# MOLECULAR CHARACTERISATION OF NOROVIRUS STRAINS CIRCULATING IN RURAL COMMUNITIES OF THE LIMPOPO PROVINCE OF SOUTH AFRICA

**BY**

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# DECLARATION:

I, JEAN-PIERRE KABUE NGANDU (Student Number 11618547), declare that the thesis hereby submitted to the University of Venda for the degree PhD (Microbiology) has not previously in its entirety or in part been submitted to any University for a degree. I am the sole author of the abstract, introduction, literature review and summative comment sections and first author of the articles included in the thesis. The articles referred are open access papers from corresponding peer-reviewed journals.

Signed _____, this the _____day of _____2018

# DEDICATION

I dedicate this work to my Redeemer and Lord, Jesus-Christ.

Glory to him, to the Holy Spirit and Heavenly Father forever.

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# ABSTRACT

Globally, one in ten child deaths before the age of 5 years is due to diarrheal disease, causing almost 800,000 mortalities worldwide, which mostly occur in Sub-Saharan Africa and South Asia. Eighty-eight percent (88%) of diarrheal deaths worldwide are attributable to unsafe water, inadequate sanitation and poor hygiene. Unsanitary environments and poor hygiene practices allow diarrhea causing pathogens including viruses, bacteria and parasites to spread more easily.

Norovirus (NoV) are now considered the most common cause of outbreaks of nonbacterial gastroenteritis. However, the factors which control the genetic diversity, the sources of sporadic NoV infections, the transmission and persistence of infection are poorly understood. Limited data are available for NoVs strains in South Africa, especially in rural and peri-urban areas. Despite the excessive burden of diarrhea disease in developing countries, NoVs outbreaks have been to date mostly reported in developed countries. Given that the contribution of the various pathogens to diarrhea may differ substantially between regions depending on local meteorological, geographic, and socio-economic conditions, there is a need to investigate intensively the role of viral agents associated with diarrhea in different settings in Africa continent.

How would poor living conditions in rural setting impact the prevalence and genetic characteristics of Norovirus strains circulating Limpopo province is the research question of this study.

To determine the prevalence and genetic diversity of NoVs strains circulating in the rural communities in the Limpopo Province, South Africa and investigate the genetic relationship between NoVs strains, a cross-sectional study was performed on human stools collected from rural communities. We used qualitative variables of poor living environmental conditions including type of water used at the household of child's parent or guardian, use of toilet seat, presence of livestock at the household and parent employment status to assess possible environmental risk factors of NoV infection within the study area.

Prior to this prospective study, we conducted a systematic review of the PubMed and EMBASE databases for published articles of Human NoVs in Africa between 1990 and 2013 in order to assess the contribution of Human NoVs to diarrhoeal diseases in Africa. This review provides a picture of Human NoVs studies in Africa and reveals that unreported sporadic gastroenteritis cases of Human NoVs are common in Africa. Most are community-associated infections reported from urban settings. Possible environmental transmission routes have been documented. Combined environmental and clinical studies are required for targeted actions to control transmission of Human NoVs in Africa.

Between July 2014 and April 2015, outpatient children under 5 years of age from rural communities of Vhembe district, South Africa, were enrolled for the study. A total of 303 stool specimens were collected from those with diarrhea (n=253) and without (n=50) diarrhea. NoVs were identified using real-time one-step RT-PCR. Nucleotide sequencing methods were performed to genotype the strains. Phylogenetic analyses

were performed to compare identified NoVs genotypes to the worldwide circulating strains. One hundred and four (41.1%) NoVs were detected. NoV detection rates in symptomatic and asymptomatic children (OR = 1.24; 95% CI 0.66 – 2.33) were not significantly different. Comparison of the median $C_T$ values for NoV in symptomatic and asymptomatic children revealed significant statistical difference of estimated GII viral load from both groups, with a much higher viral burden in symptomatic children to our knowledge this is the first study reporting on the differences in estimated viral load of GII and GI NoV positive cases and controls. The study findings may have implications for the diagnosis of NoV disease and future vaccine development, which may only need to consider GII as the genogroup associated with diarrhea in the South African population.

Sequence analyses demonstrated multiple NoV genotypes identified in rural communities of Vhembe district. The most prevalent NoV genotypes were GII.4 Sydney 2012 variants (n=7) among the capsid genotypes, GII.Pe (n=9) among the polymerase genotypes and GII.Pe/GII.4 Sydney 2012 (n=8) putative recombinants among the RdRp/Capsid genotypes. Two unassigned GII.4 variants and an unusual RdRp genotype GII.P15 were found. With note, the rare GII.P15 identified in this study, has a common ancestor with GII.P15 strain from Japan previously reported as GII / untypeable recombinant strain implicated in a gastroenteritis outbreak. To our knowledge this is the first report of this unusual genotype in the African continent.

Though not proven predictive of diarrhea disease in this study, the high detection rate of NoV reflects the substantial exposure of children from rural communities to enteric

pathogens possibly. However in this study no risk factor has been found between NoV positive and qualitative environmental variables of poor living conditions in rural setting. The results also suggest that the difference between asymptomatic and symptomatic children with NoV may be at the level of the viral load of NoV genogroups involved.

The findings highlighted NoV genetic diversity and revealed continuous pandemic spread and predominance of GII.Pe/GII.4 Sydney 2012, indicative of increased NoV activity. An unusual RdRp genotype GII.P15 and two unassigned GII.4 variants were also identified from rural settings of the Vhembe district/South Africa. NoV surveillance is warranted to help to inform investigations into NoV evolution and disease burden, and to support on-going vaccine development programmes.

**Keywords:** Human Norovirus; Common; Symptomatic; Asymptomatic; Viral load; Norovirus genetic diversity; GII.4 variants; Out patients; Sporadic gastroenteritis; Africa; Rural communities.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **Calicinet** | Calicivirus Network |
| **CDC** | Centers for Disease Control and Prevention |
| **CHDC** | Children's Hospital National Medical Center in Washington, DC |
| **CL** | A single cysteine proteinase similar to the Picornavirus 3C. |
| **DC** | Dendritic cells |
| **DRC** | Democratic Republic of Congo |
| **EIA** | Enzyme Immunoassay |
| **ELISA** | Enzyme-linked Immunosorbent Assays |
| **EM** | Electron Microscopy |
| **FUT-2** | Fucosyltransferase 2 |
| **GI** | Genogroup I |
| **GII** | Genogroup II |
| **GIII** | Genogroup III |
| **GIV** | Genogroup IV |
| **GLASS** | Global Analysis and Assessment of Sanitation and Drinking-Water |
| **GV** | Genogroup V |
| **HBGAs** | Histo-Blood Group Antigens |
| **HIV** | Human Immunodeficiency Virus |
| **IFN-ɣ** | Interferon ɣ |
| **IgA** | Immunoglobulin A |

| | |
|---|---|
| **IgM** | Immunoglobulin M |
| **IL-2** | Interleukin 2 |
| **JMP** | WHO/UNICEF joint monitoring programme for water supply and |
| **LG** | A new matrix called after the authors, Le SQ and Gascuel O |
| **L-RT-qPCR** | A long-template one-step Taq Man assays / RT- quantitative-PCR |
| **MRC** | Medical Research Council |
| **MDG** | Millennium Development Goal |
| **NA-2** | Newbury Agent 2 |
| **NLV-BEC** | Norwalk-Like Virus-Bovine Enteric Calicivirus |
| **NoV** | Norovirus |
| **Noronet** | Norovirus network |
| **NTPase** | RNA nucleoside Triphosphatase |
| **NVRL/UCD** | National Virus Reference Laboratory / University College Dublin |
| **OH** | Ohio |
| **ORFs** | Open Reading Frames |
| **P1** | Protruding 1 |
| **P2** | Protruding 2 |
| **PCR** | Polymerase Chain Reaction |
| **PEG** | Polyethylene glycol |
| **PhML** | Phylogenetic Estimation Using Maximum Likelihood |
| **POL** | Polymerase gene |
| **PRO** | Proteinase |
| **RdRp** | RNA dependent RNA polymerase |

| | |
|---|---|
| **RNA** | Ribonucleic Acid |
| **RSA** | Republic of South Africa |
| **RT-PCR** | Reverse Transcription-PCR |
| **SRSV** | Small Round Structured Virus |
| **TH1** | T helper cell type 1 |
| **UNICEF** | United Nations Children's Fund |
| **USA** | United States of America |
| **VLPs** | Virus-like Particles |
| **VP** | Viral Protein |
| **VPg** | Viral genome-linked Protein |
| **WASH** | Water, Sanitation and Hygiene |
| **WHO** | World Health Organization |
| **WSH** | Water Sanitation and Health |

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# Chapter 1

# INTRODUCTION

A joint report on water and sanitation by WHO and UNICEF in 2012 indicated that an estimated 2.5 billion people lack improved sanitation facilities, and nearly one billion people in the developing world do not have access to safe drinking water (WHO-UN / WATER-GLASS, 2012). It is estimated that 88% of diarrheal deaths, almost all of which occur in poor countries, are attributable to unsafe water, inadequate sanitation and poor hygiene (Mara *et al*., 2010; UNICEF / WHO, 2009; Black *et al.,* 2003).

Sanitation, which is generally defined as the safe disposal of human excreta (WHO / UNICEF, 2010), is an important intervention in the prevention of disease transmission. Improved sanitation has significant impacts not only on health but also on social and economic development, particularly in poor countries (Mara *et al.,* 2010). The absence of adequate sanitation facilities forces people to defecate around their living environment and in rivers or streams resulting in pollution of the surroundings. This increases the risk of transmitting enteric pathogens (WHO, 2011; UNICEF / WHO, 2009; UNICEF, 2006). Children in households with no toilet access are twice as likely to contract diarrhea as those with lavatory provision (Mozynski, 2008). The key to controlling communicable diarrheal is hygiene, sanitation and safe

water (Bartram *et al.,* 2010). However, the provision of domestic sanitation, an important factor of health or disease in human populations, has been neglected in developing countries (Bartram *et al.,* 2010; Cairncross *et al.,* 2010; Yusuf, 1990). The implementation of better hygienic practices in developing countries can greatly reduce the burden of infectious diseases including infectious diarrhea (Gutiérrez *et al.,* 2011; Cairncross *et al.,* 2010).

Despite the significant achievements made as part of the Millennium development goal (MDG) targets worldwide between 1990 and 2015 (United Nations New York, 2015), progress has been uneven across regions and countries between urban and rural areas. There are a billion more people without improved sanitation in rural areas than in urban areas (9 out of 10 people who defecate in the open, live in rural areas (WHO, 2015) and throughout Africa alone, 115 people die every hour due to diseases linked to poor sanitation, poor hygiene and contaminated water (UNICEF, 2011; WHO, 2011; UNICEF / WHO, 2009; Boshi-Pinto *et al.,* 2008; UNICEF, 2006).

Globally, diarrhea remains the second most common cause of child mortality under five years of age, accounting for about 0.6 million of child deaths each year. Diarrhea kills more young children than malaria and measles combined (Figure 1.1; Liu *et al.,* 2015) and more than 80% of child deaths due to diarrhea occur in Africa and South Asia (Liu *et al.* 2015). Children under 5 years of age in developing countries experience the highest rates of illness and death due to diarrhea, but many cases are of unknown aetiology (Guerrant, 1994). In South Africa, unsafe water and lack of sanitation and hygiene are a major concern, particularly for children under the age of 5 years of age with 9.3% of total deaths in this age group attributable to unsuitable

water, sanitation and health (WSH) (Lewin *et al.,* 2007). In 2009, gastrointestinal tract infections were recognised as the leading cause of death among children under 5 years of age in the Limpopo Province of South Africa (National Department of Health / RSA, 2012). Ntuli *et al.* (2013) reported that diarrhea was one of the ten top leading causes of death in children under 5 at a tertiary hospital in Limpopo province of South Africa (Ntuli *et al.,* 2013).

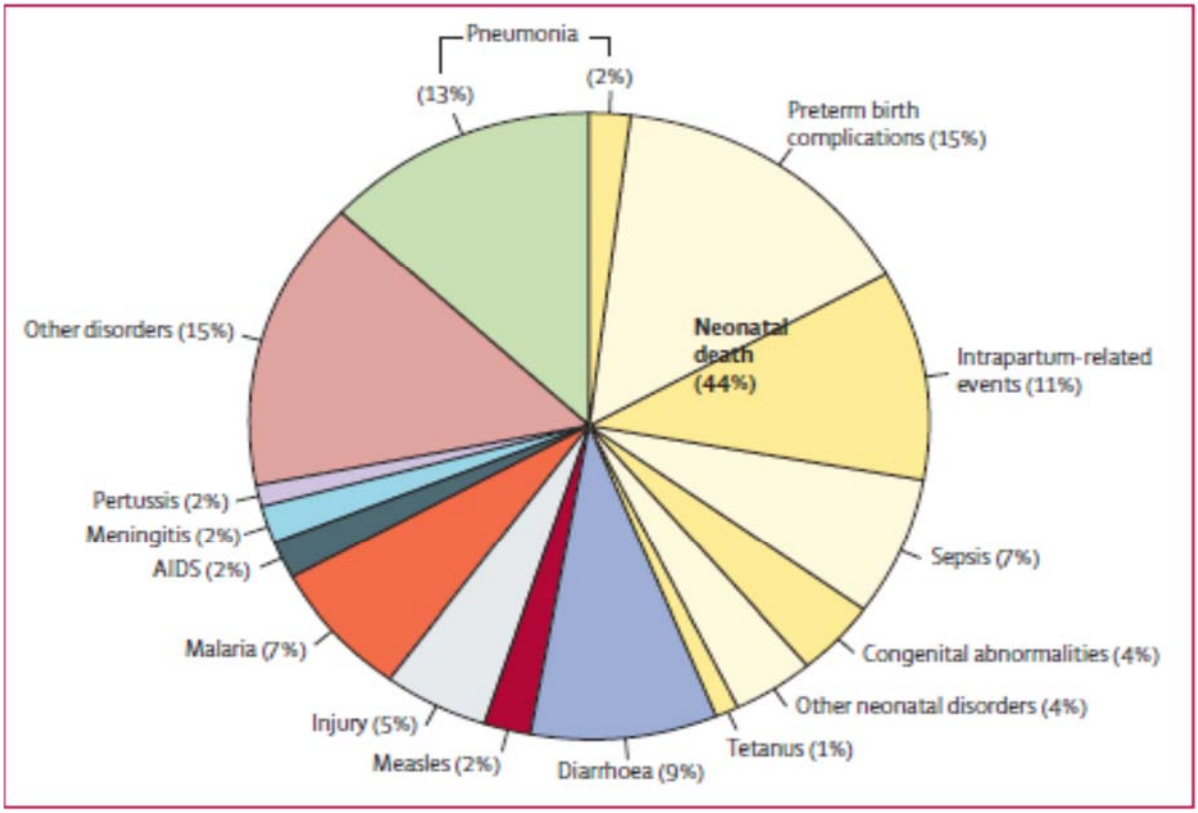



**Figure 1.1. Global causes of death among children under five years of age and neonatal cases in 2013. Proportional distribution of causes of mortality is depicted in this figure. (Liu *et al.,* 2015).**

Unsanitary environments allow diarrheal pathogens including viruses, bacteria and parasites, to spread more easily (UNICEF / WHO, 2009; UNICEF, 2006). The

viruses mainly involved in diarrheal diseases include: Rotavirus (Greenberg and Estes, 2009), Astrovirus (Mitchell, 2002); Adenovirus types 40 and 41 (Uhnoo *et al.,* 1990), and Norovirus (NoV) (Patel *et al.,* 2009). NoVs are now the leading cause of medically attended acute gastroenteritis in USA children (Payne *et al.,* 2013). With the recent success of Rotavirus vaccines, NoVs will likely become the most common cause of childhood diarrhea disease worldwide in the near future (Payne *et al.,* 2013; Bucardo *et al.,* 2011; Karst, 2010).

NoV transmission occurs usually from person-to-person via the faecal-oral route or fomites, but the importance of food and water in the disease spread is also well-recognized (Daniels *et al.,* 2000; Shieh *et al.,* 2000; Kukkula *et al.,* 1999). Contaminated water is frequently responsible for foodborne outbreaks and important in the direct dissemination of NoV to human hosts (La Rosa *et al.,* 2007; Beuret *et al.,* 2002).

Domesticated animals may also play a role in the transmission of diarrheal pathogens - the close interaction between humans and animals has been shown to be a major disease risk for humans (Grøndalen *et al.,* 2008; Matthijnssens *et al.,* 2008; Van Der Poel *et al.,* 2000). Previous studies from Papua New Guinea have revealed the increase of childhood diarrhea incidence in households with pigs (Wyrsch *et al.,* 1998; Bukenya and Nwokolo, 1991). Molecular analyses have shown a similarity between porcine, bovine and human strains, suggesting that an animal reservoir of NoV infection is possible (Scipioni *et al.,* 2008). Thus, pigs could play an important role in the potential zoonotic risk associated animal NoVs (Keum *et al.,* 2009; Wang *et al.,* 2007). This raises concerns about the possible emergence of

recombinant viruses between human and animal NoV strains as pigs are particularly ideal hosts in which viruses can easily mix and mutate (Shan *et al.,* 2011; Cheetham *et al.,* 2006). Virus recombination can affect phylogenetic groupings and increase the virulence of the virus with major implications for vaccine design (Bull *et al.,* 2007; Mattison *et al.,* 2007). Given the possible zoonotic risk from livestock, the countries where pigs and humans are kept closely should develop surveillance programs with screening of both human and animal NoVs in gastroenteritis outbreaks, water and animal samples (Mauroy *et al.,* 2008). Currently, not enough is known about the relative importance of diarrhoeal transmission from animals to humans in developing countries (Mak, 2004; Curtis *et al.,* 2000).

Presently, there is no vaccine against NoV infection and there are many challenges associated with their development. These include lack of small animal model and until very recently, an *in vitro* cell culture system for NoV (Ettayebi *et al.,* 2016). . There is also an incomplete understanding of the immune correlates of protection given the apparent lack of persistent long-term and cross-protective immunity, plus the existence of multiple genetic and antigenic types of the virus (Karst *et al.,* 2014; Herbst-Kralovetz *et al.,* 2013; Herbst-Kralovetz *et al.,* 2010; Vinje, 2010; Patel *et al.,* 2009; Tacket *et al.,* 2003). Virus-like particles (VLPs) produced by the expression and spontaneous self-assembly of the major capsid protein VP1 in recombinant systems including insect and plant cells, are being evaluated as a vaccine candidate (Ramani *et al,* 2017; Atmar, 2010; Koo *et al.,* 2010).

Due to the genetic and antigenic diversity of NoV strains, most attempts to develop diagnostic enzyme immunoassays have failed to achieve good sensitivity across the

wide range of different strains (Atmar *et al.,* 2001; Glass *et al.,* 2000). Molecular diagnostic assays based on conserved regions of the NoV genome are now broadly used in laboratories across the world (Xi *et al.,* 1990). However, the degree of genetic variability of NoV still makes it difficult to detect all strains with equal efficiency. Real-time RT-PCR for NoV is the most sensitive and specific method for rapid detection of NoV and can be used for large numbers of stools for epidemic and endemic gastroenteritis investigations (Trujillo *et al.,* 2006).

**Study rationale**

Noroviruses are the major cause of epidemic viral diarrhea affecting people of all ages (Marshall and Bruggink, 2011; Oldak *et al.,* 2009), and are responsible for at least 50 % of all gastroenteritis outbreaks worldwide, some of which may be foodborne (CDC, 2011b; Koo *et al.,* 2010). It is estimated that NoV may cause more than 1 million hospitalizations and up to 200000 deaths worldwide in children less than 5 years of age on an annual basis (Ahmed *et al.*, 2014; Patel *et al.,* 2008).

Despite the excessive burden of diarrhea disease in developing countries, NoV outbreaks have so far been mainly reported in developed countries (UNICEF, 2011; WHO, 2011). It is important to acknowledge that multiple continual exposures to enteric pathogens may reduce the chance of symptomatic disease.

Unreported sporadic infantile gastroenteritis cases of NoV are common in Africa and most of these are community-associated infections (Kabue *et al.*, 2016). In South Africa, little has been reported to estimate the actual prevalence of circulating NoV genotypes across the country (Kabue *et al.*, 2016; Platts-Mills *et al.*, 2015; Mans *et al.*, 2014; Mans *et al.,* 2013; Mans *et al.,* 2010).

The Limpopo Province in the North East region of the Republic of South Africa (RSA) is a rural region with a very poor economic status. Consequently, in this region, many communities still have inadequate sanitation and unsafe water. The close proximity of people to their livestock and the use of the same water sources with animals are common challenges to hygienic environments in these RSA rural areas (Potgieter *et al.,* 2010). Currently there is scarcity of available information on the disease burden of NoV in rural communities of RSA and the role played by unsafe water and unhygienic environment, inadequate sanitation and domesticated animals as putative reservoirs of the NoV strains.

**Research question**

The original research question of this study was to address how poor conditions in rural settings impact the molecular characteristics and transmission of circulating strains of NoV in Limpopo Province, RSA. The study hypothesis is that poor living conditions including poor hygiene practices and inadequate sanitation, unsafe water and polluted environment may influence the prevalence, genetic charcteristics and transmission of NoV strains circulating in rural communities of the Limpopo Province, South Africa. Information regarding the contribution of unsanitary environments and poor hygienic practices to NoV prevalence and diversity may help in public prevention strategies against diarrhea disease transmission with important implications for vaccine development.

**Aim and Objectives**

This study aimed to investigate the genetic characteristics of NoV strains circulating in the rural communities of the Limpopo Province / South Africa.

The objectives of the study were:

- to determine the prevalence of NoVs circulating in the rural communities of Limpopo Province;
- to determine the genetic diversity of NoV strains circulating in the rural communities of Limpopo Province;
- to examine the relatedness of NoV strains from rural communities of Limpopo Province to the worldwide circulating strains.

Using molecular techniques to detect and characterize NoV strains, this project is a cross-sectional study performed on stools collected at different clinics situated in the rural communities of Vhembe district in Limpopo Province. In this study we used the qualitative environmental variables defining poor living conditions including type of water used at the household of child's parent or guardian, use of toilet seat, presence of livestock at the household and parent employment status to explore possible risk factors of NoV infection by the means of statistical analyses (Kabue *et al.,* 2016b). To better understand the original research question of this study and motivate the rationale of the research study, this thesis also provides a review article on NoVs studies in Africa from 1990-2013. This review is presented in the third chapter of the thesis together with all other original enclosed research articles reporting the study findings. This is followed by a summative comment of the study objectives.

# Chapter 2

# LITERATURE REVIEW

## 2.1. Introduction

The advent of molecular diagnostic techniques and their extensive use in public health laboratories and research studies has considerably enhanced understanding of the burden of NoV infection. With the considerable decline of rotavirus-associated diarrhea in countries that have introduced rotavirus vaccines, NoV is increasingly documented as the leading cause of acute gastroenteritis in both children and adults (Ballard *et al.,* 2015; Pringle *et al.,* 2015; Ramani *et al.,* 2014; Hemming *et al.,* 2013; Payne *et al.,* 2013; Bucardo *et al.,* 2011). Despite this, the factors that influence genetic diversity, sources of sporadic NoV infection, transmission and persistence of infection are poorly understood and the role of non-human NoV reservoirs is still unknown.

NoV outbreaks have been widely reported in developed countries but only limited data are available for NoV strains circulating in the developing world. This overview aimed to provide a comprehensive summary of the available literature relevant to NoV infections.

## 2.2. Norovirus

### 2.2.1. History, Classification and Genomic Structure of NoV

In 1929, Zahorsky first coined the term ‘winter vomiting’ disease, characterized by a self-limited syndrome of vomiting and diarrhea, the incidence of which was noted to

peak during the cold months (Zahorsky, 1929). First recognized as an agent of viral gastroenteritis in 1969 (Adler and Zikl, 1969), NoV was later found by Kapikian *et al.* (1972), during analysis of infectious fecal filtrates derived from an outbreak of vomiting and diarrhoea in Norwalk, Ohio (Figure 2.1). Cloning of the Norwalk virus genome in 1990 led to the development of molecular diagnostic assays and a major step towards defining the global burden of NoV disease (Xi *et al.,* 1990). Norovirus (previously denoted as "Norwalk-like virus") is a genus in the family *Caliciviridae* with three other genera including Sapovirus, Lagovirus and Vesivirus (Green *et al.,* 2000). NoV is a single stranded, non-enveloped and positive-sense ribonucleic acid (RNA) virus (Jiang *et al.,* 1993; Jiang *et al.,* 1990). The genomic RNA and a subgenomic RNA, encoding the structural proteins, are formed via a negative sense intermediate (Figure 2.2; Atmar, 2010).

Structurally, NoV is a small virus with an approximate diameter of 38 nm. Its genome is 7.5 to 7.7 kb in length and contains 3 Open Reading Frames (ORFs) (Figure 2.2). ORF1 consists of six non-structural proteins that are involved in viral replication. These include p48, NTPase, p22, VPg, 3CL and an RNA-dependent RNA polymerase (RdRp) (Green *et al.,* 2007; Hardy, 2005; Figure 2.2). ORF2 translates the major structural capsid protein and ORF3 encodes a small virion-associated protein (Zheng *et al.,* 2006; Hardy, 2005). The major capsid protein referred to as Viral Protein 1(VP1) is made by the N-terminal domain (N), the shell domain (S) and the Protruding 1(P1) and Protruding 2 (P2) units (Figure 2.3; Green *et al.,* 2007).

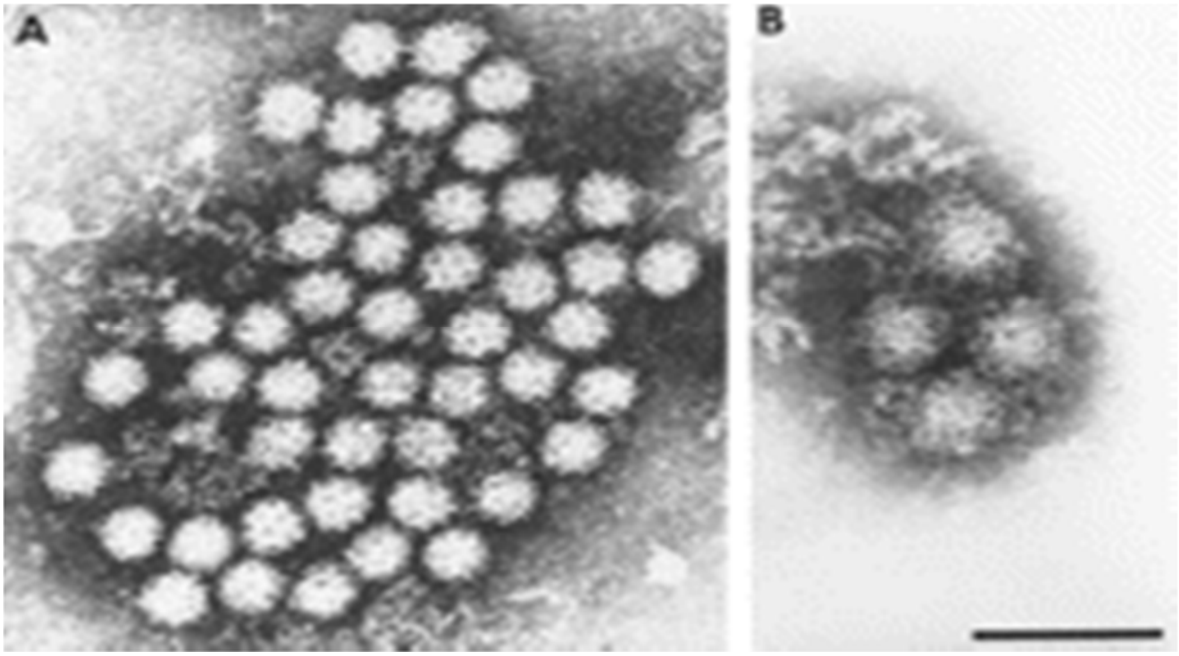



**FIGURE 2.1. Aggregate of Norwalk particles are depicted in this figure. A, Norwalk particules were observed after incubation of 0.8 ml of Norwalk stool filtrate with 0.2 ml of 1:5 dilution of prechallenge serum of volunteer A and further preparation for electron microscopy. B, Aggregate observed after incubation of 0.8 ml of Norwalk stool filtrate with 1:5 dilution of postchallenge serum from volunteer B and further preparation for electron microscopy. (Adapted from Kapikian *et al.,* 1972. J Virol 10(5): 1075-1081).**

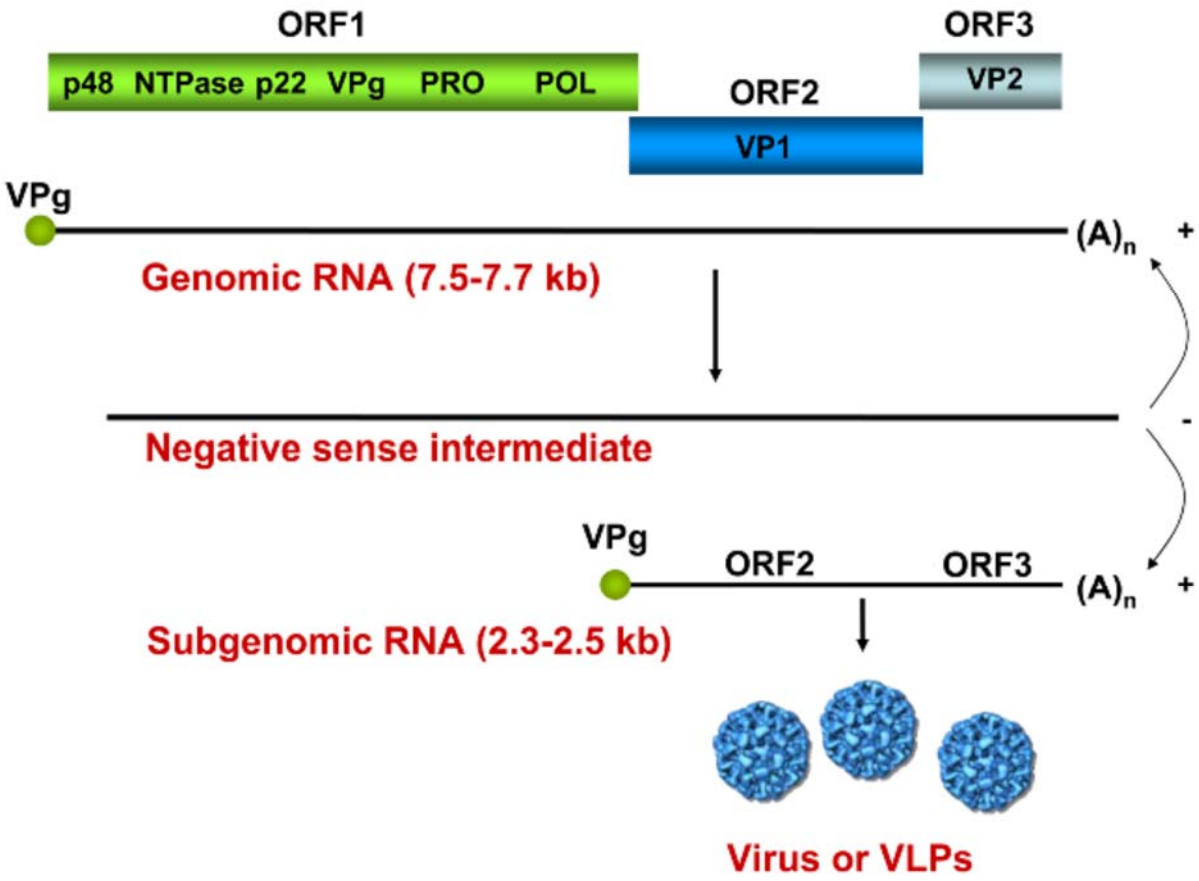



**FIGURE 2.2. Schematic illustration of NoV genomic organization and replication strategy. Depicted are: three ORFs including ORF1, ORF2 and ORF3. These ORFs encode a large polyprotein from which non-structural proteins (p48, NTPase, p22, VPg, PRO and POL) are produced by proteolytic cleavage and two structural proteins, VP1 and VP2. Replication takes place through a negative sense intermediate, from which the genomic RNA and a subgenomic RNA encoding the structural proteins are produced (Atmar, 2010. Food Environ Virol 2(3): 117-126).**

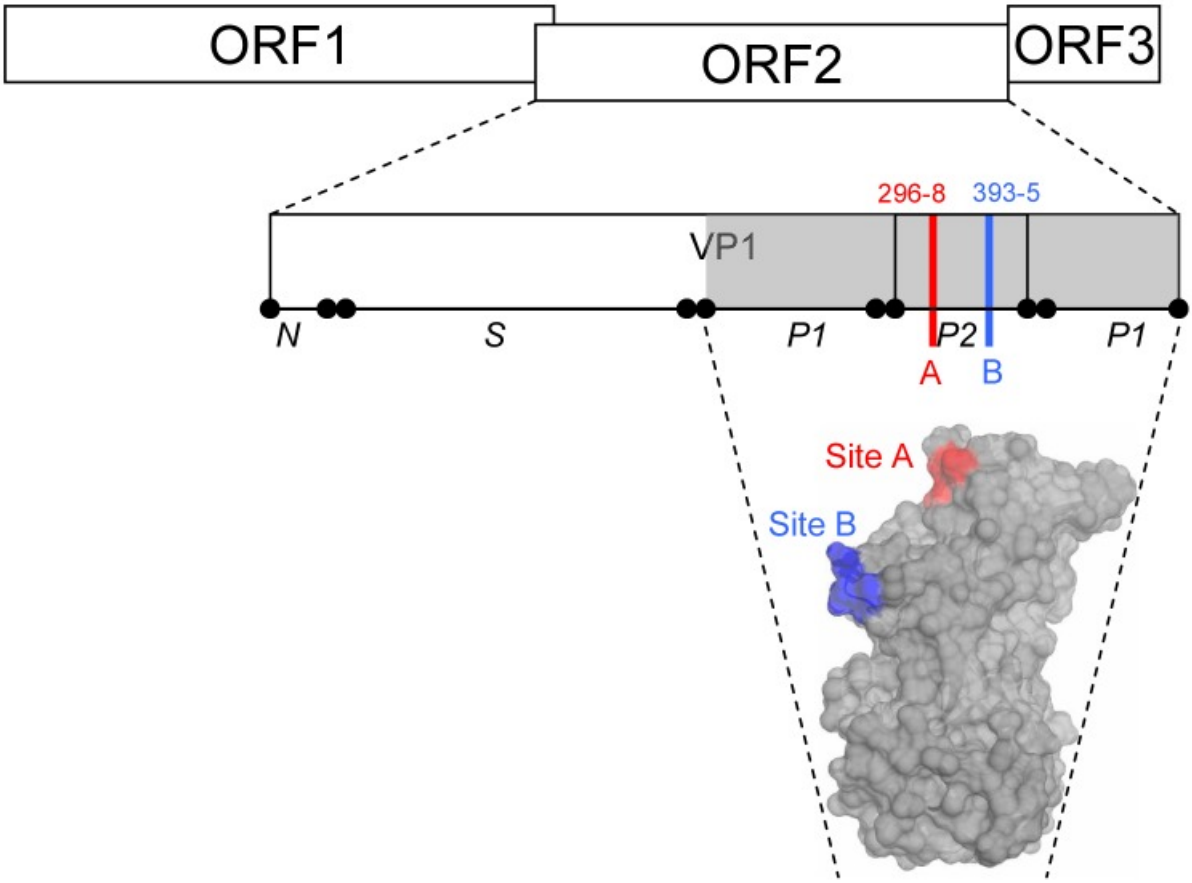



**FIGURE 2.3. Site A and Site B positions in the GII-4 Norovirus P2 domain. The major capsid protein depicted in this figure is encoded by ORF2. The VP1 protein has three main domains: an N-terminal domain (N), the highly conserved shell domain (S), and the protruding domain (P) which forms surface exposed spikes on the virus surface, which is further subdivided into hypervariable P2 domain (P2) and the more conserved P1 domain (P1) (Zakikhany *et al.*, 2012. PLoS One 7(7): e41625).**

### 2.2.2. Genetic Diversity and Recombination of Norovirus

#### 2.2.2.1. Genetic Diversity of Norovirus

The NoV genogroups and genoclusters are designated numerically, with the genogroup indicated first as a roman numeral followed by the genocluster number. Strains within a genocluster can be also further subdivided based on sequence differences in ”genotypes” (Lindesmith *et al.,* 2008). NoV strains are often named according to the location of the identified outbreak caused by individual isolates and the year they were identified, such as NV/93/USA.This is no longer recommended but a lot of isolates identified previously have been named in this way.

Based on amino acid identity in the major structural protein (VP1) (Ramani *et al.,* 2014; Zheng *et al.,* 2006), the seven genogroups of NoV are subdivided in 41 genotypes with 9 genotypes in GI, 22 in GII, 3 in GIII, 2 each in GIV-VI and one in GVII (Vinje, 2015; Ramani *et al.,* 2014; CDC, 2011b; Green *et al.,* 2007; Zheng *et al.,* 2006; Figure 2.4). The strains that infect humans (referred to commonly as ‘’human Norovirus’’) are found in GI, GII, GIV whereas strains infecting cows, mice and dogs are typically GIII, GV and GVI (Ramani *et al.,* 2014; CDC, 2011b; figure 2.4). GII NoV has been also linked to pigs and cattle; GIII NoV to cattle and sheep; GIV NoV to a lion and dogs and GV NoV to mice (Patel *et al.,* 2008).

Based on RNA-dependent RNA polymerase and capsid gene sequences, 31 polymerase genotypes and 22 capsid genotypes of GII NoV have been determined to date (Kroneman *et al.,* 2013). Of these, GII.4 is the predominant, causing at least

six epidemics of gastroenteritis worldwide over the past 20 years (1995–1996, 2002, 2004, 2006, 2009, and 2012) with the emergence and rapid global spread of viral variants (Karst and Baric, 2015). However, it has been also documented that there is a high degree of genetic diversity worldwide with non GII.4 genotypes such as GII.6, GII.3, GII.2 and GII.8 becoming more common in young children with sporadic NoV gastroenteritis (Chan *et al.,* 2015; Hoa Tran *et al.,* 2013).

Full-length genomic sequence analyses of NoV strains have shown that within a genogroup the viral strains share 69-97 % nucleotide similarity, while strains in different genogroups are only 51-56 % similar (Kojima *et al.,* 2002). More interestingly, the ORF2 major capsid protein sequence can diverge by as much as 60% between genogroups and ~20-30% between genotypes within a genogroup while partial viral capsid sequences are often very similar within a genocluster misjudging the degree of genetic diversity (Donaldson *et al.,* 2008; Lindesmith *et al.,* 2008). Phylogenetic analyses of NoV strains are usually performed using the full-length capsid sequence (Lindesmith *et al.,* 2008; Katayama *et al.,* 2002) the partial sequence may underestimate the degree of heterogeneity.

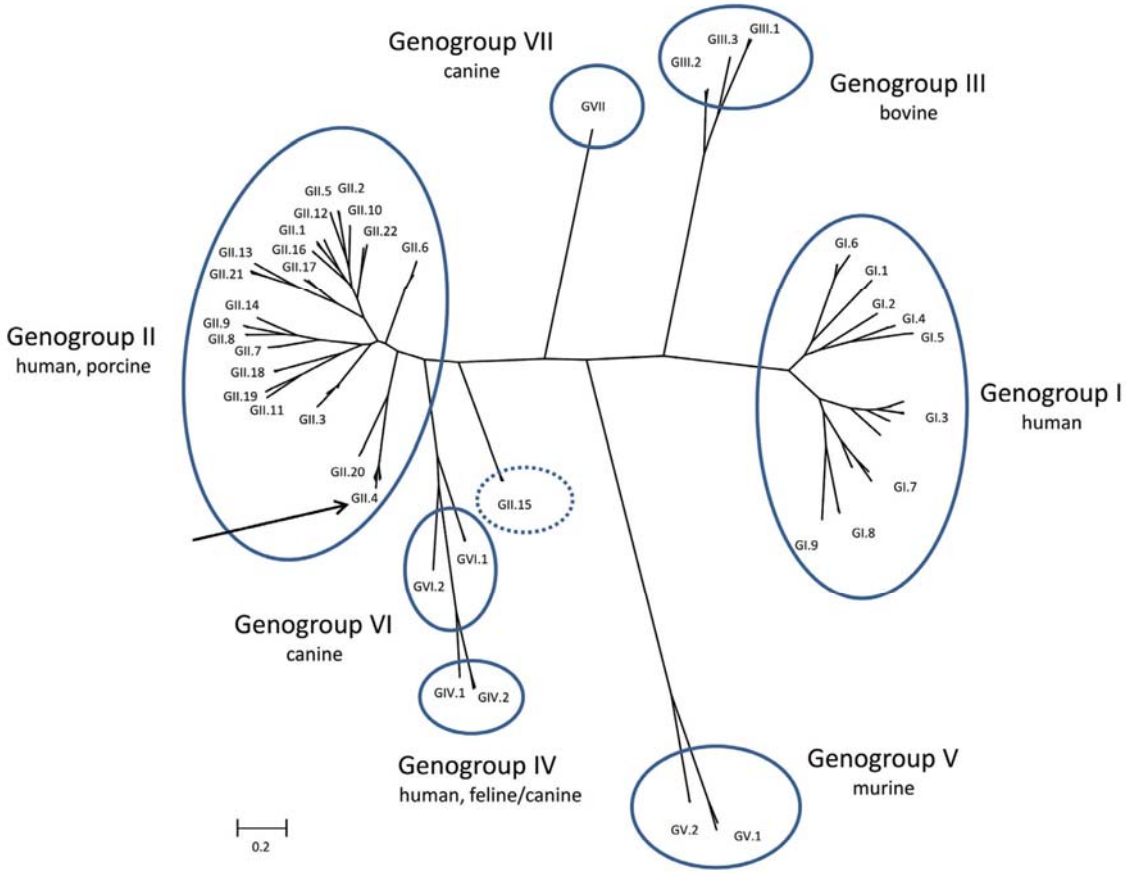



**Figure 2.4. Classification of noroviruses into 7 genogroups (GI to GVII) based on amino acid sequence diversity in the complete VP1 capsid protein. Adapted from Vinjé, 2015. J Clin Microbiol 53(2): 373-381.**

### 2.2.2.2. Recombination of Norovirus

The NoV genome varies frequently by mutation (Dingle *et al.,* 2004) and recombination (Rohayem *et al.,* 2005). Recombination takes place when "donor" nucleotide sequence is introduced into a single, "acceptor" RNA molecule to produce new RNA-containing genetic information from more than one source (Worobey and Holmes, 1999). A recombinant NoV is defined as one that clusters with two distinct groups of NoV strains when two different regions of the genome (often the capsid

and polymerase) are subjected to phylogenetic analysis (Bull *et al,* 2005). Recombination is a common event in RNA viruses and drives their evolution by genetic variation and by generating new viruses that can escape an early immune recognition (Bull *et al.,* 2007; Nayak *et al.,* 2008; Phan *et al.,* 2006a; Tsugawa *et al.,* 2006; Bull *et al.,* 2005; Worobey and Holmes, 1999).The recombination point of NoV is commonly found at the ORF1/2 overlap region and the capsid gene (Bull and White, 2011; Motomura, 2010; Bull *et al.,* 2007; Ambert-Balay *et al.,* 2005; Rohayem *et al.,* 2005; Han *et al.,* 2004).

Virus recombination can change phylogenetic groupings, confound molecular epidemiological investigations and hamper vaccine development (Bull *et al.,* 2007). Increased awareness has led to several reports of novel recombinants worldwide (Ambert-Balay, 2005; Bull *et al.,* 2005; van den Berg *et al.,* 2005), however, there is no accepted classification system for recombinant NoV. This has led to difficulties in defining the number and types of circulating recombinants (Bull *et al.,* 2007).

In addition to recombination, GII strains frequently develop genetic polymorphisms and these altered forms are called “variants” or “subtypes” (Bull *et al.,* 2006). The amino acid sites A and B of GII.4 capsid sequences (Figure 2.3) show both significant and neutral amino acid changes according to Zakikhany *et al* (2012) in an epidemiological study conducted in England between 2000 and 2011. Amino acid substitutions can occur concurrently at sites A and B, which results in cluster transition and the emergence of epidemiologically significant variants with the potential to cause outbreaks and epidemics (Allen *et al.,* 2008; Siebenga *et al.,* 2008) possibly what is later referred to as antigenic drift (Zhang *et al.,* 2015).

### 2.2.3. Host susceptibility, Immunology and Pathogenesis of NoV

Most pathogenesis and immunology data relating to NoV infection is from human volunteer studies because of the lack of animal models and difficulty of NoV *in vitro* cultivation (Ettayebi *et al* 2016). Currently, knowledge on pathogenesis of NoV infection comes from physical, histological and biochemical studies of infected human volunteers (Karst, 2010).

Karst (2010) reports that pathogenesis of NoV infection is characterized by:

1. Histological alterations of the intestine: Infected human volunteers that become ill after NoV challenges exhibit an intact intestinal mucosa with specific histological alterations. NoVs may also cause apoptosis of enterocytes in humans, pigs and mice.
2. Physical and biochemical manifestations: There is a considerable delay in gastric emptying due to the high incidence of vomiting episodes and reduced activity of specific enterocyte enzymes such as alkaline phosphatases, sucrases and lactases.
3. Systemic infection: It is possible that NoV uses dendritic cell (DC) infection to facilitate extra-intestinal spread.

Early volunteer studies revealed that upon NoV infection, infected volunteers develop immunity that appeared short-lived (from 8 weeks to 6 months) (CDC, 2011; Karst, 2010; Patel *et al.,* 2009; Johnson *et al.,* 1990) and strain-specific (Vinje, 2010; Estes *et al.,* 2006; Huang *et al.,* 2005). After a NoV challenge, virus-specific serum IgG is induced and persists for months while IgA and IgM responses are more short-

lived (Karst, 2010). Cell-mediated immunity may be important for clearance of NoV infection (Green, 2014; Lindesmith *et al.,* 2011b; Saif *et al.,* 2011; Wingfield *et al.,* 2010) - a predominant T helper cell 1 (TH1) immune response, characterised by a predominance of interferon gamma (IFN-ɣ) and interleukin 2 (IL-2) cytokines, has been demonstrated in human hosts infected with NoV. However, because cell culture systems for human NoV infection have only recently been achieved (Ettayebi *et al* 2016), the immune correlates of protection are not well defined (Atmar *et al.,* 2011; Vinje, 2010). It has not been possible to determine if the presence of NoV-specific antibodies relates to the susceptibility of some previously exposed individuals to repeat NoV infections (Karst, 2010; Agus *et al.,* 1974).

NoV infection involves the lamina propria in the proximal portion of the small intestine (Hutson *et al.,* 2002). Most NoV genotypes recognize the type 1 and 2 carbohydrate cores of the HBGAs (histo-blood group antigens) (Shirato, 2011), expressed on the surfaces of red blood cells and commonly in the gut (Hutson *et al.,* 2002). Thus, differing host HBGA genotypes can determine susceptibility to NoV infection (Lindesmith *et al.,* 2003). Several enzymes are critical in the synthesis of HBGAs, including FUT-2 and it has been shown that changes in the FUT-2 gene can render them non-functional and make a person resistant to NoV infection (Vinje, 2010). HBGAs which include H type, ABO blood group, and Lewis antigens have been proposed as candidate receptors for NoV (CDC, 2011b; Tan and Jiang, 2011; Hutson *et al.,* 2004; Huang *et al.,* 2003). The strain-specific binding and the variable expression of the HBGA receptors may explain the unpredictable host susceptibility observed in NoV outbreaks (Huang *et al.,* 2005; Hutson *et al.,* 2003; Hutson *et al.,* 2002) and confound investigations relating to NoV immunity, since persons without antibody to a particular NoV may lack genetic susceptibility to the infection (Patel *et*

*al.,* 2009). Sequence analysis of the NoV binding region indicates that the polymorphic human HBGAs are likely to have been a driving force for the divergence of human NoV genotypes (Tan and Jiang, 2010).

The major capsid protein VP1 is involved in the recognition of the host receptor (Tan and Jiang, 2010; Scipioni *et al.,* 2008). The P2 ligand of the viral capsid is the natural binding site with human HBGA, which may be the point of initial viral attachment (Donaldson *et al.,* 2008; Cao *et al.,* 2007). Amino acid changes in the P2 domain allow the virus to re-infect an individual (for example, by host cell receptor switching) and thereby escaping immunity (Donaldson *et al.,* 2008; Green, 2007). There is now some evidence that a small genetic change in the capsid region can have a big influence on NoV virulence; using an infectious cDNA clone of the attenuated virus, Bailey *et al*. (2008) restored murine NoV *in vivo* virulence by a glutamate-to-lysine substitution at position 296 in the capsid protein (VP1). Antigenic drift and receptor switching may act synergistically in GII.4 NoV to overcome human herd immunity (Bull and White, 2011; Lindesmith *et al.,* 2008).

### 2.2.4. Transmission of NoV

Humans are the only known reservoir for human NoV infections (CDC, 2011b) and transmission occurs by three general routes: person-to-person, foodborne, and waterborne. Person-to-person transmission might occur directly through the faeco-oral route, by ingestion of aerosolized vomitus, or by indirect exposure via fomites or contaminated environmental surfaces (Lopman *et al.,* 2012). Evidence of contamination of environmental surfaces with NoV has been documented suggesting

that this may serve as a reservoir in outbreak situations (CDC, 2008). Foodborne transmission occurs as a result of contamination from infected food handlers or contamination with human waste, in the case of filter-feeding shellfish (Le Guyader *et al*., 2009). Finally, recreational and drinking water can serve as vehicles of NoV transmission (Yoder *et al*., 2008).

Norovirus are well adapted pathogens for transmission within human populations. Several characteristics of NoV facilitate their spread. These include:

1. An extremely low infectious dose (≥ 18 viral particles), coupled with copious viral shedding ($10^5$-$10^{11}$viral copies per gram of faeces), even in asymptomatic infections (Hall 2012; Teunis *et al.,* 2008; Atmar *et al*, 2008).
2. Environmental stability; able to survive both freezing and heating, plus resistance to many common chemical disinfectants (such as chlorine and ethanol) and can persist on surfaces for up to 2 weeks (Hall 2012; Seitz *et al.,* 2011; Park *et al.,* 2010; Patel *et al.,* 2009; Duizer *et al.,* 2004).
3. The multiple ways in which NoVs may spread, including direct contact between hosts via fecal-oral transmission, ingestion of contaminated foods or water, handling of contaminated fomites followed by hand-to–mouth contact, and ingestion of aerosolized particles (CDC, 2012; Lopman *et al.,* 2012; Patel *et al.,* 2009)
4. Genetic diversity and rapid evolution, leading to an apparent lack of prolonged cross-protective, long-term immunity following infection and repeated infections throughout life with re-exposure (Hall 2012; Patel *et al.,* 2009).
5. The wide human host range, affecting susceptible persons of all ages from neonates to the elderly (Hall *et al*., 2011; Rockx *et al.,* 2002).

Companion animals, due to the close contact with their owners, may also be a readily accessible source of NoV transmission. Molecular analyses have revealed the close genetic relationship of NoV found in animals and humans. The sequence similarity of porcine and bovine strains with human strains suggests that an animal reservoir of NoV infection is possible (Scipioni *et al.,* 2008). This raises the question whether transmission of these viruses could also be zoonotic. So far, animal NoV have not been found in humans, however, detection of human NoV in animals suggests a risk of transmission (Delward *et al.,* 2012; CDC, 2011b; Bank-Wolf *et al.,* 2010; Scipioni *et al.,* 2008).

### 2.2.5. Epidemiology and Evolution of NoV

#### 2.2.5.1. Epidemiology of NoV

NoV is the predominant cause of viral gastroenteritis outbreaks worldwide (CDC, 2011) and they are now the leading cause of acute gastroenteritis in young children in the USA (Payne *et al.,* 2013). NoV outbreaks occur usually in semi-closed communities such as food services, nursing homes, schools, hospitals, cruise ships, evacuation sites, vacation and military settings (Matthews *et al.,* 2012; Karst, 2010; Fankhauser *et al.,* 1998), and there is often wintertime seasonality, explaining why NoV disease is referred to as winter vomiting disease (Rohayem *et al*., 2009).

GII.4 variants are the predominant pandemic genotypes, responsible for approximately 55-85 % of the reported gastroenteritis cases worldwide (Desai *et al.,* 2012). From 1990's to early 2013, seven different GII.4 variants were associated with global epidemics (Ramani *et al.,* 2014). It is likely that immune-driven receptor

changes in GII.4 gives rise to the wide range of variants emerging every 2-3 years, similar to the process that is well documented for influenza A viruses (Lindesmith *et al.,* 2011a; Koopmans *et al.,* 2008). In winter 2014–15, NoV outbreaks of GII.17 strains in China and Japan (Fu *et al.*, 2015; Lu *et al.,* 2015; Matsushima *et al.*, 2015) were reported to replace the previously dominant GII.4 genotype Sydney 2012 variant. It remains to be seen if the current change of circulating NoV strains in Asia will be observed in other parts of the world (de Graaf *et al.*, 2015).

While it is widely accepted that NoVs cause the majority of epidemic gastroenteritis, (Karst, 2010), wider availability and improvements in diagnostic methods have also enhanced our understanding of the role of NoV as the leading cause of sporadic gastroenteritis, in all age groups (CDC, 2011b; Glass *et al.,* 2009). A systematic review of community, outpatient, and hospital-based studies in both developed and developing countries estimated that NoV accounted for 10-15% of severe gastroenteritis cases in children aged under 5 years of age and 9-15% of mild and moderate diarrhea cases among persons of all ages (Patel *et al.,* 2008). Recently Ahmed *et al.* (2014) assessed the role of NoVs as a cause of endemic acute gastroenteritis in a meta-analysis of selected reports between 1990-2014 and generated a pool estimate that NoV infections were associated with 18% (95% CI 17-20) of all cases of acute gastroenteritis worldwide ( Ahmed *et al.,* 2014).

2.2.5.2. Evolution of NoV

Understanding the factors that determine the rate at which genomes mutate, provides important insights into key evolutionary mechanisms. The high rate of nucleotide substitution in RNA viruses suggest that evolutionary rates in viruses are explained by diverse aspects of viral biology, such as genomic architecture and replication speed, and not only by polymerase fidelity (Duffy *et al.,* 2008). It appears certain that genetic exchange plays a key role in several virus groups and that it has formed a good arrangement of the diversity that exists in them. Thus, evolutionary knowledge about recombination influences numerous aspects of the study of RNA viruses, from the broadest investigations of virus taxonomy, to the finest details of molecular epidemiology and vaccine design (Worobey and Holmes, 1999).

NoVs have been described as quasispecies on the basis of their population diversity, and the constant generation of genetic and antigenic heterogeneity that allows them to persist in human populations by evading the immune response (Donaldson *et al.,* 2008; Biebricher and Eigen, 2006; Escarmis *et al.,* 2006). Bull *et al.* (2011) reported that minor variants at frequencies as low as 0.01% could be successfully transmitted and this suggests that transmission is an important source of diversity at the inter-host level of NoV evolution. These findings indicate that evolution of NoV may happen through numerous mechanisms (Bull *et al.,* 2011).

Four major factors have been documented to influence the rate of antigenic variation in NoV GII.4 (Bull and White, 2011). These include:

1. Host receptor switching: NoV infection susceptibility results from an increase in the number of HBGAs to which GII.4 can bind.

2. Sequence space: NoV GII.4 concentrates its amino-acid polymorphisms within VP1 at the capsid protein. This reduction of sites in the genome enables the virus to alter without a potentially negative fitness cost.
3. Duration of herd immunity; it is unclear how long immunity post NoV infection lasts but evidence suggests it may be short-term (Karst, 2010; Johnson *et al.,* 1990) allowing NoV to recycle previous antigenic determinants.
4. Replication kinetics: The lack of proof-reading repairs mechanisms of RNA polymerases leading to higher mutation and incorporation rates of the evolving GII.4 viruses.

The predominance and periodic emergence of GII.4 NoV variants associated with a significant increase in the number of reported illnesses, stimulated intense study of this genotype when it was first recognized as a major epidemic strain (Bok *et al.,* 2009; Allen *et al.,* 2008; Lindesmith *et al.,* 2008; Reuter *et al.,* 2008; Siebenga *et al.,* 2007). Lindesmith *et al.* (2008) analysed the temporal evolution and HGBA binding characteristics of representative GII.4 NoVs circulating since 1987 and proposed the existence of five clusters of GII.4 that have likely to have evolved through antigenic drift and are serially replaced over time with intermediate periods of stability. These findings suggest that it should be possible to develop vaccines against NoV infections, however the surveillance systems will need to monitor the virus variations and frequently update the vaccines to make them more effective, as is the case with influenza virus vaccine preparation.

From the analysis of Children's Hospital National Medical Center in Washington, DC

(CHDC) archival stool specimens collected in USA, Bok *et al*. (2009) identified GII.4 NoVs dating back to 1974, which enabled a high-resolution phylogenetic analysis of GII.4 NoVs spanning 34 years (1974 to 2007). Their data support the emergence of an ancestral GII.4 genotype in the 1960s that has evolved at a rate of approximately $4.3 \times 10^{-3}$ nucleotide substitutions per site per year. Modelling predictions of the GII.4 capsid showed evidence for structural shifts in the capsid architecture over time which did not affect HBGA binding but may have contributed to the emergence of new epidemic strains. Similarly, Nordgren *et al.* 2010 previously reported a secretor-independent susceptibility pattern during a NoV gastroenteritis outbreak in Sweden.

Siebenga *et al.* (2007), when studying the GII.4 NoV capsid proteins from 1995 to 2006 in the Netherlands, found a steady association between the observed genetic findings and changes in epidemiology suggesting that the population immunity plays a role in the evolution of GII.4 NoV strains. Carlsson *et al.* (2009) in Sweden also identified evolutionarily conserved capsid residues in an immunocompromised person who was chronically infected with NoV GII.3. A detailed understanding of the evolution and mechanism of persistence of these viruses is critical to developing adequate control strategies such as antiviral drugs and vaccines (Bok *et al.,* 2009).

### 2.2.6. Symptomatic and Asymptomatic NoV Infection, Prevention and Control of Gastroenteritis Outbreaks of NoV

#### 2.2.6.1. Clinical Features of Symptomatic NoV Infection

The incubation period of NoV infection ranges between 24 to 48 hours (range, 18-72 hours) and the onset of NoV disease can be gradual or abrupt (Lee *et al.,* 2013). The gastroenteritis symptoms associated with NoV infection are typically self-limiting

with nausea, vomiting, abdominal pain, non-bloody diarrhea (4-8 stools per day) but in about 50% of cases, systemic symptoms including myalgias, malaise, headache, fever (38.3-38.9$^{o}$C) are also present. The duration of NoV gastroenteritis is typically between 12-72 hours (Rutala and Weber, 2012; Patel *et al.,* 2009) and although it is usually mild, it can be incapacitating during the symptomatic phase. The illness can be severe and prolonged in the very old or young, and immunocompromised persons (van Asten *et al.,* 2011; Green, 2014). Mattner *et al.* (2006) reported on the existence of risk groups for clinical complications of NoV infections, including adult patients receiving immunosuppressive therapy or those admitted to hospital with underlying conditions. Norovirus-infected patients with cardiovascular disease or those having had a renal transplant are at risk of hypokalaemia (Mattner *et al.,* 2006). NoV has also been involved in necrotizing enterocolitis in neonates and immunocompromised hosts (Kim *et al.,* 2011; Stuart *et al.,* 2010; Turcios-Ruiz *et al.,* 2008). Thea *et al*. (1993) performed a prospective study of diarrhea and HIV-1 infection among infants in Zaire, now called Democratic Republic of Congo (DRC), and found that hospitalized children with HIV were more likely to have chronic diarrhea associated with Small Round Structured Virus (SRSV) presence in stool, than those without HIV (Thea *et al.,* 1993). Recently, several studies have reported chronic NoV infection in HIV positive patients with persistent diarrhoea (Munir *et al.*, 2014; Lambregts *et al.*, 2010; Wingfield *et al.,* 2010).

2.2.6.2. Asymptomatic NoV infection

Highly contagious, NoVs are frequently associated with outbreaks of vomiting and diarrhea and are recognized as important agents of sporadic cases and hospitalizations (Payne *et al.,* 2013; Koopmans *et al.,* 2008). However, studies have shown that not all individuals develop symptoms and a significant proportion remain asymptomatic throughout NoV infections (Bareira *et al.,* 2010; Kindberg *et al.,* 2007; Garcia *et al.,* 2006; Gallimore *et al.,* 2004; Okhuysen *et al.,* 1995). Previous studies have revealed that NoV may be shed in faeces for over 3 weeks after symptoms (Milbrath *et al.*, 2013; Atmar *et al.,* 2008; Siebenga *et al.,* 2008), although the infectivity of the virus after this time is not clear.

Ideally, NoV shedding from asymptomatic should be divided into pre-symptomatic viral shedding, primary asymptomatic shedding and post-symptomatic shedding to better understand the true rate of asymptomatic infection (Chung, 2012; Phillips *et al.,* 2010; Atmar *et al.,* 2008; Kindberg *et al.,* 2007; Garcia *et al.,* 2006; Gőtz *et al.,* 2001). During an investigation of a hospital outbreak of NoV gastroenteritis in London, identified as being caused by a recombinant GII (r GII-3a) strain, 33% of positive samples were from asymptomatic staff and patients (Gallimore *et al*., 2004). Individuals exposed to NoV infections gain short-term homologous immunity, but may still have a role in transmitting the virus if exposed (Chung, 2012; Parashar *et al.,* 1998; Johnson *et al.,* 1990). The excretion of NoV from healthy individuals promotes the transmission of these viruses, particularly given the low infectious dose (Hutson *et al.,* 2004). Garcia *et al.,* 2006, who studied asymptomatic NoV infection in Mexican children, reported that almost 30% of stool specimens collected were positive with NoV. This study also showed that children from poor communities in

developing countries with unsanitary living conditions and poor hygienic standards could facilitate the transmission of NoV (Garcia *et al.,* 2006).

There is little evidence that NoV can cause a chronic infection in a normal host, however, prolonged viral shedding has been reported in immunocompromised individuals (Siebenga *et al.,* 2008; Levett *et al.,* 1996). Reports of patients with a range of chronic conditions affecting immune function show prolonged NoV shedding, in some cases for periods longer than 1 year (Sukhrie *et al.,* 2010; Siebenga *et al.,* 2008). It is uncertain if these chronic shedders are possible sources of nosocomial infections, even after they had been infected for a number of weeks. Because *in vitro* culture of NoV has only recently been achieved (Ettayebi *et al* 2016) it has not yet been determined whether or not the viruses shed by such patients are still infectious. Detailed molecular virological data in combination with epidemiological data could be used to track possible routes of NoV transmission within the hospital, and thus help to elucidate the infectivity of NoV from chronically infected patients (Sukhrie *et al.,* 2010).

#### 2.2.6.3. Prevention and Control of Gastroenteritis Outbreaks of NoV

Given that diarrhoeal diseases are mostly of faecal origin, interventions that prevent contamination of the domestic environment with faecal material are critical for the prevention of outbreaks (Curtis *et al.,* 2000). The safe handling and disposal of stool with adequate personal protective equipment (such as gloves and aprons) and hand washing (Figure 2.5) are key barriers to the transmission of enteric pathogens (Curtis and Cairncross, 2003).

**Figure 2.5. Hand washing; a barrier to transmission of enteric pathogens (Curtis and Cairncross, 2003. Lancet Infect Dis 3(5): 275-281).**

The prevention of NoV outbreaks currently relies on an awareness of the modes of transmission, a high standard of hand hygiene, precautions against potentially infectious material, regular effective cleaning and the control of contamination of food and water (Patel *et al.,* 2009). Strict personal hygiene and the proper disinfection of environmental surfaces are critical for the prevention of food-handler associated transmission. It is recommended that food-handlers be excluded from work during illness, and for 48–72 hours after recovery from suspected NoV gastroenteritis to prevent transmission of virus (Patel *et al.,* 2009; Parashar *et al.,* 2001). No specific antiviral therapy is currently available (Atmar, 2010).

During NoV outbreaks, in most infection-prevention and control activities, several strategies are suggested concurrently. There is no particular intervention that might be influential than others. Thus a combination of prudent interventions that reduce disease transmission is advised (CDC, 2011a; Harris *et al.,* 2010). The following interventions have been proposed in formative guidelines on outbreaks of NoV gastroenteritis in healthcare settings: Hand hygiene, personal protective equipment,

leave policies for staff, isolation / cohorting of symptomatic patients, staff cohorting, ward closure, visitor policies, education, surveillance, environment disinfection (CDC, 2011a).

Recombinant virus-like particles (VLP), which lack nucleic acid and as such are unable to replicate, are currently under evaluation as a vaccine candidate and appear to be immunogenic and safe when administered orally to people (Koo *et al.,* 2010; Atmar, 2010; Tacket *et al.,* 2003). These VLPs are antigenically and morphologically similar to native viruses. Norwalk VLP vaccine administered intra-nasally also seems to be safe and immunogenic (Herbst-Kralovetz *et al.,* 2010; Vinje, 2010). This is an important step in developing a vaccine. Next steps should include larger clinical trials to assess whether this vaccine protects against a live virus challenge.

There remain several uncertainties regarding the utility of NoV vaccination (Herbst-Kralovetz *et al.,* 2010), including an incomplete understanding of the immune correlates of protection. The frequency of NoV infections in the community suggests that natural infection does not provide long-term immunity, which raises doubts that vaccines will be any different. The lack of an animal model and the difficulty of NoV *in vitro* cultivation (Patel *et al.,* 2009) make the assessment of efficacy and disease risk more difficult,. Concerns about weakening immunity and the influence of pre-existing antibodies need to be considered in both the design and use of vaccines. There is also little cross-protection among strains from different genogroups; therefore, multivalent vaccines will likely be needed. The lack of cross-protection among strains is compounded by the apparent rapid evolution of NoVs (Vinje, 2010). Comprehensive strain surveillance is needed to identify and evaluate the most

prevalent strains or common antigenic determinants that need to be included in a vaccine. Electronic NoV surveillance systems, such as CaliciNet and NoroNet, may help detect and, perhaps in the future, predict the most prevalent strains prior to each NoV season (Vinje, 2010).

## 2.3. Diagnosis of NoV infection:

NoV infection can be suspected as the cause of an outbreak of gastroenteritis based on clinical and epidemiological criteria (Kaplan *et al.,* 1982). The criteria include the following: (1) vomiting in >50% of ill persons; (2) mean incubation period of 24–48 hours, (3) mean duration of illness of 12 to 60 hours, and (4) no bacterial pathogen identified. These criteria are highly specific (98.6%) and have a high positive predictive value (97.1%) (Turcios *et al.,* 2006). Successful intervention to prevent spread is required prior to diagnosis in situations that may result in an outbreak i.e. hospital settings or care homes (MacCannell *et al.,* 2011; Atmar, 2010; Patel *et al.,* 2009).

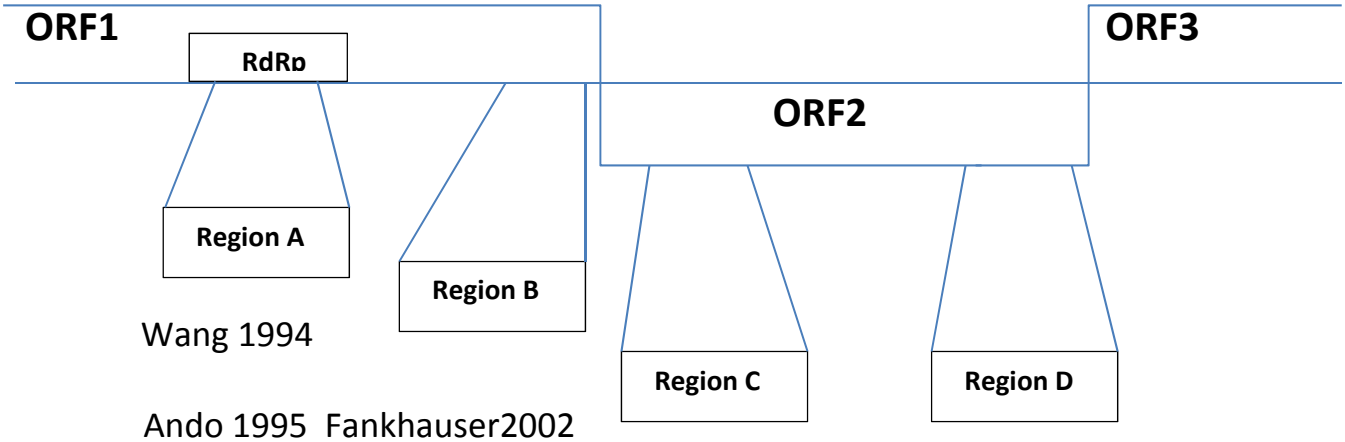
Stool specimens are commonly used to detect NoV although vomit swabs have also been demonstrated to contain high levels of virus (Kirby *et al.*, 2012; Atmar, 2010).

In the 1970s and 1980s, the primary means of NoV detection was by electron microscopy (EM) (Atmar and Estes, 2001; Kapikian *et al.,* 1972), but this method is vastly less sensitive than the molecular assays currently used. Reverse Transcription PCR (RT-PCR) assays, which first became available in the 1990s, are much easier to standardise and amenable to high-throughput testing. These assays target conserved areas in the genome, including the polymerase gene (region A), the

ORF1/ORF2 junction (region B), and areas in the VP1 gene (regions C and D) (Figure 2.6). Detection of the PCR products is also achieved by real-time PCR with the use of fluorescently labelled probes and the products can also be sequenced to confirm the identity of the target. Sequence data can be used for genotyping and in molecular epidemiologic studies (Le Guyader *et al.,* 2008; Yee *et al.,* 2007) although genotyping is also possible as part of many commercial real-time RT-PCR assays (Stals *et al.*, 2012; Atmar, 2010; Trujillo *et al.,* 2006; Kageyama *et al.,* 2004).

NoVs are very variable viruses, and no single primer set is able to amplify all strains (Vinje *et al.,* 2003; Atmar and Estes, 2001). Recently Schultz *et al.* (2011) developed a long-template one-step TaqMan assays (L-RT-qPCR) for the rapid detection and direct genotyping of GI and GII NoVs. Combining current NoV primers and probes targeting the open reading frame ORF1–ORF2 junction as well as region C at the 5'ORF2, the proposed method allowed a direct genotyping of GI and GII NoVs from clinical and environmental matrices.

Antigen detection assays are another approach to diagnosis of NoV infection. Kits using either an ELISA or immunochromatographic format are commercially available. The principal disadvantage of these kits has been their poor sensitivity, being as low as 32% in the identification of sporadic cases of NoV infection (likely due to changes in the antigenic determinants as NoVs evolves); sensitivity is also affected by NoV genotype, as some less common genotypes are not identified by these assays (Park *et al.,* 2012; Gray *et al.,* 2007). Application to multiple samples from an outbreak can improve the probability of finding a positive result (Duizer *et al.,* 2007), but problems with specificity have also been reported in certain populations, furthermore limiting the utility of this approach (Atmar, 2010; Wiechers *et al.,* 2008).




Wang 1994

Ando 1995 Fankhauser2002

Green 1995

Vinje 1996 Green 1995 Vinje 2004

Le Guyader 1996 Noel 1997

Jiang 1999 Vinje 2000a

Kojima 2002

**FIGURE 2.6. Schematic presentation of common regions used for detection and genotyping of NoV. The figure illustrates the genomic regions targeted by RT-PCR assays used for NoV detection and genotyping. These includes Region A located in ORF1 at RNA dependent RNA polymerase; Region B at the junction ORF1/ORF2; Regions C and D in the major capsid ORF2 (Adapted from Vinje *et al.*, 2004. J Virol Methods 116(2): 109-117).**

## 2.4. NoV in Food, Water and Environment

### 2.4.1. NoV in Food

NoV are currently recognised as the most important human foodborne pathogens with regard to the number of outbreaks and people affected in the Western world (Koo *et al.*, 2010; Koopmans and Duizer, 2004). In the United States of America,

NoV infections are estimated to cause more than two-thirds of all foodborne gastroenteritis outbreaks (Bresee *et al.,* 2002) with approximately 23 million cases each year (Mead *et al.,* 1999). It has been estimated that over 10% of NoV cases in England and Wales are foodborne (Adak *et al.,* 2005).

Unlike bacteria, which multiply very easily *in vitro*, viruses are very difficult to detect in foods due the very low number of viral particles required to result in human infection, and the difficulty of propagation methods. Therefore, examination of patient stool samples, which contain enormous quantities of virus, and epidemiological analysis are the best approaches for outbreak investigations. Foods can be contaminated with NoVs at any point during production and processing although the most frequent pathways are thought to be through the unhygienic practices of an infected food handler or exposure to water contaminated with fecal matter (e.g., surface water used for crop irrigation or water containing sewage discharge in shellfish habitats) (Hall, 2012; Barrabeig *et al.,* 2010; Parashar *et al.,* 2001). NoV outbreaks are commonly identified in populations including restaurant customers (CDC, 2007; Daniels *et al.,* 2000).

Bivalve molluscs, such as oysters, bioaccumulate NoVs as a result of filter feeding and selective binding mechanisms (in the same way that NoVs bind to the human ligand complex glycans HBGA) and are therefore readily contaminated when grown in NoV-containing waters (Maalouf *et al.,* 2011). Contamination of berries (e.g., raspberries) may occur prior to harvesting (e.g., due to irrigation with faecally-contaminated water), during harvesting (contamination by infected field workers) or during processing prior to distribution (contamination in the factory by infected food handlers or by washing with contaminated water). (Falkenhorst *et al.,* 2005). As

previously described, only very few viral particles are required to cause an infection and detection of virus at these levels is challenging due to the difficulty of efficient virus extraction, the presence of interfering substances that inhibit molecular detection and NoV genetic variability (Le Guyader *et al.,* 2009).

Molecular-based methods can detect viruses in shellfish but are not yet available for other foods. The applicability of the methods currently available for monitoring foods for viral contamination is unknown due to several challenges including the low doses and unequal distribution of virus in foods as well as the need to prove viability of detected viruses in foods toward the causal relationship between food consumption and illness (Koopmans and Duizer, 2004). Due to the low concentration and persistence of some inhibitors in food, the use of several primer sets is essential (Boxman *et al.,* 2006; Le Guyader *et al.,* 2004; Le Guyader *et al.,* 2003) also the use of concentration methods and internal amplification controls are needed.

### 2.4.2. NoV in Water

Similar to other viruses causing gastroenteritis, NoV multiplies in the intestines and are excreted in large quantities in human feces, which may then be discharged into sewage streams and coastal environments. As these viruses are very resistant to inactivation, the sanitary consequences can include contamination of drinking water, vegetables, and bivalve molluscan shellfish as previously described (Le Guyader *et al.,* 2010). Human waste is processed in sewage treatment plants, but the treatment procedures do not completely remove enteric viruses from the water effluents leaving the plant (Iwai *et al.,* 2009; da Silva *et al.,* 2007).

Contaminated water poses a significant health risk (Reynolds *et al.,* 2008; Karim *et al.,* 2004; Schaub and Oshiro, 2000). NoV have been detected in surface water, ground-water, drinking water, and mineral water and are a well-documented cause of waterborne outbreaks of acute gastroenteritis. Consumption of contaminated drinking water has been reported to be associated with outbreaks of NoV gastroenteritis and outbreaks linked to swimming water have also been reported (Karim *et al.,* 2004). However, because of the lack of a reliable method of detection and a reliable surveillance system, the actual disease burden of water-related NoV infection might have been underreported. Collaboration among local, state, and national public health authorities would help to define the actual disease burden that might be attributed to waterborne NoV (Matthews *et al.,* 2012; MacCannell *et al.,* 2011; Maunula *et al.,* 2005).

Unlike many bacterial pathogens, which have been controlled largely by water and wastewater treatment practices, the incidence of water-related viral diseases has remained virtually unchanged over the past several decades (Aw and Gin, 2010; Figueras and Borrego, 2010; Metcalf *et al.,* 1995). Waterborne outbreaks may also arise from direct exposure by ingestion of contaminated tap water or water-containing products, e.g., ice cubes and salads. In waterborne outbreaks, a very high proportion of the population can be affected, leading to several to hundreds of cases of gastroenteritis, followed by secondary spread and resulting in significant economic impact (CDC, 2011b; Parshionikar *et al.,* 2003; Leclerc *et al.,* 2002).

NoVs have been detected in raw urban sewage (Mans *et al.,* 2013; Blanco Fernandez *et al.,* 2011; van den Berg *et al.,* 2005). During heavy rainfall, contamination of water can also occur via run-off from crop irrigation sources or

animal droppings. The latter may also give rise to public health problems relating to exposure to zoonotic pathogens (Koopmans *et al.,* 2000). Drinking water may be produced from groundwater or surface waters. Surface waters are more heavily contaminated with pathogenic microorganisms, although viruses have also been detected in groundwater (Karim *et al.,* 2004). Bruggink and Marshall (2010) reported a significant positive correlation between monthly NoV outbreak incidences and average rainfall in Australia (2002-2007). Contaminated river waters have been reported from several countries such as Japan, Thailand, Singapore, Korea, Brazil, Sweden, and USA (Kishida *et al.,* 2012; Kittigul *et al.,* 2012; Kitajima *et al.,* 2009; Aw *et al.,* 2009; Lee and Kim, 2008; Miagostovich *et al.,* 2008; Westrell *et al.,* 2006; Borchardt *et al.,* 2004).

Although enteric viruses have been described in a range of waters since the 1970s, identification of the virus in the implicated water sample can be difficult because virus levels are often low, requiring concentration from large volumes of water (Haramoto *et al.,* 2009). In addition, RT-PCR inhibitory factors may be co-extracted and purified with the viral targets, potentially causing false-negative PCR results (Shulman *et al.,* 2012; Hata *et al.,* 2011; Wilson, 1993). In 1997, the first identification of NoV in both water and fecal samples from epidemiologically linked cases in an outbreak investigation occurred (Beller *et al.,* 1997). Since then, due to the improved concentration and detection methods, many investigators have shown identical NoV in both contaminated drinking water and related fecal samples from cases (Ngazoa *et al.,* 2008; Maunula *et al.,* 2005).

Several studies have reported the involvement of GI strains in waterborne outbreaks (Lee and Kim *et al.,* 2008; Hill *et al.,* 2005; Parshionikar *et al.,* 2003; Greening *et al.,* 2001). Maunula *et al.,* 2005 found that while 13% of total number of NoV outbreaks in Finland between 1998 and 2003 were caused by NoV GI, approximately half of the waterborne outbreaks were attributable to NoV GI strains (either GI/3 or GI/6) and postulated that there may be differences in virus stability between genotypes (Maunula *et al.,* 2005). With note, fecal samples containing NoV GI have been shown to have a viral load 100 times lower compared with the viral level in NoV GII-positive samples. This difference suggests that transmissibility via the fecal-oral route may be lower for GI than GII (Chan *et al.,* 2006) and may explain why secondary cases in the community were not a major feature of this outbreak. This hypothesis does not, however, explain why Norovirus GI appears to be predominant in waterborne outbreaks.

2.4.3. NoV in the Environment:

Environmental detection of viral pathogens requires the identification of the presence and/or quantity of virus relevant to human health in environmental matrices (air, food, soils, and water). Major challenges in environmental virus detection include low assay sensitivity, sample inhibition and an assessment of viability. These challenges can result in an underestimation of exposure risks; and difficulties in the determination of the impact of interventions against the full range of viral pathogens. (Julian and Schwab, 2012). Widespread contamination of environments during NoV outbreaks has been reported, particularly in hospital settings (Xerry *et al.,* 2010; Gallimore *et al.,* 2008; Gallimore *et al.,* 2006), food service facilities e.g., restaurants and hotels (Boxman *et al.,* 2011; Greig *et al.,* 2007; Todd *et al.,* 2007a), and bare

hand contact with food followed by improper hand washing (Todd *et al.,* 2007b). NoVs are hardy and have also been detected on environmental surfaces during non-outbreak periods, so the role of this contamination is not fully understood (Lopman *et al.,* 2012). The difficulties of isolating viral particles and producing an *in vitro* model for NoV means that no good assessments of virus viability have been made.

By shedding in feces and vomit, NoV can be transmitted directly through a range of routes: person-to-person, water, food or environmental contamination. The relative importance of environmental transmission of NoV is yet to be fully quantified but is likely to be an important feature that complicates outbreak control (Lopman *et al.,* 2012). Several factors may facilitate environmental transmission of NoV including the availability of a large pool of susceptible human hosts (Hall *et al.,* 2011; de Wit *et al.,* 2001), copious shedding in feces (Atmar *et al.,* 2008), widespread and effective dissemination by spontaneous vomiting (Gallimore *et al.,* 2008; Marks *et al.,* 2003; Cheesbrough *et al.,* 2000), prolonged shedding (Atmar *et al.,* 2008; Rockx *et al.,* 2002), environmental stability (Seitz *et al.,* 2011; Lopman *et al.,* 2009), resistance to chemical disinfection (Park *et al.,* 2011; Park *et al.,* 2010), and the diverse range of fomites that can be contaminated (Boxman *et al.,* 2011; de Wit *et al.,* 2007; Cheesbrough *et al.,* 2000). Seitz *et al*. (2011) who challenged 13 individuals at different time points with groundwater spiked with the prototype human NoV, found that Human NoVs in groundwater remains infective after 2 months and can stay detectable for over 3 years. An environmental reservoir for NoVs may initiate the spread of the NoVs in the community (Bruggink and Marshall, 2010; Hewitt *et al.,* 2007; Nygard *et al.,* 2003). This begs the question as to whether there are

environmental reservoirs for NoVs that allow persistence of infection sources and the generation of new strains throughout the year (Bull and White, 2011).

PCR is the main technique for detecting Norovirus in food, water and environmental samples. However, for a variety of reasons, including the diversity of surfaces, the heterogeneous distribution of viral contamination and the possible presence of PCR inhibitors, detection in food and environmental samples is difficult and restricted to specialist laboratories. A crucial limitation of current methods is that they detect fragments of viral RNA, which may not indicate infectious virus. Thus, PCR results from environmental samples should be interpreted with caution and in the context of available epidemiological or clinical information (Lopman *et al.,* 2012; Boxman *et al.,* 2011; Boxman *et al.,* 2009; Gallimore *et al.,* 2008). Sterile swabs can be used for environmental sampling, after which viral RNA is extracted from the swabs and subject to testing. These methods require delicate optimisation to maximise the sensitivity and accuracy. (Lopman *et al.,* 2012; Park *et al.,* 2011; Gallimore *et al.,* 2005).

Existing evidence suggests that NoV is a common environmental contaminant both within and outside of outbreak settings owing to its environmentally stability and resistance to common disinfectants. (Lopman *et al.,* 2012; Seitz *et al.,* 2011; Lopman *et al.,* 2009; CDC, 2008). Studies confirming the importance of environmental transmission, where risk of disease can be linked to exposure to a contaminated environment, are needed to firmly establish the role of this mechanism of spread, especially in healthcare settings. Eventually, evidence is needed for effectiveness of

control measures that target environmental transmission (Fernández *et al.,* 2012; Lopman *et al.,* 2012).

## 2.5. Animal NoV

NoV has been detected in a variety of animals including pigs, cattle, mice, dogs, sheep and lions (Patel *et al.,* 2008). GII NoV has been linked to humans, pigs and cattle; GIII NoV to cattle and sheep; GIV NoV to humans, lions and dogs; and GV NoV to mice (CDC, 2011; Patel *et al.,* 2008). The unanswered question in this area is whether animals can act as a reservoir for human NoVs. Phylogenetic analyses (Zheng *et al.,* 2006) revealed that animal and human strains are closely related, especially porcine NoVs which are included in the same GII as some human strains (Scipioni *et al.,* 2008). Although an animal reservoir and zoonotic transmission could exist, genetic distances between viruses (Oliver *et al.,* 2003) and difference between receptors (Farkas *et al.,* 2005; Hutson *et al.,* 2003) do not support this hypothesis. The lack of evidence that the same strains circulate in both human and animal species suggests an absence of risk to human health (Scipioni *et al.,* 2008; Oliver *et al.,* 2003). However the recent detection of sequences close to GII.4 human NoV in swine and cattle in Canada questions this (Mattison *et al.,* 2007). Animal NoVs have not been detected in human stool samples to date, suggesting that they are unable to replicate in the human GI tract. Nevertheless, zoonotic transmission of NoVs cannot be excluded (Bank-Wolf *et al.,* 2010).

NoV recombinants have been identified between NoV belonging to the same genogroup and from the same animal species within bovine (Han *et al.,* 2004; Oliver

*et al.,* 2004), porcine (Wang *et al.,* 2005) and human species (Katayama *et al.,* 2002; Vinje and Koopmans, 2000b; Jiang *et al.,* 1999). A natural co-infection with GI and GII NoV has already been described in humans (Chan *et al.,* 2006). Furthermore, simultaneous presence of human and animal NoV has been detected in shellfish (Costantini *et al.,* 2006). These observations raise concern about the risk of co-infection of humans and animal NoV, resulting in possible recombination and emergence of new strains. The emergence of such recombinants is most likely to arise in countries with high densities of animal and human populations where the close animal-human associations enhance the risk for cross species transmission (Scipioni *et al.,* 2008).

## 2.6. NoV infection in South Africa

In RSA, the first study on NoV outbreaks reported GI.1 and GII.1 strains as the causative agent in two successive outbreaks in 1993 (Taylor *et al.,* 1993). In 1995, GII.3 was identified in paediatric stool specimens from sporadic gastroenteritis (Wolfaardt *et al.,* 1995). Smit *et al.* (1997) who studied Norwalk-like virus and Mexico Virus infections at Ga-Rankuwa Hospital, Ga-Rankuwa, RSA, found that 9.2 % of stool specimens were positive for SRSVs by EM and 25 % of these SRSVs gave a positive result by RT-PCR. A later study in 1999, revealed NoV antibody prevalence levels of 94-96% among both urban and rural South African populations (Smit *et al.,* 1999). Since then, no data on NoV prevalence or circulating genotypes was reported for RSA until the recent studies by Mans *et al.* (2010) who described the emerging NoV GII.4 2008 variant detected in hospitalized paediatric patients (Mans *et al.,* 2010) and the diversity of NoVs in sewage-polluted river water in South Africa (Mans

*et al.,* 2013). There is a need to determine the prevalence and genetic diversity of NoV infections in rural and urban populations of South Africa and to compare the circulating genotypes with NoV strains found worldwide (Mans *et al.,* 2010).

## 2.7. Genetic Relationship between NoVs

A comparison of sequence for NoVs may help to find out the extent of antigenic variation between the strains, to understand the transmission route of the virus as well as their origins. To analyse the genetic relationship between the NoV genotype variants reported worldwide, the sequences of ORFs and the whole genome are processed with multiple sequence alignment (Vinjé *et al.,* 2004; Guindon and Gascuel, 2003). Phylogenetic analysis and full genome sequencing of ORF1 or ORF2 are usually performed to find out the genetic relationship between different NoV strains and the emergence of new variants (Shen *et al.,* 2012; Oliver *et al.,* 2003; Smiley *et al.,* 2003; Buesa *et al.,* 2002; Sugieda and Nakajima, 2002; Liu *et al.,* 1999). Smiley *et al.,* 2003, when studying the genetic relationships among bovine enteric caliciviruses and human caliciviruses, performed phylogenetic and sequence identity analyses of each NoV genome region to demonstrate how these viruses were most closely related to the enteropathogenic bovine enteric caliciviruses (NLV-BEC) Jena and Newbury Agent-2 (NA-2) strains.

Phylogenetic analysis can trace the evolutionary history of organisms by studying relatedness and patterns of variation in genetic material. In terms of clinical significance these methods can be used to type and characterise strains used for vaccines (Srinivasan *et al.,* 2012), by tracking genetic developments over time and

any genetic relationships between infected patients (Verhoef *et al.,* 2012; Coughlan *et al.,* 2002). Evolutionary relationships are typically illustrated using a phylogenetic tree (Bok *et al.,* 2009; Duffy *et al.,* 2002) as displayed in the figure 2.7.

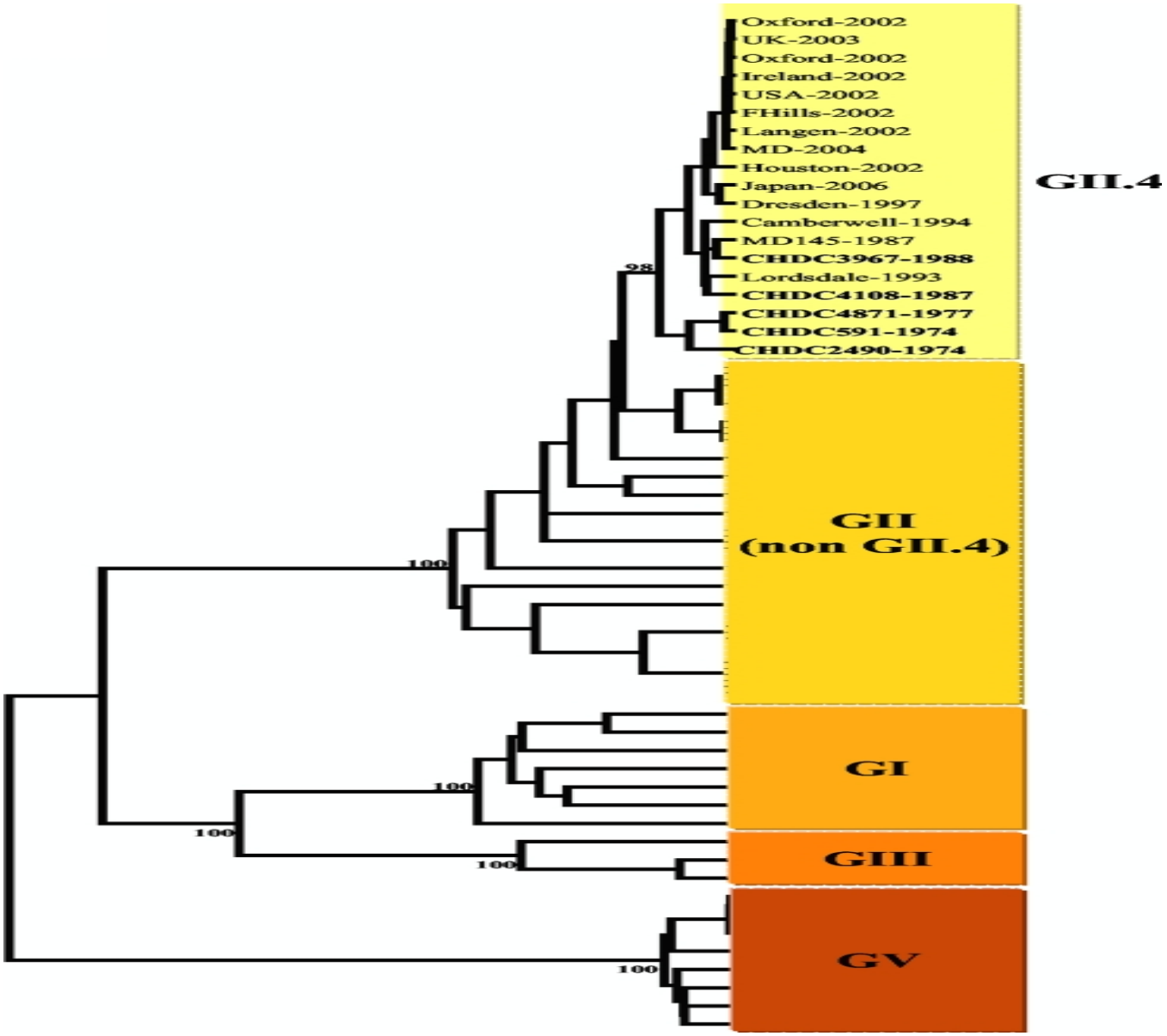



**Figure 2.7. Maximum likelihood analysis of complete genome sequences from noroviruses of different genogroups. Complete genomic sequences of norovirus genogroups I, II, III, and V obtained from the GenBank database (see Table S1 in the supplemental material) were compared with five GII.4 CHDC samples from this study (shown in boldface type). Bootstrap values are shown at the nodes of each selected phylogenetic group only. FHills-2002, Hu/NoV/GII.4/Farmington Hills/2002/US; Camberwell-1994, Hu/NoV/GII.4/Camberwell/1994/AUS; CHDC3967-1988, Hu/NoV/GII.4/CHDC3967/1988/US; CHDC4108, Hu/NoV/GII.4/CHDC4108/1987/US. (From Bok *et al.,* 2009. J Virol 83(22): 11890-11901.**

## 2.8. Summary

NoVs are now considered a major cause of outbreaks of nonbacterial gastroenteritis but the factors which impact the genetic diversity and the persistence of infection, the sources of sporadic NoV infections and asymptomatic carriage are poorly understood (Bucardo *et al.,* 2011; CDC, 2011; Marshall and Bruggink, 2011; Karst, 2010). Little is known about circulating NoV strains in RSA, especially in rural and periurban areas.

The literature has indicated that NoV are well adapted viruses for transmission within human populations, as previously described. NoV outbreaks occur usually in semi-closed communities mainly during the cool seasons (Rohayem *et al.,* 2009) and affect people of all ages (Matthews et al., 2012; CDC, 2011b). Since 2002, GII.4 variants were globally recognised as the predominant strains circulating (Siebenga *et al.,* 2009; Lopman *et al.,* 2004). NoV recombinant VLPs are being evaluated as a vaccine candidate because they are immunogenic and safe when administered orally to people (Tacket *et al.,* 2003). However NoV are genetically diverse group that rapidly change and have a short incubation period leading to an apparent lack of prolonged cross-protective immunity following infections and repeated infections throughout life with re-exposure (Hall, 2012; Patel *et al.,* 2009).

Literature has revealed that the NoV genus includes more than 35 distinct virus strains which are divided into seven genogroups based on sequence similarity. Each genogroup is further subdivided into genoclusters, based on sequence similarity and phylogenetic analysis (Patel *et al.,* 2009; Donaldson *et al.,* 2008; Zheng *et al.,* 2006). Previous reports indicated that NoV strains evolve through antigenic drift and those

strains are successively replaced over time (Bok *et al.,* 2009; Lindesmith *et al.,* 2008; Siebenga *et al.,* 2007; Dingle *et al.,* 2004). The NoV genome also undertakes frequent change by recombination (Rohayem *et al.,* 2005), when its polymerase sequence and capsid sequence forms distinct clusters with its closest neighbours in the phylogenetic tree (Bull *et al.,* 2007; Ambert-Balay *et al.,* 2005; Bull *et al.,* 2005). GII.4 strains frequently undergo genetic variation and these altered forms are called "variants" or "subtypes" (Bull *et al.,* 2006). Currently increased awareness and identification of naturally occurring recombinants worldwide have been reported (Ambert-Balay *et al.,* 2005; Bull *et al.,* 2005; van den Berg *et al.,* 2005). However there is no classification system for recombinant NoV and no widely accepted recombinant genotyping system (Bull *et al.,* 2007).

When NoV infections were first recognised, diagnostic testing of stool was achieved by electron microscopy (Atmar and Estes, 2001; Kapikian *et al.,* 1972). RT-PCR assays are now widely available and are currently the most sensitive methods for detection of NoV in clinical samples. NoVs are currently recognised as the most important human foodborne pathogens mainly in the Western world (Koo *et al.,* 2010; Koopmans and Duizer, 2004). As the viruses are very difficult to detect in food, the majority of outbreaks are only determined by the virological study of stool samples and epidemiological analysis of patients. Contaminated water poses a significant health risk (Karim *et al.,* 2004; Schaub and Oshiro, 2000). However, due to the lack of reliable methods of detection and surveillance systems, the actual disease burden of water-related NoV infection might have been underestimated. Because of the low viral levels in water, the identification of viruses in the implicated water samples is often difficult and requires water concentration from large volumes

(Haramoto *et al.,* 2009). Furthermore RT-PCR inhibitory factors may be coextracted and purified with viruses, potentially causing false-negative results (Shulman *et al.,* 2012; Hata *et al.,* 2011; Wilson, 1993).

Contamination of the environment during NoV outbreaks has been reported, particularly in hospital settings (Xerry *et al.,* 2010; Gallimore *et al.,* 2008; Gallimore *et al.,* 2006), food service facilities e.g., restaurants and hotels (Boxman *et al.,* 2011; Greig *et al.,* 2007; Todd *et al.,* 2007a), and bare hand contact with the food followed by failure to properly wash hands (Todd *et al.,* 2007b). PCR is the main technique for detecting NoV in food, water and environmental samples as human NoVs are difficult to grown in cell culture (Duizer *et al.,* 2004). A major limitation of current methods is that they detect viral RNA, which may not indicate infectious virus. Studies confirming the importance of environmental transmission, where risk of disease can be associated with exposure to a contaminated environment, are needed to firmly establish the role of this mechanism of spread, especially in healthcare settings (Fernández *et al.,* 2012; Lopman *et al.,* 2012).

The aim of this study is therefore to investigate the genetic characteristics of NoV strains circulating in the rural communities of the Limpopo Province, RSA. The hypothesis of the study is that poor environmental living conditions in this rural setting may influence the prevalence, genetic diversity and transmission of NoV circulating strains. The findings may confirm the potential human health risk of unsanitary environments in the rural communities of Limpopo Province. The results of this study will assist decision-makers in coming up with public prevention strategies to combat diarrhea disease transmission, particularly in NoV infections.

The outcomes of this study will provide information on NoV diversity, and possibly contribute to vaccine development.

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# Chapter 3

# ENCLOSED ARTICLES

# 3.1. REVIEW ARTICLE:

To support the rationale of this research study, we performed a systematic review which assessed the role of Human Norovirus in diarrheal diseases in Africa.

Title: "**Human Norovirus prevalence in Africa: a review of studies from 1990 to 2013**"

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## 3.2. OBJETIVE 1: To determine the prevalence of NoVs circulating in the rural communities of Limpopo Province.

### ARTICLE 2:

Title: "**Norovirus prevalence and estimated viral load in symptomatic and asymptomatic children from rural communities of Vhembe district, South Africa**"

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## 3.3. OBJECTIVES 2 and 3:

-To determine genetic diversity of NoVs circulating in the rural communities of Limpopo Province

- And to examine the relatedness of NoV strains from rural communities of Limpopo Province to the worldwide circulating strains.

N.B. The comparison of Norovirus strains from this research study with the Genbank reference strains is part of the following article.

## ARTICLE 3:

**TITLE**: "**Genetic characterisation of Norovirus strains in outpatient children from rural communities of Vhembe district / South Africa, 2014-2015**"

Running Head: Norovirus genetic diversity in rural communities of South Africa

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# Chapter 4

# SUMMATIVE COMMENT AND RECOMMENDATIONS

This prospective study investigated the genetic characteristics of Human Norovirus strains circulating in the rural communities of the Vhembe district / South Africa. The study objectives were to determine the prevalence and the genetic diversity of NoVs circulating in the rural communities of Limpopo Province, and to examine the relatedness of NoV strains from rural communities of Limpopo Province to the worldwide circulating strains. How would poor conditions in rural setting impact the genetic characteristics and transmission of circulating strains of NoV in Limpopo

province, South Africa? We hypothesized that poor living conditions including poor hygiene practices and inadequate sanitation, unsafe water and polluted environment found in rural settings may influence the prevalence, genetic characteritics and transmission of NoV strains circulating in the study area.

Human Norovirus is now recognized as the most common cause of nonbacterial gastroenteritis worldwide. Despite the fact that African continent is one of the most affected by diarrheal disease (Liu *et al.*, 2015), there are many countries of Africa without data on the contribution of Human Norovirus to gastroenteritis (Kabue *et al.*, 2016a). There is no reporting system of Human NoV infections in the primary healthcare system, suggesting that sporadic cases of Human NoV are likely to be underreported and the prevalence rate obviously underestimated. In this study, we used qualitative environmental variables related to the poor living conditions in rural setting to assess possible risk factors of NoV infection.

To motivate the rationale of the research study, we performed a systematic review of the PubMed and EMBASE databases for published articles of Human NoVs in Africa between 1990 and 2013 and found that unreported sporadic cases associated with Human NoVs are common among children in Africa (Kabue *et al.*, 2016a). There are mostly community-related infections. Most of NoV studies were carried out in urban settings probably due to the lack of laboratory capacity in rural settings (Kabue *et al.,* 2016a). The relationship between Human NoV infection and disease in Africa is complex. Systematic surveillance of Human NoVs is needed to estimate the burden of NoVs-induced gastroenteritis in Africa.

The assessment of NoV prevalence during the study period revealed that the detection rate of Norovirus in symptomatic cases was high but not statistically different when compared to the controls. Significant statistical difference of estimated GII viral load from both groups was found, with higher viral load burden in symptomatic children. The results suggest that the difference between asymptomatic and symptomatic children with NoV may be at the level of the viral load of NoV genogroups involved. In this study, most of stool specimens were collected ≤ 3 days of interval between the onset of diarrhea and collection of stool (Kabue *et al.,* 2016b). The duration of symptoms due to NoV infections, such as vomiting and diarrhea usually ranges from 1 to 3 days (Lopman *et al.,* 2004; Graham *et al.,* 1994), but patients may continue to shed virus for some weeks (Atmar *et al*., 2008). Stool collection within the duration of symptoms will favour the detection of Norovirus infections in clinic samples. Though the viral loads are dynamic, our stools collection was done on time (when the viral replication is supposed to reach the peak) to avoid possible change due to the long duration after the onset of diarrhea.

Though not confirmed predictive of diarrhea disease in this study, the high detection rate of NoV reveals the substantial exposure of children to enteric pathogens in rural setting with poor environmental living conditions. Most of the children in the study population were from households with a very low income and poor living conditions. In addition children aged 13 to 24 months had the highest rates of NoV positivity relative to those of other age groups in this study. This finding is similar to other previous studies of outpatient children in developing countries (Shioda *et al.,* 2015; Zou *et al.,* 2015; Jia *et al.,* 2014). At the age of 10 to 12 months, young children usually become more active and interact more with their surroundings.

Young children may have more opportunities to be contaminated by NoV-infected environments that children of other age groups (Zou *et al.,* 2015), coupled with the absence of toilet training. Though we could not find significant correlation between the qualitative environmental variables and NoV infection, all the indicators lean toward poor hygiene practices as previously discussed in our publications from this research study (Kabue *et al.,* 2017; Kabue *et al.,* 2016b).

**Further hygiene education would help to adopt behaviour in communities in order to prevent fecal oral transmission of enteric pathogens.**

This study determines the NoV genetic diversity among children under five with diarrhea, attending PHC centres in rural communities. Considering that all the children with gastroenteritis disease may not present to any healthcare facilities, what we have reported could be the tip of iceberg. The finding of high levels of NoV prevalence rate in this study area and the predominance of GII.4 Sydney 2012 genotype observed in the current investigation, which has been largely associated with increased NoV outbreaks (van Beek *et al*., 2013), suggest that there was

increased NoV activity in the study region. Unfortunately, this has not been reported before since there is no surveillance of NoV in South Africa, and this finding may also be the case throughout Africa. Characterisation of NoV genotypes in Africa may be useful to assess the role of the NoV in diarrheal disease across the continent and support the on-going NoV vaccine development. **Further efforts should be deployed to promptly investigate epidemic cases of NoV. This requires the implementation of systematic surveillance of NoV associated gastroenteritis in the rural communities of South Africa.**

GI.4 found in this study have been previously reported in waterborne NoV outbreaks and environmental sources in several countries including Thailand, Italy and South Africa (Inoue *et al.,* 2016; Di Bartolo *et al.,* 2015; Mans *et al.,* 2013). Also the phylogenetic tree based on 330-nucleotide sequence of GI capsid (Figure 3.3.4; Kabue *et al.,* 2017) reveals that GI.4 genotypes identified in this study were closely related to other GI.4 genotypes identified elsewhere in waterborne NoV outbreaks.

The presence of these GI capsid genotypes in clinical specimens from children in rural communities with poor hygiene practice and polluted environments suggests water-borne NoV transmission despite the absence of direct molecular evidence of NoV contamination of water in this study.

**Environmental NoV studies are warranted to ascertain the environmental transmission route of NoV in rural communities of Vhembe district / South Africa.** The low GI NoV rate found in clinical samples from this study is consistent with previous studies. We speculate on that in our publication on page 124 (Kabue *et al.,* 2

016b) to mention that similarly to Chan *et al.* (2006) the low rate of GI in clinical specimens may be due to the lower transmissibility via fecal-oral route and viral infectivity.

The rare GII.P15 polymerase genotype has also been found in this study and to our knowledge this is the first report of this unusual genotype in the African continent. The existence of numerous GII.4 recombinant variants circulating with the uncommon genotypes ensures enormous potential for novel viral recombinations (Bull and White, 2011). **Continuous monitoring of the strain distribution in the population will allow the identification of epidemic-causing strains in real-time.**

The aim of this research study was to determine the prevalence and genetic characteristics of Norovirus strains in rural setting with poor environmental living conditions. The majority of NoV studies in Africa to date have been extensively reported from urban settings and therefore, we speculate that the poor living conditions offered in rural setting may impact the prevalence and genetic characteristics of circulating Norovirus strains. Unfortunately, the qualitative environmental variables (subject to the reports provided by the parents of children) could not help to fully assess possible association between NoV infection and poor environmental living conditions. The logistic regression analyses performed on all the qualitative environmental variables used in this study and the presence of NoV did not reveal significant correlation (Kabue *et al.,* 2016b; Appendix B and C). This study has some limitations. First, we were not able to work on environmental samples because of cost limit. Also the study duration was within less than one year. However within 8 months of study period for samples collection, we find high level of

NoV prevalence rate consistent with previous NoV study reported in the same study area (Platts-Mills *et al.,* 2015). Second, we were not able to perform the whole genome sequencing that would help to entirely assess the NoV genetic diversity. However 9 of NoV genotypes were identified in 21 specimens from which partial RdRp and capsid regions could be amplified and sequenced. The results suggested NoV genetic diversity in the study area (Kabue *et al.,* 2017). Despite the fact we could not fully evaluate in this study the impact of poor environmental living conditions on the prevalence, genetic diversity and transmission of NoV strains circulating in the rural communities of Vhembe district / South Africa, we did achieve the study objectives which were to determine the prevalence (Kabue *et al.,* 2016b) and genetic diversity of NoV strains in the rural South Africa and to examine their relatedness to the worldwide NoV strains (Kabue *et al.,* 2017). Futher investigations that could combine the clinical and environmental samples within a long study period are needed to assess entirely different risk factors of NoV infection in rural setting of South Africa.

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# APPENDIX

## Appendix A:

### A.1. Map of study site locations:

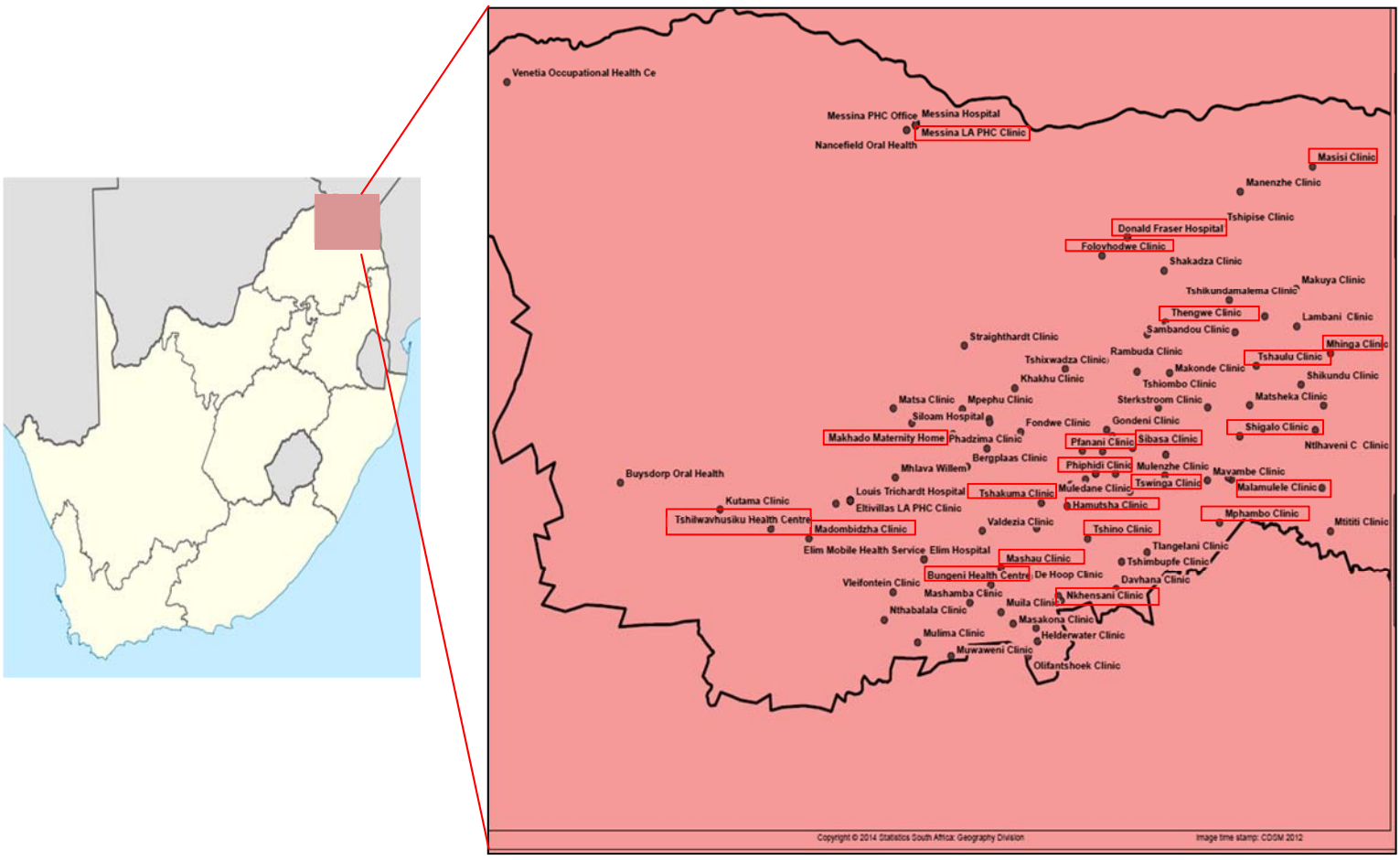


**Figure 1.** Map of the health facilities (with bullet) of Vhembe district in Limpopo / South Africa showing the selected clinics (squared). (Map of Vhembe district obtained from Statistics of South Africa, geography division, 2014; South Africa map from http://upload.wikimedia.org/wikipedia/commons/thumb/3/38/Map_of_South_Africa_with_provincial_borders.svg/500px-

## A.2. Table 1. Summary of selected clinics within the Vhembe region of Limpopo province / South Africa

| Clinic name | Number of Stool specimens collected | Number of Norovirus positive |
|---|---|---|
| MAELULA | 2 | 0 |
| TSHILWABUSIKU | 4 | 2 |
| LWAMONDO | 2 | 0 |
| SIBASA | 1 | 1 |
| MUKULA | 1 | 1 |
| THOHOYANDOU | 8 | 5 |
| MALAMULELE | 3 | 1 |
| VHUFULI / DONALD FRASER | 22 | 12 |
| MUSSINA NANCEFIELD | 1 | 0 |
| RABALI | 24 | 10 |
| TSHILIDZINI | 4 | 0 |
| TSHINO | 3 | 0 |
| TSHAULU | 2 | 0 |
| MUKULA | 1 | 0 |
| THENGWE | 19 | 8 |
| MPHAMBO PHC | 7 | 2 |
| WATER VAAL | 9 | 3 |
| MADOMBIDZA | 11 | 7 |
| MAKADHO PHC | 18 | 5 |
| TIYANI PHC | 3 | 2 |
| BUNGENI | 1 | 1 |
| HAMUTSHA | 1 | 1 |

| | | |
|---|---|---|
| MASHAU | 7 | 3 |
| MASISI PHC | 2 | 1 |
| MAJOSI | 56 | 19 |
| PHIPHIDI | 6 | 2 |
| VYEBOOM | 3 | 1 |
| NKHENSANI | 7 | 2 |
| FOLOVHODWE | 1 | 0 |
| PFANANI | 9 | 1 |
| MBOKOTA | 1 | 1 |
| GUYUNI | 2 | 1 |
| TSHIFIFI | 3 | 2 |
| DZINGAHE | 6 | 2 |
| TSHAKUMA | 3 | 1 |
| TWINGA | 3 | 1 |
| SHIGALO | 4 | 2 |
| MHINGA PHC | 1 | 0 |

## A.3. Approvals from University of Venda, Province and District health departments

**UNIVERSITY OF VENDA**

**OFFICE OF THE UNIVERSITY REGISTRAR**

TO: MR. L.D DONGOLA
SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

FROM: MR. J.M. FOURIE
HEAD: COMMITTEE ADMINISTRATION

DATE: 15 OCTOBER 2012

**DECISION TAKEN BY SENEX OF 10 SEPTEMBER 2012**

Application for approval of PhD (Microbiology) research proposal: **J.P. Kabue Ngandu** (11618547)

At its meeting held on 10 September 2012, Senex approved the PhD research proposal.

Topic: *Molecular Characterisation of Norovirus Strains Circulating in Rural Communities of the Limpopo Province of South Africa*

| | | |
|---|---|---|
| Promoter: | Prof. N. Potgieter | (University of Venda) |
| Co-promoters: | Prof. A. Dalsgaard | (University of Copenhagen) |
| | Dr. A.C. Schultz | (University of Cpenhagen) |

Mr. J.M. Fourie
Head: Committee Administration

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

S177

**NAME OF RESEARCHER/INVESTIGATOR:**
**KABUE NJP**
**Student No: 11618547**

**PROJECT TITLE: MOLECULAR CHARACTERISATION OF NOROVIRUS STRAINS CIRCULATING IN RURAL COMMUNITIES OF LIMPOPO PROVINCE, SOUTH AFRICA**

**PROJECT NO: SMNS/13/MBY/0212**

**SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS**

| NAME | INSTITUTION & DEPARTMENT | ROLE |
|---|---|---|
| Prof N Potgieter | University of Venda, | Supervisor |
| Prof A Dalsgaard | University of Copenhagen | Co-supervisor |
| Mr Kabue NJP | University of Venda, | Investigator - Student |

**ISSUED BY:**
**UNIVERSITY OF VENDA, HEALTH, SAFETY AND RESEARCH ETHICS COMMITTEE**

Date Considered: February 2013
Decision by Ethical Clearance Committee Granted
Signature of Chairperson of the Committee: .....
Name of the Chairperson of the Committee: Prof. X.G. Mbhenyane

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
TELEPHONE (015) 962 8504/8484 /8313 FAX (015) 962 8439
*"A quality driven financially sustainable, rural-based Comprehensive University"*

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Enquiries: Selamolela Donald

Ref:4/2/2

Kabue NJP
University of Venda
Thohoyandou
0950x

Greetings,

**Re: Molecular characterization of Norovirus Strains circulation in rural communities of Limpopo Province, South Africa.**

1. The above matter refers.
2. Permission to conduct the above mentioned study is hereby granted.
3. Kindly be informed that:-
   - Further arrangement should be made with the targeted institutions.
   - In the course of your study there should be no action that disrupts the services.
   - After completion of the study, a copy should be submitted to the Department to serve as a resource.
   - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.

Your cooperation will be highly appreciated.

Head of Department

DEPARTMENT OF HEALTH
HEAD OF DEPARTMENT
DR KABANE S.
02 -12- 2013
SIGNED:.....
PRIVATE BAG X9302 POLOKWANE 0700
Date LIMPOPO PROVINCE

18 College Street, Polokwane, 0700, Private Bag x9302, POLOLKWANE, 0700
Tel: (015) 293 6000, Fax: (015) 293 6211/20 Website: http/www.limpopo.gov.za

The heartland of Southern Africa – development is about people

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

**DEPARTMENT OF HEALTH**
**VHEMBE DISTRICT**

Enq: Nengudza FR
Tel: 015 9622 273 Fax: 015 9622 2373 E-mail: fhatuwani.nengudza@gmail.com
Date: 09. July. 2014

To: Kabue NJP
University Of Venda

From: Acting Senior Manager PHC

**Re: PERMISSION TO CONDUCT RESEARCH STUDY. MOLECULAR CHARACTERIZATION STRAINS CIRCULATION IN RURAL COMMUNITIES OF LIMPOPO PROVINCE, SOUTH AFRICA.**

1. The above matter refers.
2. The Department of Health Vhembe District acknowledges receipt of the communique regarding the permission to conduct the above study.
3. Permission to access Primary Health Care facilities is granted for you, however during the course of your study there should be no action that disrupts day to day service to patients.
4. During your stay you will be expected to comply with policies, rules and regulation that govern public institutions.
5. Wishing the best on your studies.

Hoping you will find this in order

**Acting District Executive Manager** **Date** 09/07/14

Private Bag X5009 THOHOYANDOU 0950
Old Parliamentary Building Tel: (015) 962 1848, (015) 962 1852, (015)962 1001/2/3/4/5/6
Fax: (015) 962 2373/ (015) 9622274/ 4623.

*The heartland of Southern Africa – development is about people!*

**A.4. Consent forms in Venda, Shangayi and English:**

**FOMO YA THENDELO NA BAMBIRI ḼA ZWIDODOMBEDZWA ZWA MUDZHENELELI**

**ṰHOHO YA THANDELA YA ṰHOḒISISO:**
**ZWIṰALULI ZWA MOLUKHULI DZA VIRASI YA NORO DZINE DZA MONOLODZA VHUPONI HA MAHAYANI KHA VUNDU ḼA LIMPOPO, AFRIKA TSHIPEMBE**

Nomboro ya Referentsi: SMNS/12/MBY/07

Vhasedzulusi: **Mr. Jean Pierre Kabue Ngandu (Mutshudeni wa PhD)**

**Prof. Natasha Potgieter (Muṱoli)**

ḒIRESI: Department of Microbiology

School of Maths and Natural Sciences

University of Venda

**Luṱingo: 015 962 8256**

Ṅwana wavho kana tshifuwo tshavho zwi khou humbelwa u dzhenelela kha u vha tshipiḓa kha thandela hei ya ṱhoḓisiso. Vha khou humbelwa uri vha dzhie tshifhinga vha vhale mafhungo o ṉetshedzwaho hafha, ane a ḓo ṱalutshedza nga u pfufhifhadza thandela hei. Kha vha vhudzise mushumi wa ngudo iyi mbudziso dziṅwe na dziṅwe nga ha tshipiḓa tshine vha sa khou tshi pfesesa zwavhuḓi. U dzhenelela havho **ndi ha u tou funa nahone vha a tendelwa u hana arali vha sa funi u dzhenelela.**

Ngudo hei yo tendelwa nga komiti ya Human Research Yunivesity ya Venḓa nahone i ḓo itwa ho sedzwa maitele na milayo ine fanela u tevhedzwa nga vha International Declaration of Helsinki, na nga maitele a mashumele avhuḓi a Afrika Tshipembe na maitele a ṱhoḓisiso ano fanela u tevhedzwa a Medical Research Council (MRC).

**Thandela iyi ya ṱhoḓisiso ndi ya mini?**

**.** Ngudo hei i ḓo katela mafhambuwa a ṅwana wa vhukale ha miṅwaha miṱanu ane a khou dinwa nga u tshuluwa, zwifuwo zwa hayani, sambula dza virasi dzi no wanala kha tshidzulo tsha bungani na sambula dza maḓi**.** Ngudo iyi i ḓo katela vhana vha 1000, zwifuwo zwa hayani zwa 400, sambula dza maḓi dza 400 na sambula dza virasi dzi no wanala kha tshidzulo tsha bungani dza 300.

. Ṱhoḓisiso hei yo livhiswa kha u ṱoḓulusa vhushaka vhune ha vha hone kha virasi dza Noro dzi no wanala kha vhathu na virasi dza Noro dzi no wanala kha zwifuwo dzine dza monolodza vhuponi ha mahayani kha Vundu ḽa Limpopo.

Mafhungo haya a ḓo thusa hu tshi dzhiwa tsheo kha maitele a tshitshavha a u thivhela phiriselo ya vhulwadze ha u tshuluwa nga maanḓa kha u kavhiwa nga virasi ya Noro na kha u khwinifhadza fhethu hune ha vha na mabunga kha zwitshavha zwa mahayani.

Mawanwa a ngudo iyi a ḓo ṋetshedza mafhungo nga ha u phaḓalala ha virasi ya Noro ho sedzwa kha u bveledza dzilafho.

Mafhungo oṱhe ane a khou ṱoḓea a ḓo waniwa kha vhone, zwi tshi katela na nḓila ine ra nga vha kwama ngayo, vhukale, mbeu, ḓuvha ḽa u tshuluwa, tshiimo tshavho tsha

HIV na maṅwe malwadze. Mafhambuwa a linganaho 10g a ḓo dzhiwa kha ṅwana wavho kana kha tshifuwo tshavho, zwenezwo zwi ḓo iswa laborothari u senguluswa.

**Ndi ngani vhone vho humbelwa u dzhenelela?**

Ṅwana wavho kana tshifuwo tshavho zwo nangiwa kha ngudo iyi ho sedzwa u tshuluwa hune a khou vha khaho.

**Vhone vha fanela u ita zwifhio?**

U dzhenelela kha ngudo iyi ndi zwa u tou funa nga iwe muṋe. Vha nga hana u ṋekedza mafhungo kana mafhambuwa.

**Vha ḓo vhuelwa nga u vha tshipiḓa tsha thandela iyi ya ṱhoḓisiso?**

A huna ndiliso ya tshelede ine vha ḓo ṋewa nga u dzhenelela havho. Fhedzi zwa konadzea vha ḓo wana mvelelo dza tsenguluso ya zwisinisa na dza tsenguluso ya zwitshili.

**Hu na khohakhombo dzine vha nga dzi wana nga u vha tshipida kha ṱhoḓisiso iyi?**

A huna khohakhombo dzine vha nga dzi wana nga u dzhenelela havho. U kuvhanganya mafhambuwa zwi ḓo itwa nga murahu ha musi ṅwana a tshi khou bvisa malaṱwa nga tshifhinga tsha u tshuluwa.

**Ndi nnyi ane a ḓo kona u swikelela rekhodo yavho ya dzilafho?**

Mafhungo avho nga ha dzilafho ḽavho zwi ḓo kona u swikelelwa fhedzi nga dokotela, nese na tshigwada tsha ṱhoḓisiso.

Madzina avho ha nga anḓadzwi tshitshavhani nahone arali mawanwa a ngudo iyi a tshi khou tea u anḓadzwa kana u ṋetshedzwa, mafhungo avho a ḓo vha o ṱalulwa nga nomboro ya khoudu. Madzina avho a ḓo vhulungwa lwa tshiphiri.

**Arali hu na zwiṅwe-vho zwine vha ṱoḓa u zwi ḓivha kana u zwi ita?**

Vha nga kwamana na Prof. Natasha Potgieter (Department of Microbiology / University of Venḓa) Luṱingo: 015 962 8256 arali vha na dziṅwe mbudziso dzine vha vha nadzo kana musi vha tshi khou ṱangana na thaidzo.

**Muano nga mudzheneleli:**

Nga u saina hafha fhasi, Nṋe …….ndo tenda u dzhenelela kha ngudo ya

ṱhoḓisiso ya ṱhoho hei: "**Zwiṱaluli zwa Molukhuli dza Virasi ya Noro dzine dza Monolodza Vhuponi ha Mahayani kha Vundu ḽa Limpopo, Afrika Tshipembe"**

Ndi khou bula zwauri:

- Ndo vhala kana ndo vhalelwa mafhungo haya na fomo ya thendelo yo ṅwalwa nga luambo lune nda luamba na u lu pfa zwavhuḓi.
- Ndo vha na tshifhinga tsha u vhudzisa dzimbudziso nahone mbudziso dzanga dzoṱhe dzo fhindulwa zwavhuḓi.
- Ndo zwi pfesesa uri u dzhenelela kha ngudo iyi ndi **u tou funa iwe muṋe** nahone a tho ngo tou kombetshedzwa u vha tshipiḓa kha ngudo hei.

Tsaino yo itwa (Fhethu)…….nga ḽa (datumu)……..2012.

_____ _____

**Tsaino ya mudzheneleli** **Tsaino ya ṱhanzi**

**Muano nga musedzulusi:**

Nṋe (dzina) ……ndi khou ana uri:

. Ndo ṱalutshedza mafhungo a re nga ḽiṅwalo heḽi kha Vho - ……

. Ndo vha ṱuṱuwedza uri vha vhudzise mbudziso, nahone nda dzhia tshifhinga tsho teaho u dzi fhindula.

. Ndo fushea ngauri vho pfesesa zwipiḓa zwoṱhe zwa ṱhoḓisiso sa zwe zwa ṱalutshedzwa afho nṱha.

. Ndo shumisa / kana a thon go shumisa muḓologi. (Arali muḓologi o shumiswa, muḓologi u fanela u saina muano u re afho fhasi.

Tsaino yo itwa (fhethu)……nga ḽa (datumu)……2012.

_____ _____

**Tsaino ya musedzulusi** **Tsaino ya ṱhanzi**

**Muano nga muḓologi:**

Nṋe(dzina)……..ndi khou ana uri:

- Ndo thusedza musedzulusi (dzina)……u ṱalutshedza mafhungo kha a re kha ḽiṅwalo heḽi kha (dzina ḽa mudzheneleli)……hu tshi khou shumiswa luambo lwa Tshivenḓa.
- Ro muṱuṱuwedza u vhudzisa dzimbudziso ra dzhia tshifhinga tsho teaho u dzi fhindula.
- Ndo ṋekedza mafhungo one one o teaho a sina tshiṱahe kha zwe zwa vha zwi tshi khou elana na nṋe
- Ndo fushea ngauri mudzheneleli o pfesesa nga vhuḓalo zwine zwa vha kha ḽiṅwalo ḽa thendelo ḽo tevhelaho maga a mulayo o ṋetshedzwaho nahone mbudziso dzawe dzoṱhe dzo fhindulwa lu fushaho

Tsaino yo itwa (fhethu)……. nga ḽa (datumu)…..……2012.

_____ _____

**Tsaino ya Muḓologi** **Tsaino ya ṱhanzi**

FOMO YA THENDELO NA BAMBIRI ḼA ZWIDODOMBEDZWA ZWA MUDZHENELELI

ŢHOHO YA THANDELA YA ŢHOḌISISO:
ZWIŢALULI ZWA MOLUKHULI DZA VIRASI YA NORO DZINE DZA MONOLODZA VHUPONI HA MAHAYANI KHA VUNDU ḼA LIMPOPO, AFRIKA TSHIPEMBE

Nomboro ya Referentsi: SMNS/12/MBY/07

Vhasedzulusi: Mr. Jean Pierre Kabue Ngandu (Mutshudeni wa PhD)

Prof. Natasha Potgieter (Muţoli)

ḌIRESI: Department of Microbiology

School of Maths and Natural Sciences

University of Venda

Luţingo: 015 962 8256

Ṅwana wavho kana tshifuwo tshavho zwi khou humbelwa u dzhenelela kha u vha tshipiḓa kha thandela hei ya ţhoḓisiso. Vha khou humbelwa uri vha dzhie tshifhinga vha vhale mafhungo o ṅetshedzwaho hafha, ane a ḓo ţalutshedza nga u pfufhifhadza thandela hei. Kha vha vhudzise mushumi wa ngudo iyi mbudziso dziṅwe na dziṅwe nga ha tshipiḓa tshine vha sa khou tshi pfesesa zwavhuḓi. U dzhenelela havho ndi ha u tou funa nahone vha a tendelwa u hana arali vha sa funi u dzhenelela.

Ngudo hei yo tendelwa nga komiti ya Human Research Yunivesity ya Venḓa nahone i ḓo itwa ho sedzwa maitele na milayo ine fanela u tevhedzwa nga vha International Declaration of

Helsinki, na nga maitele a mashumele avhuḓi a Afrika Tshipembe na maitele a ṱhoḓisiso ano fanela u tevhedzwa a Medical Research Council (MRC).

Thandela iyi ya ṱhoḓisiso ndi ya mini?

. Ngudo hei i ḓo katela mafhambuwa a ṅwana wa vhukale ha miṅwaha miṱanu ane a khou dinwa nga u tshuluwa, zwifuwo zwa hayani, sambula dza virasi dzi no wanala kha tshidzulo tsha bungani na sambula dza maḓi. Ngudo iyi i ḓo katela vhana vha 1000, zwifuwo zwa hayani zwa 400, sambula dza maḓi dza 400 na sambula dza virasi dzi no wanala kha tshidzulo tsha bungani dza 300.

. Ṱhoḓisiso hei yo livhiswa kha u ṱoḓulusa vhushaka vhune ha vha hone kha virasi dza Noro dzi no wanala kha vhathu na virasi dza Noro dzi no wanala kha zwifuwo dzine dza monolodza vhuponi ha mahayani kha Vundu ḽa Limpopo.

Mafhungo haya a ḓo thusa hu tshi dzhiwa tsheo kha maitele a tshitshavha a u thivhela phiriselo ya vhulwadze ha u tshuluwa nga maanḓa kha u kavhiwa nga virasi ya Noro na kha u khwinifhadza fhethu hune ha vha na mabunga kha zwitshavha zwa mahayani.

Mawanwa a ngudo iyi a ḓo ṋetshedza mafhungo nga ha u phaḓalala ha virasi ya Noro ho sedzwa kha u bveledza dzilafho.

Mafhungo oṱhe ane a khou ṱoḓea a ḓo waniwa kha vhone, zwi tshi katela na nḓila ine ra nga vha kwama ngayo, vhukale, mbeu, ḓuvha ḽa u tshuluwa, tshiimo tshavho tsha HIV na maṅwe malwadze. Mafhambuwa a linganaho 10g a ḓo dzhiwa kha ṅwana wavho kana kha tshifuwo tshavho, zwenezwo zwi ḓo iswa laborothari u senguluswa.

Ndi ngani vhone vho humbelwa u dzhenelela?

Ṅwana wavho kana tshifuwo tshavho zwo nangiwa kha ngudo iyi ho sedzwa u tshuluwa hune a khou vha khaho.

Vhone vha fanela u ita zwifhio?

U dzhenelela kha ngudo iyi ndi zwa u tou funa nga iwe muṋe. Vha nga hana u ṋekedza mafhungo kana mafhambuwa.

Vha ḓo vhuelwa nga u vha tshipiḓa tsha thandela iyi ya ṱhoḓisiso?

A huna ndiliso ya tshelede ine vha ḓo ṋewa nga u dzhenelela havho. Fhedzi zwa konadzea vha ḓo wana mvelelo dza tsenguluso ya zwisinisa na dza tsenguluso ya zwitshili.

Hu na khohakhombo dzine vha nga dzi wana nga u vha tshipida kha ṱhoḓisiso iyi?

A huna khohakhombo dzine vha nga dzi wana nga u dzhenelela havho. U kuvhanganya mafhambuwa zwi ḓo itwa nga murahu ha musi ṅwana a tshi khou bvisa malaṱwa nga tshifhinga tsha u tshuluwa.

Ndi nnyi ane a ḓo kona u swikelela rekhodo yavho ya dzilafho?

Mafhungo avho nga ha dzilafho ḽavho zwi ḓo kona u swikelelwa fhedzi nga dokotela, nese na tshigwada tsha ṱhoḓisiso.

Madzina avho ha nga anḓadzwi tshitshavhani nahone arali mawanwa a ngudo iyi a tshi khou tea u anḓadzwa kana u ṋetshedzwa, mafhungo avho a ḓo vha o ṱalulwa nga nomboro ya khoudu. Madzina avho a ḓo vhulungwa lwa tshiphiri.

Arali hu na zwiṅwe-vho zwine vha ṱoḓa u zwi ḓivha kana u zwi ita?

Vha nga kwamana na Prof. Natasha Potgieter (Department of Microbiology / University of Venḓa) Luṱingo: 015 962 8256 arali vha na dziṅwe mbudziso dzine vha vha nadzo kana musi vha tshi khou ṱangana na thaidzo.

Muano nga mudzheneleli:

Nga u saina hafha fhasi, Nṋe .....ndo tenda u dzhenelela kha ngudo ya ṱhoḓisiso ya ṱhoho hei: "Zwiṱaluli zwa Molukhuli dza Virasi ya Noro dzine dza Monolodza Vhuponi ha Mahayani kha Vundu ḽa Limpopo, Afrika Tshipembe"
Ndi khou bula zwauri:

- Ndo vhala kana ndo vhalelwa mafhungo haya na fomo ya thendelo yo ṅwalwa nga luambo lune nda luamba na u lu pfa zwavhuḓi.
- Ndo vha na tshifhinga tsha u vhudzisa dzimbudziso nahone mbudziso dzanga dzoṱhe dzo fhindulwa zwavhuḓi.
- Ndo zwi pfesesa uri u dzhenelela kha ngudo iyi ndi u tou funa iwe muṋe nahone a tho ngo tou kombetshedzwa u vha tshipiḓa kha ngudo hei.

Tsaino yo itwa (Fhethu).....nga ḽa (datumu).....2014.

_____ _____

Tsaino ya mudzheneleli Tsaino ya ṱhanzi

5

Muano nga muḓologi:

Nṋe(dzina).....ndi khou ana uri:

- Ndo thusedza musedzulusi (dzina).....u ṱalutshedza mafhungo kha a re kha liṅwalo heḽi kha (dzina ḽa mudzheneleli).....hu tshi khou shumiswa luambo lwa Tshivenḓa.
- Ro muṱuṱuwedza u vhudzisa dzimbudziso ra dzhia tshifhinga tsho teaho u dzi fhindula.
- Ndo ṋekedza mafhungo one one o teaho a sina tshiṱahe kha zwe zwa vha zwi tshi khou elana na nṋe
- Ndo fushea ngauri mudzheneleli o pfesesa nga vhuḓalo zwine zwa vha kha liṅwalo ḽa thendelo ḽo tevhelaho maga a mulayo o ṋetshedzwaho nahone mbudziso dzawe dzoṱhe dzo fhindulwa lu fushaho

Tsaino yo itwa (fhethu)..... nga ḽa (datumu).....2014.

_____ _____

Tsaino ya Muḓologi Tsaino ya ṱhanzi

6

# PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

## Molecular Characterization of Noroviruses circulating in rural communities of Limpopo province: Role of Water, Sanitation and Animals domesticated

Reference Number: SMNS/12/MBY/07

Investigators:

**Mr Jean Pierre KABUE NGANDU (PhD Student)**

**Prof NATASHA POTGIERTER (Supervisor)**

Address:
Department of Microbiology
School of Maths and Natural Sciences
University of Venda
**Contact Number: 0159628256**

Your baby /or animal is being invited to take part in a research project. Please take some time to read the information presented here, which will explain briefly the project. Please ask the study staff any questions about any part that 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?**

**.** This study will include stools from young children under 5 years with diarrhea, domesticated animals, toilet seat samples and water samples**.** About 500 children, 400 domesticated animals, 100 water samples and 200 toilet seats will be included in this study.

. The project aimed to investigate the relationship between Human Norovirus and Animal Norovirus circulating in the rural communities of the Limpopo province.
This information will help decisions making in public prevention strategies against diarrhea disease transmission particularly in Norovirus infection also in the improvement of sanitary

environments in the rural communities. The findings of this study will also provide information on Norovirus diversity with implications on vaccine development.

General information will be taken from you, including contact details, age, gender, use of toilet, date of diarrhea, HIV status and others illnesses, ect. A total of 10 g of stools will be collected from your baby /or animal and will be transported to the laboratory for analysis.

**Why have been invited to participate?**

Your baby /or animal was selected for this study on the basis of the diarrhea he is suffering from.

**What will your responsibilities be?**

Participation on in this study is completely voluntary. You may refuse to provide information or stool.

**Will you benefit from taking part in this research project?**

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

**Are there in risks involved in your taking part in this research?**

There are no risks involved in participating. Collection of stools will be done after or when the baby is eliminating waste during diarrhea episodes.

**Who will have access to your medical records?**

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

**Is there anything else that you should know or do?**

You may contact Prof Natasha Potgieter (Department of Microbiology / University of Venda) at tel 0159628256 if you have any further queries or encounter any problems.

**Declaration by participant:**

By signing below, I ……..agree to take part in a research study entitled "**Molecular Characterization of Noroviruses circulating in rural communities of Limpopo province: Role of Water, Sanitation and Animals domesticated**"

I declare that:

- I have read or had to 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 pressurized to take part.

Signed at (place)……..on
(date)……..2014.

_____
_____
**Signature of participant** **Signature of witness**

**Declaration by investigator:**

I (name) ……declare that:

. I explained the information in this document to …….

. I encouraged him/her to ask questions and took adequate time to answer them.

. I am satisfied that he/she 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)……2014.

_____
_____
**Signature of investigator** **Signature of witness**

**Declaration by interpreter:**

I(name)……..declare that:

. I assisted the investigator(name)……to explain the information in this document to (name of participant)……using the language medium of Venda.

. We encourage him/her to ask questions 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 had all his/her question satisfactorily answered.

Signed at (place)……..on
(date)…….2014.

_____
_____

**Signature of Interpreter** **Signature of witness**

**A.5. Capture forms: Asymptomatic (AS) and Symptomatic (S) cases**

**Department of Microbiology, School of Mathematical and Natural Sciences**

**University of Venda:**

**RESEARCH PROJECT DATA CAPTURE FORM AS:**

Subject Number:……

**Consultation Details:**

Date:……Visit Number:……Site / Clinic name:……

**Patient Information:**

Name:……Date of Birth:……Gender:……

Contact Details:……Parent status:……

Water source: Tap ☐ Spring / River / Wells / Boreholes: ☐ Toilet seat use: ☐

Livestock: ☐

**Medical History:**

No Symptoms of: Diarrhea ☐ Vomiting ☐ Fever ☐

Acute respiratory infections ☐

Reported clinical immunodeficiency ☐

No symptoms within the last 4 weeks: ☐ Within two days after collection ☐

Rota Vaccine dose received: ☐

**Sample Collection:**

Date of Collection:……

Type of Stools: Watery ☐ Sausage ☐ Mushy ☐

**Treatment:**

Current:……...

Previous:……

**Laboratory Results:**

**Real-time RT-PCR:……**

**Sequencing:……**

**Department of Microbiology, School of Mathematical and Natural Sciences**

**University of Venda:**

**RESEARCH PROJECT DATA CAPTURE FORM S:**

Subject Number:……

Consultation Details:

Date:……Visit Number:……Site / Clinic name:……

Patient Information:

Name:……Date of Birth:……Gender:……

Contact Details:……Parent status:……

Water source: Tap ☐ Spring / River / wells / Boreholes: ☐ Use of Toilet seat: ☐

Livestock: ☐

Medical History:

Date of Onset:…… Rota Vaccine dose received: ☐

Source of Contamination: Community ☐ Outbreak ☐ Hospital ☐

Symptoms: Diarrhea ☐ Vomiting ☐ Fever ☐ Abdominal pains ☐

Dehydration ☐ Reported clinical immunodeficiency ☐

Sample Collection:

Date of Collection:……

Type of Stools: Watery ☐ Sausage ☐ Mushy ☐

Treatment:

Current:……

Previous:……

Laboratory Results:

Real-time RT-PCR:……

Sequencing:……

# APPENDIX B: DATA USED FOR STATISTICAL ANALYSIS

# Appendix C: Logistic regression and other statistic tests

## Logistic regression being positive for noro in case

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | Positive for Noro | .216 | .321 | .452 | 1 | .502 | 1.241 | .661 | 2.329 |

a. Variable(s) entered on step 1: Positive.

## Logistic regression with different Genotypes

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | Neg | | | 1.820 | 3 | .611 | | | |
| | GI | .541 | .775 | .488 | 1 | | 1.718 | .376 | 7.846 |
| | GII | .392 | .406 | .929 | 1 | | 1.479 | .667 | 3.282 |
| | GI+GII | -.226 | .468 | .233 | 1 | | .798 | .319 | 1.997 |

a. Variable(s) entered on step 1: Results.

# Logistic regression with genotypes as predictors

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | GIPos | -.098 | .405 | .058 | 1 | .809 | .907 | .410 | 2.006 |

a. Variable(s) entered on step 1: GIPos.

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | GIIPos | .125 | .331 | .143 | 1 | .705 | 1.133 | .593 | 2.167 |

a. Variable(s) entered on step 1: GIIPos.

## Mann-Whitney U

**Ranks**

| | Case | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| cT GI | 0 | 9 | 30.44 | 274.00 |
| | 1 | 42 | 25.05 | 1052.00 |
| | Total | 51 | | |
| cT GII | 0 | 16 | 75.56 | 1209.00 |
| | 1 | 88 | 48.31 | 4251.00 |
| | Total | 104 | | |

**Test Statistics[a]**

| | cT GI | cT GII |
|---|---|---|
| Mann-Whitney U | 149.000 | 335.000 |
| Wilcoxon W | 1052.000 | 4251.000 |
| Z | -.988 | -3.324 |
| Asymp. Sig. (2-tailed) | .323 | .001 |

a. Grouping Variable: Case

b. Not corrected for ties.

## t test compaing ct values in cases and control

**Group Statistics**

| | Case | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| cT GI | 0 | 9 | 30.5533 | 7.12466 | 2.37489 |
| | 1 | 42 | 28.0669 | 7.39289 | 1.14075 |
| cT GII | 0 | 16 | 34.1925 | 6.10478 | 1.52619 |
| | 1 | 88 | 27.7032 | 7.04411 | .75090 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| cT GI | Equal variances assumed | .151 | .699 | .921 | 49 | . |
| | Equal variances not assumed | | | .944 | 11.993 | . |
| cT GII | Equal variances assumed | .342 | .560 | 3.453 | 102 | . |
| | Equal variances not assumed | | | 3.815 | 22.910 | . |

## Logistic regression of predictors for specific symptoms

Vomiting

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | GIPos | .084 | .400 | .044 | 1 | .833 | 1.088 | .497 | 2.383 |
| | GIIPos | .151 | .316 | .228 | 1 | .633 | 1.163 | .626 | 2.159 |

a. Variable(s) entered on step 1: GIPos, GIIPos.

## Fever

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|
| Step 1[a] | GIPos | .226 | .431 | .274 | 1 | .601 | 1.253 | .538 | 2.919 |
| | GIIPos | -.126 | .352 | .129 | 1 | .720 | .881 | .442 | 1.758 |

a. Variable(s) entered on step 1: GIPos, GIIPos.

## Dehydration

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|
| Step 1[a] | GIPos | .030 | .596 | .003 | 1 | .959 | 1.031 | .321 | 3.312 |
| | GIIPos | -.077 | .470 | .027 | 1 | .869 | .926 | .368 | 2.326 |

a. Variable(s) entered on step 1: GIPos, GIIPos.

## Asthenia

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|
| Step 1[a] | GIPos | -.158 | .583 | .074 | 1 | .786 | .854 | .272 | 2.678 |
| | GIIPos | -.394 | .449 | .770 | 1 | .380 | .675 | .280 | 1.625 |
| | Constant | -1.864 | .234 | 63.239 | 1 | .000 | .155 | | |

a. Variable(s) entered on step 1: GIPos, GIIPos.

# Logistic regression of cT value for GI on other symptoms

## Vomiting

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGI | -.071 | .055 | 1.690 | 1 | .194 | .931 | .837 | 1.037 |

a. Variable(s) entered on step 1: cTGI.

## Fever

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGI | -.100 | .064 | 2.440 | 1 | .118 | .904 | .797 | 1.026 |

a. Variable(s) entered on step 1: cTGI.

## Dehydration

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGI | -.043 | .078 | .303 | 1 | .582 | .958 | .821 | 1.117 |

a. Variable(s) entered on step 1: cTGI.

Asthenia

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGI | -.132 | .101 | 1.701 | 1 | .192 | .876 | .719 | 1.069 |

a. Variable(s) entered on step 1: cTGI.

## Logistic regression of cT value for GII on other symptoms

Vomiting

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGII | -.071 | .041 | 3.063 | 1 | .080 | .932 | .860 | 1.009 |

a. Variable(s) entered on step 1: cTGII.

Fever

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGII | -.005 | .040 | .016 | 1 | .898 | .995 | .920 | 1.076 |
| | Constant | -1.363 | 1.131 | 1.453 | 1 | .228 | .256 | | |

a. Variable(s) entered on step 1: cTGII.

Dehydration

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Odds Ratio | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGII | -.019 | .055 | .118 | 1 | .731 | .981 | .880 | 1.094 |
| | Constant | -1.782 | 1.541 | 1.338 | 1 | .247 | .168 | | |

a. Variable(s) entered on step 1: cTGII.

Asthenia

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | cTGII | .007 | .052 | .017 | 1 | .897 | 1.007 | .909 | 1.115 |

a. Variable(s) entered on step 1: cTGII.

# Logistic regression of norovirus on whether or not watery stool

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | Positive | .454 | .243 | 3.508 | 1 | .061 | 1.575 | .979 | 2.535 |

a. Variable(s) entered on step 1: Positive.

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | GIPos | .589 | .324 | 3.300 | 1 | .069 | 1.802 | .955 | 3.403 |
| | GIIPos | .130 | .263 | .246 | 1 | .620 | 1.139 | .681 | 1.906 |
| | Constant | -.713 | .154 | 21.541 | 1 | .000 | .490 | | |

a. Variable(s) entered on step 1: GIPos, GIIPos.

# Appendix D:

## D.1. Conference abstract of the oral presentation at

## 8th INTERNATIONAL WATER & HEALTH SEMINAR

## CANNES, June 27th - 29th, 2016

**Title: "**Molecular characterization of Norovirus strains circulating in rural communities of the Limpopo province of South Africa"

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**Background:** Norovirus is now one of the emerging and commonest waterborne enteric pathogens, causing both sporadic and outbreak-related gastroenteritis worldwide. High detection rate of NoV in rural communities reflects the substantial exposure of children from rural communities to enteric pathogens possibly due to unsafe water, poor sanitation and hygiene practices.

**Objectives**: Therefore this study reports on Human NoV-infection prevalence and viral burden of NoV in children in rural South Africa.

**Methods**: Between July 2014 and April 2015, young children from rural communities of Vhembe district, South Africa, were randomly enrolled for the study. A total of 304 stool specimens from outpatient children under 5 years of age with diarrhea (n=253) and without (n=51) diarrhea were collected. NoVs were identified using real-time one-step RT-PCR.

**Results**: One hundred and four (41.1%) NoV infections were detected (62[59.6%] GII, 16[15.4%] GI, and 26[25%] GI/GII mixed) in symptomatic children and 18 (35.3%) including 9(50%) GII, 2(11.1%) GI and 7(38.9%) GI/GII mixed in asymptomatic children. NoVs were predominantly detected from children with a single episode of diarrhea (54.8%, 57/104) presenting with liquid stool (50%, 52/104). Comparison of the median $C_T$ values for NoV in symptomatic and asymptomatic cases revealed a considerable difference of estimated GII viral load from both groups, with a much higher viral burden in symptomatic children.

**D.2. Conference abstract of the oral presentation at**

## 20th INTERNATIONAL CONFERENCE ON INFECTIOUS DISEASES

## SYDNEY, AUSTRALIA, January 29th - 30th, 2018

**Title:** "Genetic Diversity of Norovirus strains in outpatient children from rural communities of Vhembe district, South Africa, 2014-2015"

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***Keywords**: Asymptomatic, norovirus genetic diversity, South african rural communities, symptomatic.*

Norovirus are now considered the most common cause of outbreaks of nonbacterial gastroenteritis. Limited data are available for Norovirus strains in Africa, especially in rural and peri-urban areas. Despite the excessive burden of diarrhea disease in developing countries, Norovirus infections have been to date mostly reported in developed countries. There is a need to investigate intensively the role of viral agents associated with diarrhea in different settings in Africa continent.

To determine the prevalence and genetic diversity of Norovirus strains circulating in the rural communities in the Limpopo Province, South Africa and investigate the genetic relationship between Norovirus strains, a cross-sectional study was

performed on human stools collected from rural communities. Between July 2014 and April 2015, outpatient children under 5 years of age from rural communities of Vhembe district, South Africa, were recorded for the study. A total of 303 stool specimens were collected from those with diarrhea (n=253) and without (n=50) diarrhea. NoVs were identified using real-time one-step RT-PCR. Nucleotide sequencing methods were performed to genotype the strains. Phylogenetic analyses were performed to compare identified NoVs genotypes to the worldwide circulating strains.

One hundred and four (41.1%) NoVs were detected. NoV detection rates in symptomatic and asymptomatic children (OR = 1.24; 95% CI 0.66 – 2.33) were not significantly different. Comparison of the median $C_T$ values for NoV in symptomatic and asymptomatic children revealed significant statistical difference of estimated GII viral load from both groups, with a much higher viral burden in symptomatic children to our knowledge this is the first study reporting on the differences in estimated viral load of GII and GI NoV positive cases and controls. The most prevalent NoV genotypes were GII.4 Sydney 2012 variants (n=7) among the capsid genotypes, GII.Pe (n=9) among the polymerase genotypes and GII.Pe/GII.4 Sydney 2012 (n=8) putative recombinants among the RdRp/Capsid genotypes. Two unassigned GII.4 variants and an unusual RdRp genotype GII.P15 were found. With note, the rare GIIP15 identified in this study, has a common ancestor with GIIP15 strain from Japan previously reported as GII / untypeable recombinant strain implicated in a gastroenteritis outbreak. To our knowledge this is the first report of this unusual genotype in the African continent.

Though not confirmed predictive of diarrhea disease in this study, the high detection rate of NoV reflects the substantial exposure of children from rural communities to enteric pathogens possibly due to poor sanitation and hygiene practices. The results suggest that the difference between asymptomatic and symptomatic children with NoV may be at the level of the viral load of NoV genogroups involved. The findings highlighted NoV genetic diversity and revealed continuous pandemic spread and predominance of GII.Pe/GII.4 Sydney 2012, indicative of increased NoV activity. An unusual RdRp genotype GII.P15 and two unassigned GII.4 variants were also identified from rural settings of the Vhembe district/South Africa. NoV surveillance is warranted to help to inform investigations into NoV evolution and disease burden, and to support on-going vaccine development programmes.