ☐ Dehydration ☐
	Abdominal pain/cramps ☐		
Date of Onset: _____			
How many days of presenting with diarrhea before consulting:			
Sample collection			
Date of collection:	_____ _		
Type of sample:	Type of Stool: Watery ☐	Sausage ☐	Mushy ☐
	Respiratory Swab: Nasal ☐	Throat ☐	
Treatment			
Current :			
Previous :			
Laboratory Results			
PCR:			
Sequencing:			

APPENDIX 8

BRISTOL STOOL CHART

APPENDIX 9
(BOOM METHOD FOR NUCLEIC ACID EXTRACTION)

Pea-sized amount faeces in 500 µl PBS (or less if small volume)

↓

Centrifuge for 15 sec @ 12,000 rpm

↓

Transfer supernatant in 1.5 ml sterile micro-tube + 900 µl L6 buffer

↓

Vortex for 1 min and centrifuge for 15 sec @ 12,000 rpm

↓

Transfer the supernatant to a new 1.5 ml tube

↓

Add extraction matrix (100 µl Silica beads)

↓

{***note*: Vortex the extraction matrix thoroughly before use**}

↓

Vortex the solution for 15 sec and shake softly for 15 min (or on Rocking platform)

Centrifuge the tube @ 2,000 rpm for 15 sec and discard the supernatant

↓

Add 500 µl of L2 buffer and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**X2**)

↓

Add 500 µl of 70% Ethanol and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**X2**)

↓

Add 500 µl of Acetone and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**X1**)

↓

Dry the Silica pellet by placing the opened tube in a heat block @ 50 °C for 5 min

↓

Re-suspend the Nucleic acid with 150µl PCR grade water,Mix well & heat@56°C for 5 min

↓

Centrifuge @ Maximum speed for 20 min

↓

Change the glove and collect carefully 100 µl of supernatant

(avoid collecting the silica pellet)

↓

Store the extracted Nucleic acid @ -80°C (or -20°C).

APPENDIX 10
(AGAROSE GEL FOR SEPARATING DEOXYRIBONUCLEIC ACID)