NOROVIRUS IN CHILDREN 5 YEARS AND BELOW PRESENTING WITH DIARRHOEA IN KWAZULU-NATAL

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PREFACE

In South Africa (SA), data on the specific cause of viral diarrhoea in children less than 5 years are mostly on rotavirus (RoV) and from hospitalized patients. A few studies have been carried out on out-patient populations in SA to evaluate the relative importance of other enteric viruses like Norovirus (NoV). Hence, the precise epidemiology of these other virus-associated diarrhoea in children from the community in KwaZulu-Natal remains unknown. Epidemiological data are required to understand the contribution of other enteric pathogens, especially NoV, to diarrhoeal diseases in children. Studies on the characterization of NoV continue to produce important insight regarding the pathogenesis of NoV infection as well as in vaccine development and immunity. The introduction of commercial assays based on antigen detection by monoclonal antibodies and highly sensitive molecular techniques such as the real-time reverse-transcription polymerase chain reaction (RT-PCR) for NoVs in stool has improved the rate of detection of this pathogen. The study presented here was designed to detect human noroviruses among children 5 years and below attending King Edward VIII Hospital in KwaZulu-Natal (KZN), South Africa.

DECLARATIONS

This work was carried out by me, Nelisiwe Veronica Nxele. It has not been submitted in any form to the University of KwaZulu-Natal or any other tertiary institution for the purpose of obtaining a degree or any other academic qualification. All experiments were carried out in the Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal under the supervision of Professor A Willem Sturm and co-supervised by Dr. Osaretin Emmanuel Asowata.

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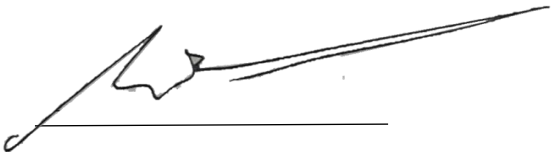

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CONFERENCE PRESENTATIONS

- Nxele NV, Asowata, OE, Ashiru OT, Sturm AW and Moodley P (2016). Detection of Human Noroviruses among children 5 years and below in KwaZulu-Natal. **College of Health Sciences Symposium,** University of KwaZulu-Natal, Durban, South Africa. Oral presentation (08-09 September 2016).

- Nxele NV, Asowata, OE, Ashiru OT, Sturm AW and Moodley P (2016). Detection of Human Noroviruses among children 5 years and below in KwaZulu-Natal. **Laboratory Medicine and Medical Science Annual Research day,** University of KwaZulu-Natal, Durban, South Africa. Oral presentation (05 August 2016).

ACRONYMS

BMI	Body mass index
C	Cytosine
DBS	Dry blood spot
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FUT	Fucosyltransferase
G	Guanine
HBGA	Histo-blood group antigen
IDEIA	Individual with disability education act
IF	Immunofluorescent
kDa	kilodaltons
KZN	KwaZulu-Natal
$M_{\phi}s$	Microphages
NLV	Norwalk like virus
NoV	Norovirus
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVA	Polyvinyl alcohol
RdRp	RNA dependant RNA polymerase
RNA	Ribonucleic acid
RoV	Rotavirus
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	real-time polymerase chain reaction
SA	South Africa
SPSS	Statistical Package for the Social Sciences

UTR	Untranslated region
USA	United States of America
VLP	Virus like proteins
VP	Virion Protein

ABSTRACT

Background - NoVs are single stranded RNA viruses belonging to the family *Caliciviridae*. They cause gastroenteritis in all age groups but mostly in young children and the elderly. They are classified into seven genogroups (GI – GVII) and only GI, GII and GIV infect humans. They cause self-limiting infection that resolves in approximately 10 – 50 hours after exposure. Symptoms include diarrhoea, vomiting, cramps, chills and headaches. Despite a high rate of vaccine coverage in KZN through the ….., mortality among infants remains high. Therefore a study was conducted to describe the role of non-bacterial aetiologies of diarrhoea in children 5 years and under in KZN. This work investigates the contribution of NoV infection to this pathology.

Methods - Stool specimens were collected between June 2014 and August 2014 from children 5 years and below presenting with diarrhoea to a regional hospital. Written informed consent was obtained from their parents or guardian. Demographic information was collected using a structured questionnaire. The specimen were tested for NoV antigen using Enzyme Linked Immunosorbent Assay (ELISA) and Real-time Polymerase Chain Reaction (RT-qPCR) was used to detect viral RNA. The two methods were than compared with each other.

Results - One hundred and eighty-two stool specimens were collected and tested for NoV. The prevalence of NoV when specimens were tested by ELISA and RT-qPCR was 10.4 % and 22.5 % respectively. The sensitivity of the ELISA in comparison to RT-qPCR was 24.4 % (95 % Cl: 12.4 – 40.3 %) and the specificity was 93.6 % (95 % Cl: 88.2 – 97 %). The infection rate was highest in children within the age group of 12 – 24 months and all the NoV detected were of the GII genogroup

Conclusion - NoV is a common cause of diarrhoeal illness in children presenting to the King Edward VIII Hospital. Genogroup GII dominated with 100 % of all positive NoV cases belonging to this group. Taking into consideration the low sensitivity of the ELISA test, the RT-qPCR would be more suitable for routinely testing stool specimens for NoV. An effective NoV vaccine is urgently needed.

CHAPTER 1

INTRODUCTION

NoVs are an important cause of acute gastroenteritis in all age groups, with the highest incidence among young children, elderly and immunocompromised individuals (Ahmed *et al.*, 2014; Belliot *et al*., 2014). NoVs are small, non-enveloped single stranded RNA viruses that belong to the family *Caliciviridae* (Lopman *et al.*, 2008). The infection is self-limiting in both healthy and immunocompromised individuals and resolves within 10 to 51 hours after exposure (Mans *et al*., 2016). The clinical presentation of NoV infection is characterized by watery diarrhoea along with vomiting, cramps, chills and headache (CDC, 2002). No therapeutic treatment is currently available against NoV infection and the possibility of a vaccine is elusive as NoV could not be cultured until recently (Ettayebi *et al.*, 2016; Ahmed *et al.*, 2014). The introduction of RoV vaccine in many parts of the world has allowed NoV to become the leading cause of gastroenteritis in young children in most of these areas (Bucardo *et al*., 2008; Payne *et al.*, 2013) .

NoVs are phylogenetically classified into seven genogroups (GI – GVII) based on the amino acid sequences (Payne *et al.*, 2013; Zheng *et al*., 2006). Three of the genogroups are known to infect humans (GI, GII and GIV). Most human NoV outbreaks are caused by GI and GII, which are the genogroups with the largest genetic diversity. Genogroup GII-genotype 4 (GII.4) is the most prevalent type causing more than 95% of human NoV infections. Genotyping is based on the genetic makeup of an organism and human populations develope immunity against a specific genotype. This has caused the fast evolution and dominance of GII.4 with new strains evolving every two to three years replacing previously overriding strains (Vinjé, 2015; Hasing *et al*., 2013; Lopman *et al.*, 2004). There has been major improvement in understanding the diversity and evolution NoV in the past decade (Eden *et al*., 2014; Eden *et al*., 2013). Surveillance systems have been successfully implemented in developed countries including the United States of America and European countries resulting in a sharp increase in the reported NoV prevalence and diversity. On the other hand, low income countries like those in Africa have few NoV surveillance systems (Mans *et al*., 2016). Reports on NoV infection in Sub-Saharan (Armah *et al*., 2006; Mans *et al.*, 2010) and Northern Africa (Benmessaoud *et al*., 2015; El Qazoui *et al*., 2014) indicated the prevalence of NoV infection to be greater in children less than 5 years compared to older children (Siebenga *et al*., 2009; Smit *et al.*, 1999).

In South Africa (SA), data on the specific cause of viral diarrhoea in children 5 years and below are mostly on Rotavirus and from hospitalized patients. A few studies have been carried out on out-patient populations to evaluate the relative importance of other enteric viruses like NoV (Mans *et al*., 2010). Hence, the precise epidemiology of viral diarrhoea from children in the population remains largely unknown. Furthermore, SA studies focusing on the number and distribution of viruses responsible for diarrhoea in infants and young children are scarce. Viral and epidemiological data are required to further understand the association of diarrhoea in children to the different viral aetiologies including NoV.

Most epidemiological information on viral diarrhoea in Africa is from hospitalized children. Few reports have been published on the viral species infecting children with mild to severe diarrhoea who are usually managed in outpatient clinics. This study was designed to determine the aetiology of diarrhoea in children attending King Edward VIII Hospital KZN, SA.

1.1 Problem statement

In South Africa (SA), data on the specific cause of viral diarrhoea in children 5 years and below are mostly on Rotavirus and from hospitalized patients using surveillance systems. A few studies have been carried out on out-patient populations in to evaluate the relative importance of other enteric viruses like Norovirus (Mans *et al*., 2010). Hence, the precise epidemiology of viral diarrhoea in out-patient children remains unknown. Furthermore, South African studies focusing on the number and distribution of viruses responsible for diarrhoea in infants and young children are scarce. Viral and epidemiological data are required to further understand the association of diarrhoea in children to the different viral aetiologies especially NoV.

This study was designed to determine the prevalence detecting human NoV in children 5 years and below attending King Edward VIII Hospital in KwaZulu-Natal (KZN) as well as the best method for its detection. Studies on the characterization and discovery of NoV continue to produce important insight regarding the pathogenesis and development of immunity of NoV infection. The inability to cultivate human NoV in cell culture has hampered vaccine develoment. Of all causes of diarrhoea only RoV infection is currently vaccine preventable. Therefore, there is a need to investigate the contribution of other causes. The introduction of commercial assays based on antigen detection by monoclonal antibodies and highly sensitive molecular techniques such as the real-time reverse transcriptase polymerase chain reaction (RT-qPCR) as the diagnostic tools for determining the presence of NoV in stools, has increased the rate of epidemiological studies. It is important to determine the characteristics of circulating enteric viruses as a prerequisite to vaccine development and to the understanding of their genetic diversity in SA.

1.2 Aim

The aim of this study was to detect human Noroviruses GI and GII among children of 5 years and under presenting with diarrhoea in KwaZulu-Natal, SA.

1.3 Objectives: -

- To detect human NoV using enzyme linked immunosorbent assay (ELISA)
- To detect human NoV GI and GII using real time reverse transcriptase polymerase chain reaction (RT-qPCR)
- To compare the result of RT-qPCR with that of the ELISA

1.2.1 History and Discovery of Norovirus

In 1929, Dr John Zahorsky, a paediatrician in St Louis proposed the term winter vomiting disease for an illness that was characterized by the sudden onset of diarrhoea and vomiting and the disease peaked during the cold winter months (Zahorsk, 1929). In October 1968, an outbreak resembling "winter vomiting disease" occurred at an elementary school in Norwalk, Ohio with high prevalence among teachers and students. This illness was characterized by diarrhoea, nausea and vomiting which resolved between 12 to 24 hours from the onset (Conly & Johnston, 2003). The causative agent of the outbreak was non-bacterial and was not cultivable, therefore viral aetiology was suspected (Adler & Zickl, 1969). As the virus, could not be grown in cell culture, the only alternative was to test it in human volunteers. Consequently, clinical studies were done using faecal filtrate free of bacteria to the viral hypothesis using faeces obtained from the Norwalk, Ohio outbreak. The volunteers did develop similar symptoms as the outbreak cases and the viral aetiology was confirmed. The virus was then named Norwalk agent and later Norwalk virus after the location where the virus was detected. In 1972, Kapikan and colleagues visualized the virus for the first time using immuno-electron microscopy (IEM) (Kapikian, 2000). They described the virus as non-enveloped small round structured viruses (27-32 nm in diameter) that caused gastroenteritis (Green *et al*., 2000). These small round structured viruses could not be cultured using existing tissue culture methodology and the nature of the virus remained obscure until molecular methods became available. In 1990, Norwalk like virus (NLV) genome was described (Xi *et al*., 1990) which led to the development of PCR assays in 1992. With full access to the NLV genetic information, it became clear that, like most viruses, different geno-groups of NLV existed within a single genus, which eventually was termed NoV (Zheng *et al*., 2006). PCR assays were then introduced into clinical studies in patients with gastroenteritis and it became clear that the virus was responsible for more cases then previously described (Amar *et al*., 2007). Estimates indicate that NoV is responsible for 12% – 40% of all gastroenteritis cases worldwide (Reuter *et al*., 2005; Amar *et al*., 2007; Patel *et al*., 2008; Ahmed *et al*., 2014).

1.2.2 Virology

NoVs are a genetically diverse group of linear positive-sense single stranded RNA viruses that belong to the family *Caliciviridae* (Lopman, 2015). They contain a poly-adenylated RNA genome of ~ 7.7 kb, which is surrounded by an icosahedral non-enveloped capsid of ~ 27 nm in diameter (Jiang *et al*., 1993). The capsid comprises of single protein, arranged into 180 protein units in a T=3 symmetry. It consists of 45 % to 56 % guanine (G) + cytosine (C). NoV infect cells directly and, upon entering the cell, acts as messenger RNA (Tan *et al.*, 2006). The 3' end of the genome is poly-adenylated, resembling messenger RNA of eukaryote cells. The 5' end is attached to a virus encoded protein known as VPg, required for infectivity (Thorne & Goodfellow, 2014). NoVs have short untranslated regions (UTRs) at the end of each genome with Human NoV having 48 nucleotides at the 3' UTR (Gutiérrez-Escolano *et al*., 2000; Karst *et al*., 2003; Pletneva *et al*., 2001). The UTRs contains conserved RNA secondary structure which are found all over the genome extending to the coding regions (Simmonds *et al*., 2008). All these structures are important in the translation, viral replication and pathogenesis of the NoV. Calicivirus are positive stranded RNA virus that replicate through the synthesis of a full length anti-genomic strand using RNA dependent RNA polymerase (RdRp) that is translated initially from the RNA genome entry into the cell host (Clarke & Lambden, 1997). The minus strand then acts as a template for the synthesis of a full length genomic RNA where non-structural protein are translated including the RdRp, helicase and protease (Simmonds *et al.*, 2008).

The NoV genome contains three conserved open reading frames (ORFs), except genome of Murine NoV which has a fourth ORF (Karst *et al*., 2014; McFadden *et al*., 2011a; Thackray *et al.*, 2007). This fourth ORF has not been identified in any other NoV except Murine NoV Genogroup Five (Clarke & Lambden 2000; McFadden *et al*., 2011a). ORF1 (194kDa) encodes for a large poly protein which is cleaved by seven non-structural proteins that are involved in the replication of the genome (Sosnovtsev *et al*., 2006). The ORF1 is more than 5 kb in length making the first two-third of the genome. ORF2 (60kDa) and ORF3 (23kDa) each encode for a structural protein, Virion Protein (VP) 1 which is a major capsid protein is believed to participate in the identification of target receptors (Hardy, 2005). It contains two P domains (P1 and P2) and one S domains. The P2 domain contains an antigen presenting site and carbohydrate receptor binding region that may facilitate infection (Hardy, 2005). VP2 is a minor capsid protein that interacts with the RNA genome when virion formation occurs (Herbert *et al*., 1996; Meyers *et al*., 1991; Morales *et al*., 2004; Herbert *et al*., 1997). NoV also contains a sub-genomic RNA identical to the last 2.4 kb of the actual genome (ORF2 and ORF3). The 5' end of the sub-genomic RNA is attached to the VPg and the 3' end is also attached to a poly-A-tail (Herbert *et al*., 1997).

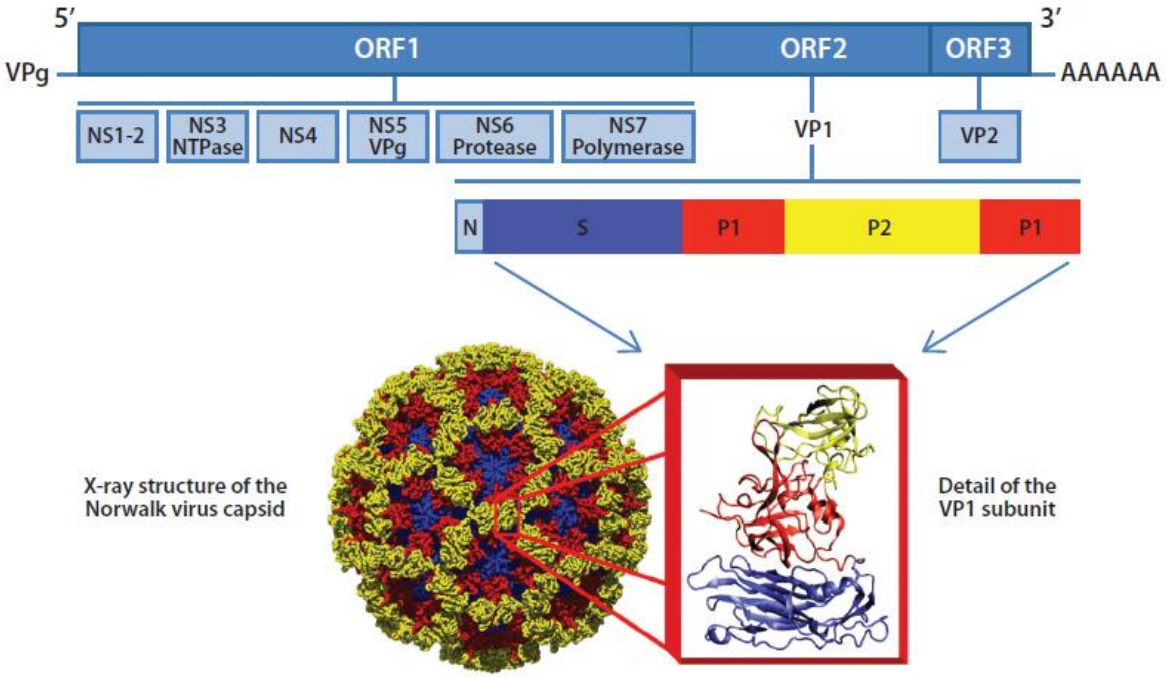

Figure 1: Human NoV genome organization (Lopman, 2015)**.**

NoV is one of 5 genera within the *Caliciviridae* family. The other genera include Sapovirus, Lagovirus, Vesivirus and the most recent Nebovirus (Clarke *et al*., 2012). Sapovirus and Norovirus are the only genera that cause human gastroenteritis. Currently NoV is phylogenetically classified into seven Genogroups (GI – GVII) (Fig.2) based on the amino acid differences in the major structural protein (VP1) (Zheng *et al*., 2006; Vinjé 2015). Each of these genogroups can be divided into genetic clusters called genotypes. Three of the genogroups are known to infect humans (GI, GII and GIV). Most Human NoVs outbreaks are caused by GI or GII, which are the genogroups with the largest genetic diversity (Hansman *et al*., 2006). GII is the most prevalent genogroup, causing more than 95 % of human NoV infections. GIV can be also found in humans, while GIII and GV strains are found in cows and mice, respectively (Vinjé, 2015). Canine NoV is a recently discovered pathogen in dogs, with strains classified into genogroups IV and VI. NoV GII has 21 genotypes, GI has nine and GIV has two (Kroneman *et al*., 2013).

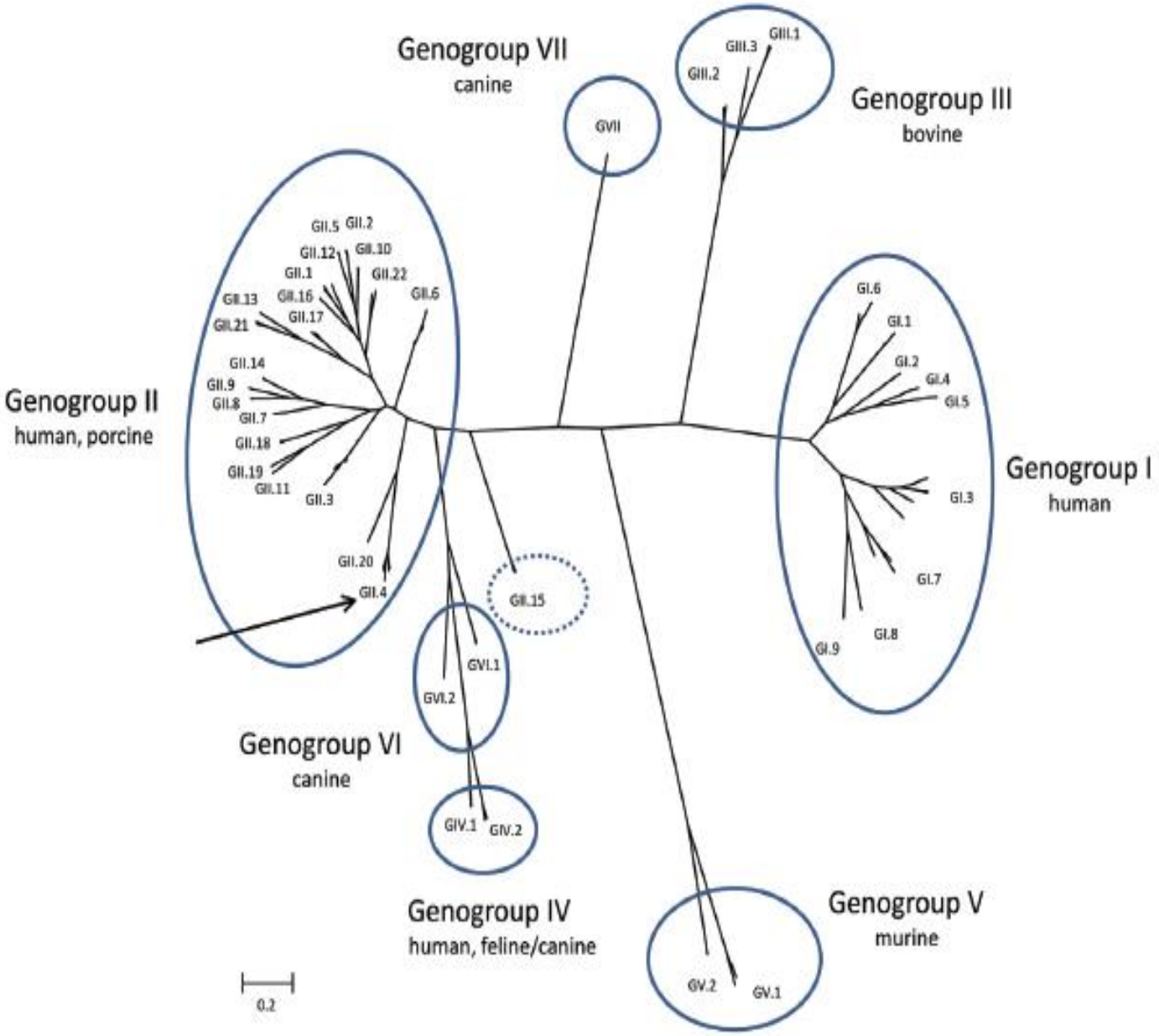

Figure 2: Classification of NoV into seven genogroups (GI – GVII) (Vinjé, 2015)

Since the mid-90s the majority of the global NoV outbreaks as well as sporadic cases have been caused by GII.4 viruses (Lopman, 2015). The evolution of GII.4 NoV and the development of new epidemic strains is caused by the mutation in the hyper variable region in the part of the genome that encodes the capsid which is the P2 domain of the 2nd ORF (Tan *et al*., 2003; Chen *et al*., 2004; Lindesmith *et al*., 2008; Boon *et al*., 2011; Bull *et al*., 2010; Bull & White 2011). These mutations cause major changes in the epitope, causing the virus to be unrecognizable by the antibodies that have been acquired from

previous exposure (Bull & White, 2011). This process is known as epochal evolution, and was first described for influenza viruses to explain the emergence of epidemic strains (Lopman, 2015).

1.2.3 Host susceptibility

The cell tropism of human NoV remains a mystery. When intestinal biopsy of human volunteers infected with GI.1 and GII.1 NoV were evaluated by immune-electron microscopy (IEM), viral particles were not detected (Dolin *et al*., 1975; Agus *et al*., 1973). This lead to the assumption that human NoV infects a small number of cells in vivo. The attempts to develop a cell culture system to grow NoVs has proven to be unsuccessful as they could not be cultured (Duizer *et al.*, 2004). However, recent advances with animal models suggest that several cell types including dendritic cells (DC), macrophages ($M_{\varphi}s$) and enterocytes support the in vivo replication of NoV at low levels (Karst, 2010) .

Histo-group blood antigens (HBGAs) are carbohydrates linked by proteins or lipids in the surface of mucosal epithelial cells that are recognized by human NoV (Tan & Jiang, 2005) . The glycosyltransferases that control HBGAs synthesis are encoded by the Lewis (FUT3) and Secretor (FUT2) gene families (Bucardo *et al.*, 2008). The association between NoVs and HBGAs has been proven to be essential for NoV strains (Karst, 2010). HBGAs are used as receptors by NoV and NoVs have adapted themselves into using different types of HBGA. 80% of the population are individuals with wild type FUT 2 gene which susceptible to Norwalk-virus infection and are referred to as secretors, while 20% of the population have a null FUT 2 gene which is completely resistance to Norwalk-virus infection and are referred to as non-secretors (Lindesmith *et al*., 2008) . Tan and colleagues described that the NoV strains had distinct binding profiles, this suggesting that strains can be differentiated into those that binds Lewis epitope or those that binds A or B epitope (Tan *et al*., 2009). GI and GII have genogroup specific differences in there receptor binding interface of the capsid that interacts with the HBGAs, therefore even if both genogroups interact with the same HBGAs, the specific residues in the binding will be different (Karst, 2010). The high prevalent GII.4 NoV strains that were circulating in the 1970s to the 1980s all displayed a similar HBGAs binding pattern (B antigen, LewisY and H type 3), a strain circulating in 1997 used the same HBGAs profile with a strain that was circulating in 2006 along with A and $Lewis_B$ antigen (Cannon *et al*., 2009). Strains isolated from a 2002 outbreak were unique from strains isolated from outbreaks in 2004 and 2005, they failed to bind to HBGAs suggesting that they bind to other carbohydrates (Bok *et al*., 2009; Lindesmith *et al*., 2008).

Although NoV strains show a distinct HBGAs binding pattern, they can all infect almost everyone because of their high genetic variation (Le Pendu *et al*., 2006). This showing the likelihood of human NoV to co-evolve together with their human host (Karst, 2010). The association with HBGAs is

important for in vivo infection even though cell lines expressing HBGAs in vitro are resistant to infection and transfection of the FUT 2 gene does not promote infection (Duizer *et al*., 2004; Guix *et al.*, 2007). It is suggested that cells expressing HBGAs are not the intended target of NoV infection, and that the binding to HBGAs that are found on the surface of enterocytes may facilitate the attachment of NoV to the intestinal wall while the binding of NoV to other neighbouring cells like dendritic cells and macrophages may be essential for the entry and replication of the virus (Karst, 2010).

1.2.4 Epidemiology

NoV affect individuals of all age groups but the highest rate of gastroenteritis is among young children (Lopman, 2015). The estimated incidence NoV for all age groups is mostly available in developed countries. With a life expectancy of 80 years, an average person will experience 3 to 8 episodes of NoV illness in their lifetime, of which one of them will occur before age 5 (Phillips *et al*., 2010). The incidence of NoV infection is 5 times higher in children under 5 years compared to the rest of the population (Phillips *et al*., 2010). The incidence of disease across all age groups in low income countries is not well documented. Therefore, comparing age specific incidence is difficult between those countries. A study in Peru demonstrated the incidence of NoV to be 50 per 100 person years in the first two years (Saito *et al*., 2014), with the highest incidence in children between 6 – 23 months suggesting that children between 0 – 5 months are protected by maternal antibodies (Lopman, 2015). Other studies, in Kenya and Guatemala estimated the incidence of NoV to be 10 times higher in children 5 years and below compared to children older than 5 years (Lopman, 2015). Generally, the incidence of gastroenteritis caused by NoV is highest in the age group 6 – 23 month (Shioda *et al*., 2015). Nearly all children are infected at least once by 5 years of age (Son *et al*., 2013; Menon *et al*., 2013; Nurminen *et al*., 2011) and multiple infections are a result of short lived immunity and no cross protection to the diverse strains of NoV. Substantial progress in understanding the diversity and evolution of NoV gastroenteritis has been made in the past decades (Eden *et al*., 2014; Eden *et al*., 2013; Siebenga *et al*., 2009). Developed countries like the United States and the European countries have implemented surveillance systems that resulted in an increase in NoV data and diversity while African continents only exhibit a few NoV surveillance studies (Mans *et al*., 2016). Those southern African studies demonstrated a mean overall prevalence of 13.5 % (range 0.8– 25.5 %) for NoV infection in young children. More recently, and reports from other parts of Africa identified similar prevalence's of NoV infection (Smit *et al*., 1999; Taylor *et al*., 1996; Mans *et al.*, 2016).

1.2.4.1 Globally

NoV is responsible for almost 50 % of all non-bacterial gastroenteritis outbreaks and 18 % of all gastroenteritis cases worldwide (Patel *et al*., 2009). This figure is skewed due to the scarcity of surveillance data from under developed countries. The estimated annual cost of Norovirus related health care is about 777 million US dollars in the USA alone. In 2009 and 2010, NoV was responsible for 14 000 hospitalizations, 281 000 emergency visits, and 627 000 outpatient visits in the USA, representing $273 million of direct health costs for each of the 2 years, nationwide, for children aged 5 years and below (Payne *et al*., 2013). Australia is estimated to have 1.8 million cases of NoV infection each year (Hall *et al*., 2005). In 2002 to 2008, more than 700 000 cases of NoV infection were reported in Germany and 26 % of those cases resulted in hospitalization (Belliot *et al*., 2014). In England, NoV was responsible for 9 % of gastroenteritis related hospitalization which increased to 19 % when elderly were included (Haustein *et al*., 2009). England estimated the annual loss of $184 million due to nosocomial gastroenteritis outbreaks conducted from a survey in 1999 and a large part was caused by NoV infection (Lopman *et al.*, 2004). Studies in England and Wales suggested death associated with NoV infection of individuals older than 65 years might reach 20 % (Belliot *et al*., 2014). Many countries have not studied the prevalence of NoV in sufficient detail to gain reliable burden estimates. There is scarcity of data from Africa where the effect of gastroenteritis probably has more severe consequences (Desselberger & Goodfellow, 2014). Therefore, additional high-quality studies in these settings is crucial to improve disease estimates.

1.2.4.2 Africa

The greatest burden of diarrheal disease occurs in developing countries, with up to 200 000 deaths each year in children less than 5 years of age alone (Patel *et al*., 2008). In 1996 and 1999 Southern African countries like Angola, Zimbabwe, Namibia and Mozambique published reports on the high prevalence of IgG antibodies GI.1 (Atmar *et al*., 2008). South Africa (SA) first encountered NoV associated gastroenteritis in 1993 where genogroups GI.1 (Hawaii strain) and GII.1 (Norwalk strain) were identified as the causative agents in outbreaks (Taylor *et al*., 1993). In 1996, a study in Pretoria investigated the effect of Norwalk virus infection in young children and adults up to the age of 40 years. They reported that 57 % of the population was exposed to NoV between age 1 – 2 years (Taylor *et al*., 1996). In 1997, the estimated prevalence for human calicivirus was 3.3 % with NoV signifying 89 % and 11% for sapoviruses (Wolfaardt *et al*., 1997) NoV GI and GII were both identified but GII was the most dominant. Another study was reported in both urban and rural population of SA where antibody prevalence levels ranged between 94 – 96 % (Smit *et al.*, 1999). NoV was also characterized in the year 2008 in hospitalized paediatric patients with acute gastroenteritis in a Pretoria hospital where the strain of GII.4 was first reported in SA (Janet *et al*., 2010). SA has no mandatory gastroenteritis outbreak

reporting system and therefore, outbreaks of NoV remain underreported and the prevalence of infection underestimated.

1.2.5 Norovirus Seasonality in Africa

In Africa, NoV seasonality is not well understood compared to the northern hemisphere (Mans *et al*., 2016). North African countries experienced NoV infection in different months. In Morocco and Libya, NoV peaked in June to September (summer months), Egypt NoV peaked in October to May (cold months) while NoV infection in Tunisia no clear seasonal pattern was observed (El Qazoui *et al*., 2014; Abugalia *et al*., 2011; Benmessaoud *et al*., 2015; Rackoff *et al*., 2013; Sdiri-Loulizi *et al*., 2009; Hassine-Zaafrane *et al*., 2013). In West Africa, Nigeria experienced NoV infections in October (rainy month) while in Ghana and Burkina Faso NoV peaked in December to April (cool dry months) (Nordgren *et al*., 2013; Armah *et al*., 2006; Krumkamp *et al*., 2015; Oluwatoyin *et al*., 2012). In East Africa, only a study in Tanzania described seasonality of NoV, which was detected in April, at the end of the rainy season (Moyo *et al*., 2014). In Southern Africa, Malawi experienced NoV at end of rainy months and in South Africa NoV peaked in September to November (Dove *et al*., 2005; Trainor *et al*., 2013; Mans *et al*., 2010). Ghana and Egypt NoV seasonality NoV was the same as for Rotavirus both falling in cool, dry months. The peak of NoV and Rotavirus did not overlap for other African countries. Climate change in Africa can be divided into wet and dry seasons with North and Southern Africa experiencing four seasons. NoV seasonality can be influenced by many factors, these include humidity, temperature, rainfall and human behaviour (Mans *et al*., 2016). Studies have shown the murine NoV favours low humidity for its survival (Colas de la Noue *et al*., 2014). Most African countries experience a peak NoV activity in cold and dry months of the year. This might be associated with improved virus survival in low humidity environments. Cultural behaviour and population densities may also be a reason for different seasonality that is experienced across the African continent (Colas de la Noue *et al*., 2014).

1.2.6 Molecular Epidemiology

Although 32 genotypes have been identified within human NoV (Kroneman *et al*., 2013), GII.4 is still the genotype frequently associated with gastroenteritis pandemics and it accounts for more than 80 % of all human NoV infections (Siebenga *et al*., 2009; Tu *et al*., 2008). Because human NoV could not be cultured, its evolution remains unknown and it is difficult to explain why GII.4 viruses are successful (White, 2014). and is currently still in research (Siebenga *et al*., 2009). GII.4 is responsible for 6 major pandemic outbreaks that occurred in the last 2 decades. These pandemic include the US 96 that occurred in the 1990s (Noel *et al*., 1999; White *et al*., 2002), Farmington Hills – 2002 (Lopman *et al*., 2004; Widdowson *et al*., 2004), Hunter – 2004 (Bull *et al*., 2006), Den Haag – 2006b (Tu *et al*., 2008; Eden

et al., 2010), New Orleans – 2009 (Yen *et al*., 2011) and the most recent Sydney – 2012 (Eden *et al*., 2014). The first four pandemics are thought to have occurred due to mutation in the P domain that resulted due to an antigenic drift from the previous GII.4 viruses (Bull *et al*., 2010; Debbink *et al*., 2012; Donaldson *et al*., 2010). Nonetheless, the recent two cities, New Orleans – 2009 and Sydney – 2012 both show the intra-genotype recombination where ORF1 and ORF2 overlaps and they have evolved through a process of antigenic shift and antigenic drift (Eden *et al*., 2013). The emerging of GII.4 corresponded with the sharp increase of gastroenteritis epidemics and Norovirus infections globally. This was shown by the Sydney – 2012 GII.4 pandemic which was first identified in Australia in March 2012 (Bull *et al*., 2010), by August 2012, it was responsible for 25 % of acute gastroenteritis associated with NoV (Eden *et al*., 2014). By November 2012 to January 2013, it had replaced most of GII.4 NoV resulting in an increase in the number of gastroenteritis outbreaks across the world. Currently Sydney – 2012 is the most prevalent NoV, although looking at past evolutional patterns of GII.4, it is expected that in a couple years to come, it will be replaced by a new GII.4 variant (White, 2014). GII.3 also causes sporadic infection in young children (Liu *et al.*, 2014). It was dominant in various countries in the late 1970 – 1980s and early 1990s. GII.17, infection of pigs, dogs, cattle and macaques emerged during 2014 – 2015 epidemic in China and South-east Asia, causing major epidemic of gastroenteritis (Huang *et al.*, 2017).

1.2.7 Norovirus in different age groups

1.2.7.1 Children

Children aged 5 years and below have the highest incidence of NoV gastroenteritis. The incidence of disease is between 15 900 to 27 700 per 100 000 population in children <5 years and this is 6,5 times the incidence of children ≥ 5 years (Phillips *et al*., 2010). Children in this age group have shown higher rates of NoV associated with outpatient's visits, hospitalization and emergency department visits. In USA where RoV vaccine is widely used (Payne *et al*., 2013), NoV is now the leading cause of gastroenteritis in children 5 years and below but data from developing countries is still lacking (Belliot *et al*., 2014). NoV is associated with 18 % all diarrheal disease worldwide in children 5 years and below and NoV has been detected in stool of diarrhoea-free children which makes it difficult to know the correct burden of disease in children from developing countries (Kotloff *et al*., 2013; Lopman *et al*., 2014).

1.2.7.2 Adults

People 65 years and older suffer more from NoV infection than younger adults. With more than 800 deaths per year in USA alone, the mortality ratio in this age group is 20 times higher than the mortality of people aged 18 – 64 years of age (Verhoef *et al*., 2013; Desai *et al*., 2012). Elderly people in general do not have a higher risk of infection. Those individuals living in old-age homes and other care facilities

that have a higher risk of being affected during outbreaks (Wikswo & Hall 2012; Kroneman *et al*., 2008). These outbreaks are caused by GII.4 virus which appears to result in more severe outcome compared to other genogroups and causes hospitalization and death (Desai *et al*., 2012). There is increasing evidence that NoV outbreaks in nursing homes causes excess mortality (Trivedi *et al*., 2012).

1.2.8 Clinical features and Treatment

NoV symptoms are described based on reports from gastroenteritis outbreaks and human volunteer studies (Moreno-Espinosa *et al.,* 2014). The clinical presentation includes diarrhoea, vomiting, nausea, fever, chills, myalgia and abdominal cramps. Diarrhoea and vomiting are the predominant symptoms of NoV infection, being present in 90 % and 75 % of cases respectively (Rockx *et al*., 2002). Adults normally experience diarrhoea as the predominant symptom while young children experience nausea and vomiting more than diarrhoea. NoV diarrhoea is characterised by the passage of loose or watery stool without blood or mucus at least three time a day (Mans *et al*., 2016). Nausea and vomiting result from the brief gastroparesis that occurs after viral infection and is resolved when the illness resolves itself in 12 – 72 hours (Meeroff *et al., 1980)*. NoV infections are mostly self-limiting and most patients recover without becoming debilitating and life-threatening. Young children and elderly people are at risk of dehydration. This can lead to electrolyte disturbance which can result in hospitalization or death if not treated (Lopman *et al*., 2003). Patients become resistant to re-infection only for 4 – 6 months, as there is no development of long term immunity. Studies involving human volunteers revealed that infection starts in the small bowel where there is expansion of the villi and shortening of the microvilli (Wilhelmi *et al*. 2003). Nearly 30% of NoV infections are asymptomatic. People with asymptomatic infection have a similar viral load and shedding duration as symptomatic individuals (Rockx *et al*., 2002)

1.2.9 Transmission

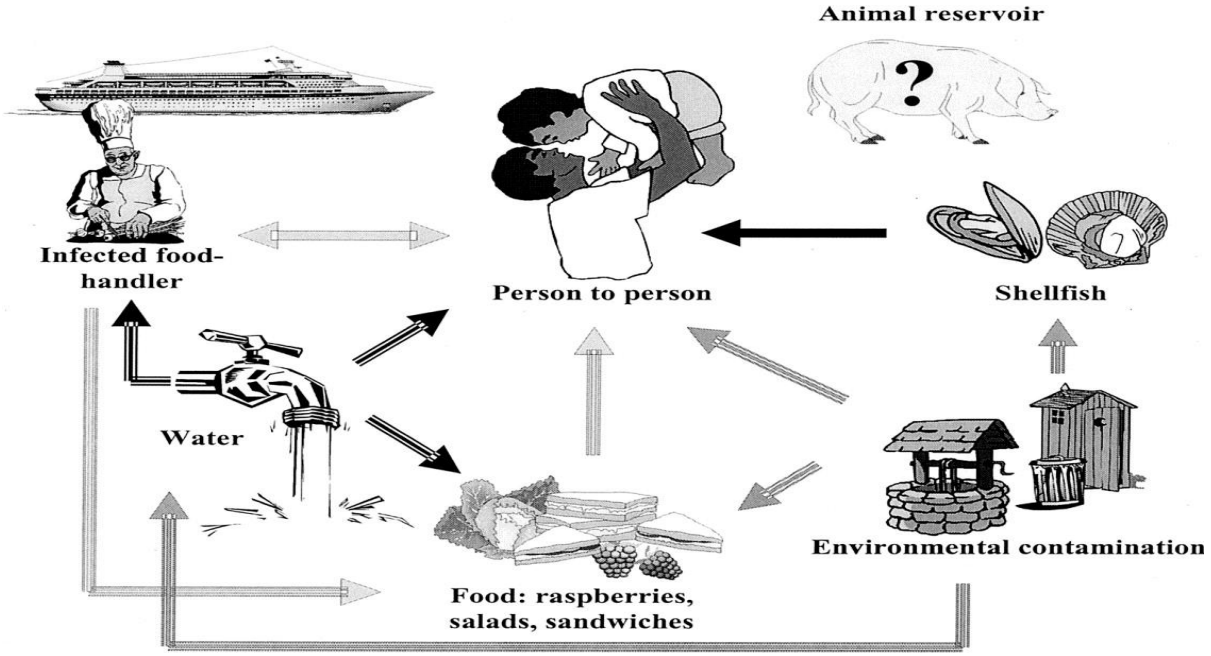

Figure 3: Transmission of Human NoV particles (Moreno-Espinosa *et al.*, 2004)

NoVs are highly transmissible and humans are the only known reservoir for human NoV (Fields, 2007). NoV can be transmitted through a variety of routes (Fig 1) but mainly through person to person contact (faecal-oral routes), aerosolized vomit and contaminated surfaces (Ester *et al*., 2004). Aerosolized vomit that contains NoV can be swallowed and inhaled but there is no evidence that the virus replicates in the respiratory tract (Marks *et al*., 2003; Marks *et al*., 2000). NoV can also be transmitted through ingestion of contaminated food, especially oysters and shellfish, and drinking contaminated water (Parashar *et al*., 1998; White *et al*., 1986) .

NoV have a low infectious dose of ~18 viral particles coupled with high viral concentration in the stools during shedding with 10 to 100 viral copies per gram of faeces even in asymptomatic infections (Teunis *et al*., 2008; Aoki *et al*., 2010). Thus suggesting, that up to 5 billion infectious doses can be shed by an individual in one gram of faeces (Hall *et al*., 2012). Prolonged shedding of the virus increases the risk of transmission and is of concern in food handlers and health care workers even after symptoms are resolved (Parashar *et al*., 1998). NoV are environmentally stable and have the ability to survive in both freezing and extremely hot temperature. They are resistant to most commonly used chemical disinfectants, like high levels of chlorine and can survive on surfaces for up to two weeks (Lopman *et al*., 2012). This make it challenging to eliminate NoV from contaminated water (Barker *et al*., 2004).

The most common settings for NoV outbreaks are closed and semi closed communities. This includes a variety of institutions, cruise ships, schools, day care centres, nursing homes, hospitals and restaurants

(Centers for Disease Control and Prevention (CDC), 2002). The size of reported outbreaks ranges from less than 10 individuals to epidemics of more than 6000 people (Fankhauser *et al*., 1998). Duration of outbreaks ranges from 24 hours to 139 days with a median of 8 days (Lopman *et al*., 2003).

Water-borne outbreaks have been reported involving drinking water, swimming pools, lake water, well water and ice (Blevins *et al*., 2004; Lawson *et al*., 1991). These outbreaks also occur when sewage contaminates water that is supplied for drinking purposes are accidently consumed during recreational activities. Foreign travelling can also be a risk factor for NoV transmission, with high risk possible resulting from the change in behaviour while traveling and being exposed to different strains of NoV (Chang *et al*., 2006; Chang *et al*., 2004).

1.2.10 Laboratory Diagnosis and Detection Methods

In 1972, NoV was first detected in stool using Immuno-Electron Microscopy (IEM). This method is labour intensive, has low sensitivity and requires highly skilled personnel (Lopman, 2015). In the 90's, Norwalk virus and Southampton virus, prototype of NoV were cloned and sequenced. This opened the way for detecting NoV directly from stool samples based on RT-PCR (Jiang *et al*., 1992; De Leon *et al*., 1992).

Similarly, NoVs have been used in cloning technology, expression of virus like particles (VLPs) and viral protein. This has led to the advancement of serological assays with antigens that may be used in the production of monoclonal antibodies and polyclonal sera (Jiang *et al*., 1992). This led to the development of immune-chromatography and antigen capture enzyme immunoassay tests that are available commercially (Lopman, 2015). However, regardless of the antigenic and genetic variation and viable viral loads in NoV stool specimens, the tests have low sensitivity and may not capture the full range of circulating viruses (Bruggink *et al*., 2013; Gray *et al*., 2007). Thus, enzyme immunoassays and other antigen detection tests may only become useful in outbreak settings (Gray *et al*., 2007; Kirby *et al*., 2010; Morillo *et al*., 2011; Derrington *et al*., 2009; Bruins *et al*., 2010).

There are many commercial EIA for NoV stool detection methods, their sensitivity and specificity depend on the diagnostic goal: clinical diagnosis versus outbreak investigation (Jiang *et al*., 1992). Generally, EIA are highly specific for the detection of NoVs but generally not sensitive enough to detect a wide range of NoVs (Jiang *et al*., 1992) . Newer EIAs have been developed based on antibodies against a wide range of viral antigens but sensitivity remains restricted (de Bruin *et al*., 2006). Their high specificity makes them useful for diagnosing NoV in outbreaks investigation, when there are many specimens available and confident detection in few cases might be adequate for confirming the cause of disease (de Bruin *et al*., 2006; Gray *et al*., 2007). However, sensitivity of such assays are genotype dependent and results differ based on the diversity of the circulating strain in the population (de Bruin

et al., 2006; Gray *et al.*, 2007). As a result, samples that are negative by EIA in outbreaks should be further tested using real-time RT-PCR. With further enhancement of these assays to improve their sensitivity, they could be useful in rapid diagnosis of NoV in sporadic diarrheal cases (Patel *et al*., 2008).

Real-time PCR using TaqMan assays is currently the most widely used method for the detection of NoV. It is highly sensitive and can detect low viral load present in an asymptomatic individual or a person that recovered within a few weeks from NoV gastroenteritis. The high sensitivity allows small amounts of virus to be detected in the samples of asymptomatic individuals and people that have recently recovered from NoV from infection (Lopman, 2015). Although inaccurate detection is a cause for concern in most assays, it is more problematic in Real-time PCR as it can detect as low copies number possibly non replicating virus (Lopman, 2015). In each of the NoV genogroups, the ORF1 – ORF2 junction is the most conserved region in the Norovirus genome, making it the main target for primers and probes for nucleic acid detection and amplification (Lopman, 2015).

Recently, many platforms that can simultaneously detect a wide range of pathogens have been developed for the detection of parasites, bacteria and viruses. These platforms include Luminex and BioFire which can detect all the pathogens and still differentiate between GI and GII NoV. With these diagnostics, understanding the cause of illness will be challenging if multiple pathogens are detected in the stool of an individual person (Lopman, 2015).

1.2.11 Immunity and Vaccine

The immunity of human NoV is not well understood which hampers the strategies for the formulation of immunization against NoV (Estes *et al*., 2000). The failure to cultivate NoV in cell culture until recently prevented researchers from studying the role of neutralizing antibodies (Fields, 2007). Recombinant VLPs expressed in transgenic plants or in Baculovirus have been shown to be immunogenic when administered to human volunteers. This raises the possibility for protection through vaccination (Ball *et al*., 1999). These vaccines could be useful to those who are at high risk of severe disease, including elders in nursing homes, young children in day care centres and immunocompromised individuals. Nonetheless, there are still many challenges in the development of NoV vaccine, including the lack of long term immunity, understanding of immune correlates and the protective cross-immunity against all the multiple existing genetic viruses (Tacket *et al*., 2003) .

Although NoV vaccine is still under development (Atmar and Mullen, 2013), it will take years before a licensed vaccine will be available (Debbink *et al.*, 2014). The protective immune correlate is not understood but it is assumed that the adaptive immune system, antibodies along with CD4 and CD8 lymphocytes are essential for protection against NoV infections (Tomov *et al*., 2013; Fang *et al.*, 2013)

. The vaccine may be of limited use in immunocompromised patients with weakened B – cell and T – cell function except for patients that are suitable for the incorporation of NoV vaccine "when available" with other recommended vaccines before or after organ or stem cell transplantation (Danziger-Isakov and Kumar, 2013). An effective NoV vaccine that will lower the overall prevalence of disease in the general population and in health care workers will benefit immunocompromised patients indirectly by limiting their exposure to the disease, thus preventing infection (Eden *et al*., 2014).

1.2.12 Prevention and Control

Infection control is still the first line of defence against the spread of NoV infection (MacCannell *et al.*, 2011*)*, and it is more important in immunocompromised individuals (Atmar and Mullen, 2013). No specific medication to treat NoV infection and therefore it cannot be treated with antibiotics. Drinking lot of liquids to replace fluids from vomiting and diarrhoea also oral rehydration fluids ((UK), 2009). Hospitals and long term care facilities should adhere strictly to infection prevention practices when caring for immunocompromised patients to limit exposure of patients, staff and visitors to NoV (Dew *et al*., 2005). The US centre of disease control and prevention (CDC, 2002) issued guidelines for the prevention of NoV infections, effective disinfectants and managing healthcare settings (Lopman, 2015). The restriction of movements between patients and staff, attention when washing hands and improved environmental cleaning has led to positive outcomes in nosocomial settings. Some NoV outbreaks were linked with health care workers handling immunocompromised patients while sick (Doshi *et al*., 2013).

NoVs are generally resistant to ethanol based cleaning solutions and detergents therefore require additional chemical disinfectants (Barker *et al*., 2004; Duizer *et al*., 2004). Effective disinfectants are those containing hypochlorite, phenolic based and hydrogen peroxide based formulations (Barker *et al*., 2004; Doultree *et al*., 1999). Special care must also be given when processing food in regards to the frequent occurrence of foodborne outbreaks (Koopmans and Duizer, 2004). Recent studies have shown that depuration of oysters does not completely remove NoV as they bind to oyster tissues using carbohydrates structure similar to that of a human histo-blood group antigen (Le Guyader *et al*., 2006). Therefore, precaution must be taken to prevent oysters from being contaminated by faeces and vomit or any treatment plant effluent. Increased purification of drinking and swimming water can also help decrease the incidence of NoV outbreaks (Lodder *et al*., 1999).

CHAPTER 2

METHODOLOGY

2.1 Ethics Approval

Ethics approval for this study was issued by the University of KwaZulu-Natal's Biomedical Research Ethics Committee (BREC), reference number BE350/15 (Sub-study of BE222/13). Caregivers of the participants provided written informed consent prior to initiation of study procedures.

2.2 Study Area and Population

The study was conducted at King Edward VIII Hospital situated at Ward 33 in eThekwini District of KwaZulu-Natal (KZN), South Africa. It is one of the largest hospitals in the southern hemisphere, providing regional and tertiary services to the whole of KZN and Eastern Cape. Children enrolled in this study were 5 years of age and below presenting with acute diarrheal disease at the paediatric outpatient department. Those eligible were admitted for in-patient care.

2.3 Study design

The study questionnaire was developed and designed by the Medical Microbiology department of the University of KwaZulu-Natal (UKZN). The questionnaire was designed in both English and IsiZulu, and outlined the importance of the study as well as questions regarding risk factors associated with diarrhoeal disease, medical history, clinical symptoms and demographic information. In June 2014, the specimen collection phase of the study began with hospitalized patients at the paediatric ward of King Edward VIII Hospital. The objective and concept of this study were clearly explained to the caregiver of each potential participant. Confidentiality was ensured by giving each patient a number. The consent forms were the only documents containing the identities of the participants and they were kept safe in a separate location.

2.4 Specimen Collection and Preservation

After written informed consent was obtained from the parent/guardian, stool specimens were collected by trained personnel from soiled diapers using an autoclaved wooden spatula. The specimens were transported in a 10 ml screw capped tube and transported at 2 – 8 ^{o}C in a cooler box with ice packs to the Medical Microbiology laboratory. Specimens were preserved by aliquoting 0.1 g of stool into 2 ml microcentrifuge tubes and stored at – 80 ^{o}C until further use.

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2.5 Enzyme Immunosorbent assay (ELISA)

The IDEIA™ Norovirus kit (Oxoid Limited, Basingstoke Hampshire, United Kingdom) was used to detect norovirus (NoV). It is a single plate with micro-wells pre-coated with monoclonal antibodies for both GI and GII NoV strains. The assay is a sandwich ELISA (figure 1) and was carried out according to the manufacturer's instructions. Briefly, 1 ml of sample diluent was added to 0.1 g of stool to make a 10% stool suspension. The suspension was then vortexed, incubated at room temperature for 10 minutes and centrifuged for 5 minutes at 5000 rpm. An amount of 100 µl of each stool supernatant as well as of the negative and positive control (provided with the kit.) were added to separate micro-wells. This was followed by the addition 100 µl of conjugate to all micro-wells. This conjugate consisted of peroxidase labelled polyclonal antibodies and monoclonal antibodies against NoV GI and GII in a buffered protein solution containing antimicrobial agent labelled with a blue dye. The content of the wells was mixed gently and then incubated at 30 ^{o}C for 60 minutes. Unbound antibodies were removed from the micro-wells by washing 5 times with 350 µl of Tris-buffered saline. After the last wash, the plate was turned upside down on an absorbent paper to remove all traces of buffer and 100 µl of the 3, 3', 5, 5'-tetramethylbenzidine (substrate) was added to all wells and incubated at 30 ^{o}C for 30 minutes. The reaction was stopped by adding 100 µl of sulphuric acid (stop solution) before reading the results. The presence of bound antibodies were indicated by the colour change which was triggered by 3,3',5,5'-tetramethylbenzidine (TMB), a visualizing agent that act as a hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase enzyme. The resulting di-imine caused the solution to take on a blue colour. The optical density (OD) readings were measured at a wavelength of 450 nm. The cut-off value was calculated as 0.10 plus the OD value for the negative control. Samples with OD values greater than the cut-off value plus 0.01 were regarded as positive, and samples with OD values lower than the cut-off value minus 0.01 were regarded as negative.

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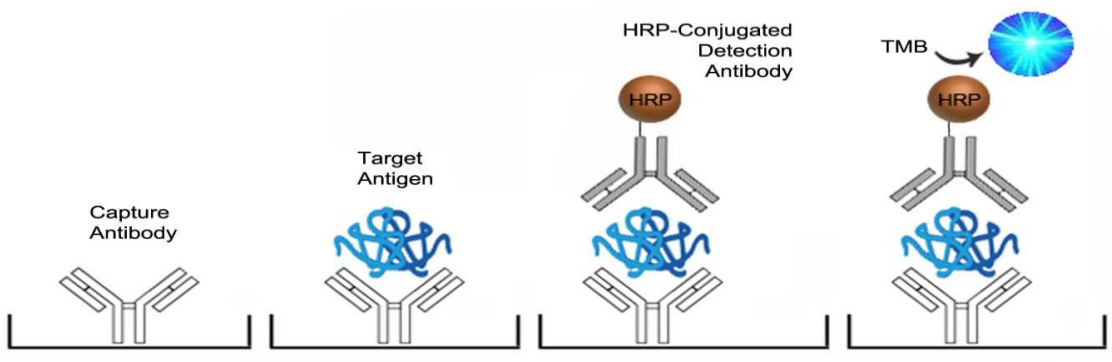

Figure 4: Sand-witch ELISA

2.6 Ribonucleic acid (RNA) extraction

RNA extraction was performed using the QIAamp® viral RNA Mini-kit (Qiagen, Valencia, CA, and USA) according to the manufacturer's instructions. One ml of PBS, pH 7.2, was added to 0.1 g of stool to make a 10 % stool suspension. The suspension was then briefly vortexed and centrifuged at 14000 x g for 1 minute. The volume of lysis buffer and -carrier RNA mix needed per batch of sample was calculated by the number of samples to be simultaneously processed using the following formula: -

$$\boldsymbol{N} \times 0,56\ mL = \boldsymbol{Y}\ mL \text{ and } \boldsymbol{Y}\ mL \times 10\ \mu L/mL = \boldsymbol{Z}\ \mu L.$$

N: number of samples to be processed, **Y**: volume of buffer AVL, **Z:** volume of carrier RNA-elution buffer.

Of the buffer AVL-carrier RNA-buffer mix 560 µL was pipetted into a 1.5 mL microcentrifuge tube followed by the addition of 140 µL of the 10 % stool suspension. The sample mixture was mixed by vortexing for 15 seconds and incubated at room temperature for 10 minutes. The sample mixture was centrifuged briefly for 30 seconds at 14000 x g, then 560 µL of molecular grade ethanol (98 %) was added followed by vortexing. The tubes were centrifuged, then 630 µL of the lysate was dispensed into the QIAamp spin mini column connected to a 2 mL collection tube and centrifuged at 6000 x g for 1 min. The spin mini column was then placed into a new 2 mL collection tube and the tube containing the filtrate was discarded. This procedure was repeated twice to filter out all the remaining volume of the lysate. Afterwards, 500 µL of wash buffer (AW1) was added to the spin mini column and centrifuged at 6000 x g for 1 min. The spin mini column was then placed into a new 2 ml collection tube and the tube containing the filtrate was discarded. Then, 500 µL of a second wash buffer (AW2) was added to the spin mini column followed by centrifugation at 14,600 x g for 3 min. Next, the spin mini column was placed into a new collection tube and centrifuged at full speed for 1 min to remove residual wash buffers. The spin mini column was then placed into a 1.5 ml microcentrifuge tube and 60 µl of buffer AVE was added to the spin mini column which was left to equilibrate at room temperature for 1 min. The RNA was eluted by centrifugation at 6000 x g for 1 min. The RNA samples were stored at – 80 ^{o}C until further use.

2.7 Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

Real time RT-qPCR was performed using the one-step RealStar® Norovirus RT-PCR kit (Altona diagnostic, Hamburg, Germany) according to the manufacturer's instruction. Briefly, 5 µL of each viral RNA or controls (positive and negative controls) was used as a template. A master-mix reaction of 20 µL containing 5 µL of Master A, 15 µL of Master B and 0.5 µL of the internal control. Master A and B contained buffer, enzyme, and primers for both genogroups and probes. After the master-mix

preparation, a PCR reaction of 25 µlLwas added to a 96 well reaction plate, the plates were covered with adhesive film cover and centrifuged at 1000 x g for 30 seconds. Amplification of NoV GI and GII were performed using an Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems™, USA). The reverse transcription reaction at 50 ºC for 10 minutes was followed by denaturation at 95 ºC for 10 minutes and amplification at 95 ºC for 15 seconds and 95 ºC for 45 seconds in 45 cycles. The positive and negative controls were used to ascertain integrity of the reaction. Samples were regarded positive when amplification occurred

2.8 Statistical analysis

The sample size was calculated using the prevalence formulae by Hajian-Tilaki (2014). Assuming Norovirus prevalence of 20 % and an ELISA sensitivity of 72.8 %. An estimated 280 stool samples or more were needed for the Norovirus prevalence study at 95 % confidence interval. MedCalc version 12.2.1 (MedCalc Software, Mariakerke, Belgium) was used to determine sensitivity, specificity and a 95 % confidence interval of the ELISA tests compared to the RT-qPCR assays. Data was analysed using SPSS 22. Categorical variables were compared using Chi-square test. A p-value less than 0.05 was considered to be statistically significant.

CHAPTER 3

3. RESULTS

3.1 ELISA versus RT-qPCR

A total of 182 stool specimens were tested for the presence of NoV using two different testing methods, the antigen detection (ELISA) and RT-qPCR. Nineteen of 182 (10.5 %) tested positive with ELISA and 41/182 (22.5 %) tested positive with RT-qPCR. When the two methods were compared, RT-qPCR detected 31/182 (17 %) more positives then ELISA and 9/182 (5 %) of the ELISA positives were negative when tested by RT-qPCR. The RT-qPCR did not only detect NoV, it was also able to differentiate between GI and GII, which causes the most NoV outbreaks in human. All positives in this study were GII (100 %).

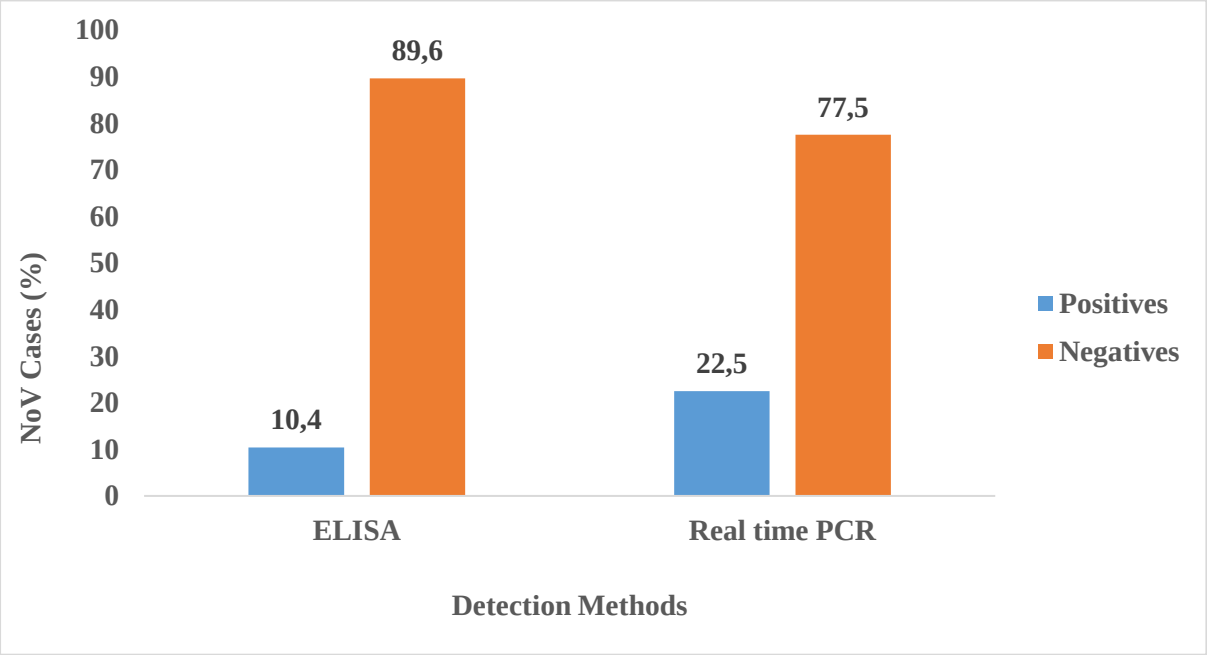

Figure 5: Detection of Norovirus using two different methods

Table 1: Comparison of the antigen detection test and RT-qPCR detection methods

ELISA Results	RT-qPCR Results		No. of Specimens
	Positive	Negative	
Positive	10 (5.5 %)	9 (5.0 %)	19 (10.5 %)
Negative	31 (17.0 %)	132 (72.5%)	163 (89.5 %)
No. of Specimens	41 (22.5 %)	141 (77.5%)	182 (100 %)

Sensitivity and Specificity

MedCalc version 12.2.1 (MedCalc Software, Maria-kerke, Belgium) was used to determine sensitivity, specificity and 95 % confidence intervals of the ELISA tests compared to the RT-PCR assays. This resulting in a sensitivity of 24 % (95 % Cl = 12 % - 40 %) and a specificity of 94 % (95 % Cl = 88 % - 97 %). The positive predictive value (PPV) was 53 % (95 % Cl = 29 % - 76 %) and the negative predictive value (NPV) was 81 % (95 % Cl = 74 % - 87 %).

3.2 Sociodemographic and clinical features

Table 2 shows a comparison of sociodemographic and clinical characteristics between NoV positive and negative cases. Ninety-three females (51 %) and eighty-nine males (49 %) were recruited. Of the 41 specimens that tested positive for NoV, 18/41 (44 %) were females and 23/41 (56 %) were males ($p = 0.3$). in the age group stratified analysis, the NoV prevalence was different between the age groups but not statistical significant ($p = 0.6$). the highest prevalence was observed in the age group 12 - 23 months (39%). This was followed by ≤ 5 months and 6 – 11 months age group, both with a prevalence of (26.8 %). This is in line with what has been found in previous studies as children older than 2 years may have developed immunity due to previous NoV infection as it is suggested that a child will experience at least one NoV infection before the age of 5 years. The distribution of NoV infection was not statistical significant ($p = 0.8$). Six (14.6 %) of the NoV positive cases were HIV positive, 20 (49 %) were HIV negative, nine (22 %) were HIV exposed and six (14.6 %) were unknown as records of their HIV status could not be found.

Table 2: Sociodemographic and clinical features of NoV positive compared to NoV negative children

	NoV positive (n = 41)	NoV negative (n = 141)	p value
Sex (n=182)			
Female	18	75	0.3
Male	23	66	
Age group (n=182)			
≤ 5 months	11	51	0.6
6 - 11 months	11	35	
12 - 23 months	16	39	
24 - 35 months	2	11	
36 - 60 months	1	5	
HIV status (n=182)			
positive	6	18	0.8
negative	20	81	
exposed	9	26	
unknown	6	16	
Duration of Diarrhoea (n=182)			
1 - 3 days	18	74	0.4
4 - 5 days	15	34	
≥ 6 days	6	28	
unknown	2	5	
Fever (n=182)			
> 38.5 ºC	7	15	0.2
≤ 38,5ºC	33	126	
unknown	1	0	
BMI (n=182)			
Underweight (< 18.5)	33	112	0.6
Healthy weight (18.5 - 24.9)	6	16	
Overweight (25 - 29.9)	0	1	
Obese (> 30)	0	4	
unknown	2	8	
Mode of Feeding (n=89)			
Breast feeding	7	33	0.5
Mixed feeding	6	43	

CHAPTER 4

4. DISCUSSION

In the province of KwaZulu-Natal (KZN), South Africa, there is a discrepancy between the level of vaccination coverage and infant mortality (van den Heever, 2012). The two major syndromes of infectious diseases that contribute to high mortality rate are lower respiratory tract infections and diarrhoea. In an attempt to uncover the reason for that discrepancy for diarrhoea, this study investigated the prevalence and detection of human NoV in children 5 years and under in a tertiary hospital in KZN, (King Edward VIII hospital) between June 2014 and August 2015.

The result of the study provided important insights into the burden of disease caused by NoV as well as the epidemiological features of disease in KZN. The study was part of a bigger study where multiple investigators were looking at different aetiologies that also cause diarrhoea like RoV, *Cryptosporidium parvum* and *Giardia lambliae*. Bacterial aetiologies and additional viruses could not be included due to lack of funding.

The NoV ELISA test and NoV RT-qPCR kit were used to determine the prevalence of NoV infection in KZN (Figure 5). The comparison between the two assays allowed us to determine the most suitable test for routine diagnostic use. In total 50 of the 182 children tested NoV positive. The RT-qPCR assay was positive for 41 and the ELISA for 19 resulting in prevalence's of 22.5 % and 10.5 % respectively (Figure 5). A study in Canakkale, Tukey also found similar result when they were detecting NoV using ELISA and RT-PCR. They found 16 (17.4 %) samples were positive for NoV, where 10/92 (10.9 %) samples were positive using ELISA and 15/88 (samples positive for real time RT-PCR (Aksu and Alper, 2016). However, nine of the 19 ELISA positives could repeatedly not be confirmed with RT-qPCR. Spiking of these stools with PCR positive specimens showed that there was no inhibition (Table 1). It is therefore likely that these tests represented false positives or contained antigen in the absence of viral RNA. Page et al, reported on a study that was done in four site in South Africa where they detected NoV in 452/3103 (15 %) of hospitalized children <5 years with diarrhoea with the majority of disease in children <2 years 417/452 (92 %) (Page et al, 2017). The ELISA tests as screening test for NoV antigens was rapid and relatively easy to perform. However, the RT-qPCR assays with increased sensitivity could detect NoV RNA in an additional 31 (17 %) of patients (Table 1). The RT-qPCR also allowed determination of NoV genogroups. Most NoV detection tests target GI and GII as they are responsible for 90% of all human NoV infections. Therefore, GIV could have been missed in this study. Genogroup two (GII) was responsible for all the NoV infections during the study but we could not determine the specific genotype as genotyping could not be done due to lack of funding. A study in Turkey also detected majority of GII where they found 93 % GII and only one sample positive for GI by RT-qPCR (Aksu and Alper, 2016). Whilst ELISA tests are quick and easy to perform, they are less

sensitive compared to molecular methods. Molecular methods, on the other hand, are more expensive, have a longer turn around time and require more technical expertise than ELISA. Taking this into consideration, together with the high prevalence of NoV detected in this study, the ELISA test would provide rapid preliminary results in individual patients and could be used to establish the aetiology of community as well as nosocomial outbreaks. During outbreaks not all cases need to be diagnosed accurately. The increased sensitivity of the RT – qPCR assay could be utilized where NoV diagnosis is critical to patient care, as with immunocompromised patients, or in single cases of diarrhoea in non-epidemic manifestations. A study by de Bruin and colleagues also compared the performance of the ELISA test to RT-PCR and found the sensitivity and specificity of ELISA to be low and propose RT-PCR to be the gold standard for routine diagnosis of NoV in specimens from patients with gastroenteritis (de Bruin et al., 2006).

Sociodemographic and clinical information were also recorded from the children enrolled in this study (Table 2). We found that there was no relationship between gender and infection as there was no statistical difference ($p > 0.05$) between the number of males 18/41 (44%) and females 23/41(56 %) with NoV infection. This is different from what was reported from a study done in Japan (Ozawa *et al.*, 2007), where they found males (10.8 %) to have a higher prevalence of NoV infection compared to females (6.4 %). In the present study, the highest rate of NoV infection was observed in children less than 24 months 38/41 (93 %). The peak detection rate occurred between 12 - 23 months which as was also shown in a French study (Tran *et al*., 2010). These findings indicate that the age of children was closely associated with the rate of NoV infections and that this peak might be due to poor patient hygiene and to frequent contact between young infants and other household and community members in a community with poor sanitation. Moreover, the weak immune systems of younger children might contribute to their higher susceptibility to NoVs than that of older children. Our findings confirm NoV infection usually occur in early childhood, which may indicate that protective immunity due to earlier infection is present after the age of 2 years (Sai *et al*., 2013). It also highlights the importance of implementing prevention strategies in the early years of life, although this might result in a shift in age category with the highest prevalence. Children older than 24 months had the lowest prevalence of NoV infection 3/41 (7.3 %). This suggest that older children may have protective immunity due to previous infection as studies have shown that a child will experience at least one NoV infection before age 5 years.

Infection with HIV is common among children of sub-Saharan Africa countries, and diarrhoeal disease is a leading cause of morbidity and mortality in HIV-infected children in these areas (Pavia *et al*., 1992; Thea *et al.*, 1993). In this study, we found significantly lower incidence of NoV infection in HIV infected children compared to HIV uninfected cases. Only 24/182 (13 %) of all children enrolled in this study were HIV positive and from that six (25 %) were NoV infected. These findings differ from those

in a previous study (Cegielski *et al*., 1994), which reported a strong association between HIV infection and NoV infection. The lack of such an association in the current study can partly be explained by the fact that 6/41 (14 %) of our NoV positive patients had an unknown HIV status and 9/41 (22 %) were exposed to HIV by having an infected mother. Understanding the association between causative agents of diarrhoea in HIV infected and exposed children needs further investigation.

Diarrhoea and low-grade fever have been reported as important clinical features associated with NoV infection. In an attempt to find the duration of diarrhoea associated with NoV infection, we found that only 18/41 (44 %) of NoV positive children had diarrhoea for about 3 days at the time of specimen collection while 15/41 (36.5 %) had diarrhoea for 4 to 5 days and only 6/41 (14.6 %) have had diarrhoea for 6 days or more. The duration of diarrhoea did not differ between NoV positive and NoV negative patients ($p > 0.05$). In the current study, fever was defined as > 38.5 ºC and we found that only 22/182 (12 %) of all patients enrolled had fever with most of these (8 %) being NoV negative children. There was therefore no statistical significance ($p > 0.05$) between NoV positive and NoV negative individuals regarding fever. Height and weight was used to calculate body mass index (BMI) of the children to compare their nutritional status. Malnutrition generally results not only in a reduced ability to control infections but also in a reduced ability to develop protective immunity toward sub-sequent re-infections (Chandra, 1983; von Bubnoff, 2011). This was also reflected in our study, where malnourished (underweight) children made up 145/182 (80 %) of our study participants. The prevalence NoV infection was with 80 % higher in malnourished as compared to children (14.6 %) with normal BMI. We could not determine whether BMI was associated with the rate at which NoV infection cleared as this was a cross-sectional study.

Multiple preventive and therapeutic interventions have been designed to decrease mortality and disability in children. Among these, early and exclusive breastfeeding is one of the most important interventions to reduce neonatal and infant mortality (Walker *et al*., 2011). Breastfeeding is widely promoted (Bhutta *et al*., 2013) and is the most cost effective intervention for protecting children against diarrhoea and all other causes of mortality (Jones *et al*., 2003). Human breast milk helps protect infants by serving as a source of nutrition uncontaminated by environmental pathogens in addition to the direct protection due to its multiple anti-microbial, anti-inflammatory and immune-regulatory components (Morrow & Rangel, 2004). Breastfeeding demonstrates a dose–response relationship of protection against morbidity and mortality in infancy by diarrheal disease. Exclusive breastfeeding, defined as feeding only human milk with no other liquids or foods, is known to offer maximum protection against diarrhoea in infants younger than 6 months of age, whereas partial breastfeeding offers intermediate protection compared with no breastfeeding. The proportion of partially breastfed infants increases with age (Arifeen *et al*., 2001). Improvements in breastfeeding practices can be achieved through various educational and promotional strategies. Paediatricians play a critical role by supporting and motivating

a successful mother-infant breastfeeding dyad (Section on Breastfeeding, 2012). Breastfeeding promotion remains an intervention of enormous public health potential. It is one of the most cost-effective child health interventions currently available and does not require extensive health system infrastructure. These characteristics, along with the large disease burden associated with suboptimal breastfeeding, indicate that breastfeeding promotion has the potential to improve child health outcomes (Roberts *et al.*, 2013). NoV infection occurs less often in infants of mothers with higher levels of lacto-N-difucohexaose (a 2-linked fucosyl-oligosaccharide) in their milk (Morrow *et al.*, 2005), as this protects against this virus by blocking the binding to the histo-blood group antigen receptors (Morrow *et a*l., 2005). We observed no difference between children that were exclusively breastfed 7/13 (54 %) and children that were mixed fed 6/13 (46 %). This might be on the account of the small numbers.

As this study was part of a bigger study, where other non-bacterial aetiologies were like RoV were investigated, the result of those studies were compared with ours. We discovered a prevalence of 79/182 (43.4 %) for RoV compared to 41/182 (22.5%) for NoV. This prevalence was consistent with what has been found in other settings (Mans *et al*., 2016). The peak incident of infection for RoV was in children ≤ 5 months 25/79 (31.6 %) followed by age group 12 - 23 months 23/79 (29.1 %), then age group 6 - 11 months 18/79 (22.7 %) in children less than 24 months compared to children older with a prevalence of 13/79 (16.4 %). This was different from what was observed in children with NoV infection where the highest prevalence was in the age group 12 - 23 months 16/41 (39.0 %) followed by the age group ≤ 5 months and 6 - 11 months each with 11/41 (26.8 %). Other clinical signs and symptoms like fever and nutritional status did not differ between NoV and RoV. Both NoV and RoV infected children had the highest prevalence in children that were underweight compared to children with healthy weight. Only 16/182 (8.8 %) of the children were co-infected with RoV and NoV and from those, 9/16 (56 %) of them were in the 12 – 23-month age group.

Our study has several limitations. This includes the duration of the study, considering that a 3 months study period is too short to observe seasonal variability with consequent risk of over- or underestimating the annual burden of infection. ELISA detected 9 positives that were not detected by RT-qPCR, these positives could be false positive as RT-qPCR did not detect them. This may be due to primers specific for GI and GII not including GIV.

4.1 CONCLUSION

The aim of this study was to detect NoV in children 5 years and under with diarrhoea in KZN. NoVs is a public health threat among children, in both developed and developing countries. Our findings showed that RT-qPCR is superior to ELISA when detecting NoV in stool specimen. Our findings also reveiled that the circulating genogroup was GII and therefore further investigation on genotyping should be done to better understand circulating genotypes. The highest prevalence of NoV was seen in children younger

than 24 months compared to older children suggesting protective immunity from previous infection in the latter.

CHAPTER 5

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Non-bacterial aetiology of diarrhoea in children of 5 years and under in KwaZulu-Natal

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ABSTRACT

Objective

To investigate the non-bacterial aetiologies of diarrhoea in children 5 years and under in KwaZulu-Natal.

Methods

Stool specimens were collected between June 2014 and August 2014 from children ≤ 5 years presenting with diarrhoea to a regional hospital. Written informed consent was obtained from their parents or guardian. Patient information was collected using a structured questionnaire. Enzyme-linked immunosorbent assay (ELISA) and Real-time Polymerase chain reaction (RT-PCR) were used for detecting rotavirus and norovirus. Protozoa were detected microscopically using the modified trichrome stain and the modified Ziehl-Neelsen stain. The data were analysed using SPSS.

Results

One hundred and eighty-two stool specimens were collected. A total of 114 (62.6%) children were positive for at least one pathogen. Rotavirus had the highest prevalence of 43.3% followed by Norovirus with 22.5%, then *Cryptosporidium parvum* with 9.9% and *Giardia lambliae* with 2.7%. Co-infection with two or more enteric pathogens was detected in 26 (14.2%) children. Rotavirus-Norovirus (7.1%) was the most common co-infection, followed by Rotavirus-*C. parvum* (3.3%) and Rotavirus- *G. lambliae* (0.5%).

Conclusion

This study highlights the burden of non-bacterial aetiologies of diarrhoea among children of 5 years and under in KwaZulu-Natal. Single infection of viral aetiology was observed in more than half of the study population. An effective Norovirus vaccine is urgently needed while Rotavirus vaccination needs to be optimised. The potential synergistic effect of multiple infection on the severity of diarrhoea especially among children less than 2 years old need further exploration.

Key words: Diarrhoea; Children; Norovirus; Rotavirus; *Cryptosporidium parvum; Giardia lambliae*

INTRODUCTION

Acute gastroenteritis is the leading cause of mortality in young children globally and an important cause of morbidity and hospitalization in developing countries (Kosek *et al*., 2003; Parashar *et al.,* 1998). It affects people of all age but children 5 years and under tend to be more severely ill, especially children below 2 years of age because of their immature immunity and rapid dehydration. The prevalence of bacterial gastroenteritis diminished along with improved hygiene, sanitation, food and water handling, but non-bacterial gastroenteritis remained with viruses as the most important cause in children (Mäki *et al*., 1978; Mäki *et al*., 1981; Vesikari *et al.,* 1981). These viruses include rotavirus (RoV), norovirus (NoV), Astrovirus, Sapovirus and Adenovirus serotype 40 and 41 (Higgins *et al*., 2011). Of these RoV and NoV are most frequently diagnosed. Both are highly infectious with an infective dose between 10 and 100 virions and both are transmitted via the faecal oral route and via respiratory droplets, (Ward *et al*., 1986; Said *et al.,* 2008; Mandell *et al.,* 2010). RoV infection can be diagnosed using ELISA antigen test, polymerase chain reaction (PCR) and RT-PCR (Pang *et al*., 1999). Since antigen detection tests are relatively insensitive, the importance of NoV in outbreaks of acute gastroenteritis was only appreciated with the introduction of RT-PCR (Chen & Hu, 2016).

Giardia lamblia and *Cryptosporidium parvum* are protozoan parasites that cause acute gastroenteritis. Transmission occurs mainly through contact with contaminated food, water, direct contact with infected animals or exposure to water contaminated by animal faeces. *C parvum* are obligate intracellular parasites. They are secreted in the intestinal epithelial cells in a form of an oocyst (Laurent *et al*., 1997). In immune deficient individuals such as those infected with HIV and/or malnourished, *C. parvum* causes chronic or persisting diarrhoea and can also infect the biliary tract (Hunter and Nichols, 2002; Pantenburg *et al*., 2008).

G. lamblia can be found in two distinct forms, as cyst and as trophozoite (Faghiri & Widmer, 2011). Outside the host it exists as a cyst which is resistant to many environmental stresses except soil deposition and cold water in winter (Robertson and Gjerde, 2004). The trophozoites are responsible for disease manifestations. They colonize the small intestine and attach to the intestinal wall with a ventral disk (Adam, 2001). Trophozoites encyst in the jejunum after nuclear replication. This encystment is triggered by host factors like high bile concentration, basic pH and low cholesterol in that part of the gut (Lauwaet *et al.,* 2007). *C. parvum* are intracellular coccoid protozoa that belong to the family *Cryptosporididae*. They have a wide range of hosts including dogs, cats, cattle, mice, deer and fowl. Sporulated oocysts of *Cryptosporidium parvum* are excreted by the infected host with the faeces and through other routes such as respiratory secretions. We report on the aetiology other than bacteria of acute gastroenteritis in children of 5 years and under, attending a tertiary health care center in KwaZulu-Natal.

METHODOLOGY

Children of 5 years and below presenting with diarrhoea at King Edward VIII Hospital (KEH) in the eThekwini district of KwaZulu-Natal were recruited from Monday to Friday from 8:00 till 14:00 between 1 June 2014 and 30 August 2014. Diarrhoea was defined as 3 or more loose stool within a 24 hour period (Howard *et al.*, 2017). Informed consent was obtained from parents or guardians. Demographic and clinical data captured on a questionnaire and a stool specimen was collected from soiled diapers using a wooden spatula. Based on the calculated BMI children were classified as underweight, healthy weight, overweight or obese. The body temperature was measured using an infrared ear thermometer (HuBDIC Co., Limited, Korea). Fever was defined as a temperature of ≥38.5 ^{o}C (Howard *et al*., 2017). Blood specimens for HIV testing were collected by means of, the dry blood spot (DBS) collection kit. Infant HIV polymerase chain reaction (PCR) was performed using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche, South Africa). HIV testing was done in the laboratory of KEH using PCR.

Stool specimens were transported in a cooler box to the Medical Microbiology laboratory at the Nelson R Mandela School of Medicine, adjacent to KEH. The specimens were aliquoted on arrival and stored at -80^{o}C until further use. One aliquot was suspended in 10 % formalin for parasitological investigations. The ProSpecT™ Rotavirus antigen detection kit and the IDEIA™ Norovirus kit (Oxoid Limited, Basingstoke Hampshire, United Kingdom) were used to diagnose the respective viral infections. A 10% stool suspension was prepared using sample diluent provided with the kit. The suspension was allowed to stand at room temperature for 10 minutes, following which the top layer was used for antigen detection according to the manufacturer's instructions.

Viral ribonucleic acid (RNA) was extracted from a 10% (w/v) stool suspension made in phosphate buffered saline (PBS) (PH 7.2) using the QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RT-qPCR was performed using the Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems™, USA)

Viral RNA from the stool specimens were subjected to quantitative PCR (qPCR) specific for Norovirus. This qPCR was performed using the RealStar® Norovirus RT-PCR kit (Altona diagnostics, Hamburg, Germany). This is a one-step reverse transcription qPCR (RT-qPCR) kit and tests were performed according to the manufacturer's instructions.

	Stage	Cycle Repeats	Acquisition	Temperature	Time
Reverse Transcription	Hold	1	-	50	10:00 min
Denaturation	Hold	1	-	95	10:00 min
Amplification	Cycling	45	-	95	0:15 min
			√	98	0:45 min

In addition to detecting NoV, the qPCR kit also differentiated between the most prevalent NoV genogroups which are genogroup I (GI) and genogroup II (GII).

Smears prepared with the formalin preserved aliquot were stained with modified trichrome stain to diagnose protozoal infections (Hale *et al*., 1996) and modified Ziehl-Neelsen stain for the diagnoses of *C. parvum* (Hooja *et al.,* 2011). Smears were viewed using light microscopy (Olympus, Japan) at 400X magnification for the detection of protozoal parasites.

Statistical Analysis

The association between the prevalence of the viral, parasitic and co-infections and the categorical variables was assessed using the Fisher's exact test. A p-value <0.05 was considered to be statistically significant. The data was analysed using SPSS (version 22).

RESULTS

A total of 182 children were enrolled in this study. Of these, 91 (50 %) and 23 (12.6 %) were infected with viruses and protozoa respectively, giving a total of 114 (62.6 %) positives for at least one pathogen. Table 1 shows the age and gender distribution of the children together with their clinical features. Of the positives, 53 (46.5 %) were male and 61 (53.5 %) female. RoV was with an overall prevalence of 43.4 % highest using EIA. This was followed by NoV with an overall prevalence of 22.5 % from the RT-PCR. All NoV detected were of Genogroup II. *C. parvum* was found in 18 (9.9 %) and *G. lambliae* in 5 (2.7 %) of the patients. With regards to single infection, RoV was most common (30.8 %), followed by NoV (12.1 %). In addition, 3.3 % and 2.2 % of single infection were attributed to *C. parvum* and *G. lamblia* species respectively. None of the other protozoal causes of diarrhoea were found. Co-infection with two or more enteric pathogens was detected in 26 (14.3 %) children. These co-infections were stratified as virus-virus, virus-protozoan and protozoan-protozoan types. The most common co-infection was the Norovirus-Rotavirus, detected in 13 (7.1 %) of the study population. This was followed by the rotavirus-*C. parvum* combination (3.3 %). The lowest coinfection rate was recorded for Rotavirus-*G. lamblia* (0.5 %). No co-infection was observed with orovirus and *G. lamblia*.

Most patients (163; 89.6 %) were less than 2 years old. The prevalence of diarrhoeal pathogens was higher in this age group as compared to children above this age [99 (54.4 %) vs. 15 (8.2 %), p = 0.04]. Further analysis revealed that the presence of multiple infections was not related to age (p = 0.3). Most (87.4 %) of the children did not have fever at the time of specimen collection and of these, 62.9% had at least one pathogen. Of the (12 %) children that had fever, 60 % has at least one pathogen. There was no relationship between the prevalence of pathogen detected among children who had fever and those who did not (p = 0.6). In addition, fever was not related to infection with multiple pathogens (p = 0.2). Of the total number of children enrolled in the study, 145 (79.7 %) were underweight and 65.5% of these had at least one pathogen. Children underweight are more likely to have infection. However, there was no relationship between the species of pathogen detected among underweight and healthy weight children (p = 0.2). Nighty two (50.5 %) children had a diarrhoeal duration between 1- 3 days and about 45.6% of children had diarrhoea which lasted for more than 3 days. We observed that there was a greater chance of detecting pathogens within the first 3 days of illness. After the 3 days, the rate of detection decreased, however this observation was not significant within our study population (p = 0.5). Just over half of the patients were HIV negative (55.5 %) and about 57 % of this group harboured at least one pathogen. In contrast, only 11 % of children who were HIV positive had one or more pathogens. Thus, the prevalence of diarrhoeal pathogens in these children was not influenced by their HIV status (p = 0.2). When we merged the HIV exposed children with the HIV positive children, we found no statistical significance between the prevalence of diarrhoeal pathogens detected in this group and that in the HIV negative children (p = 0.09).

Information regarding mode of feeding was collected from children of 6 months or younger. Of the 89 children who fell into this age group 40 (44.9 %) were exclusively breast fed (Table 1). Within the exclusively breastfed study population, 21 (52.5 %) harboured at least one pathogen. In contrast, 34 (69.4 %) of the mixed fed children had at least one pathogen. This difference was not statistically significant ($p = 0.13$).

Table 1: The percentage clinical characteristics for Norovirus, Rotavirus and Protozoa for mono-infection, co-infection and triple infection in children 5 years and under

	NoV only	RoV only	Giardia	Crypro	NoV and RoV	NoV and Giardia	NoV and Crypto	Rov and Giardia	RoV and Crypto	NoV, RoV and Giardia	NoV, RoV and Crypto	None	Total
	22 (12.1%)	56 (30.8%)	4 (2.2%)	6 (3.3%)	13 (7.1%)	0	3 (1.6%)	1 (0.5%)	6 (3.3%)	0	3 (1.6%)	68 (37.4%)	182
Age group (n = 182)													
≤ 5 Months	6 (27.3%)	19 (34%)	2 (50%)	1 (16.7%)	3 (23.1%)	0	2 (66.7%)	0	3 (50%)	0	0	26 (38.2%)	62 (34.1%)
6 – 11 Months	7 (31.8%)	14 (25%)	0	2 (33.3%)	1 (7.7%)	0	1 (33.3%)	0	1 (16.7%)	0	2 (66.7%)	18 (26.5%)	46 (25.3%)
12 – 23 Months	7 (31.8%)	13 (23.2%)	2 (50%)	3 (50%)	8 (61.5%)	0	0	0	1 (16.7%)	0	1 (33.3%)	20 (29.4%)	55 (30.2%)
24 – 35 Months	1 (4.5%)	6 (10.7%)	0	0	1 (7.7%)	0	0	1 (100%)	1 (16.7%)	0	0	3 (4.4%)	13 (7.1%)
36 – 60 Months	1 (4.5%)	4 (7.1%)	0	0	0	0	0	0	0	0	0	1 (1.5%)	6 (3.3%)
Sex													
Male	10 (45.5%)	25 (44.6%	3 (75%)	2 (33.3%)	7 (53.8%)	0	0	1 (100%)	4 (66.7%)	0	1 (33.3%)	40 (58.8%)	93 (51.1%)
Female	12 (54.5%)	31 (55.4%)	1 (25%)	4 (66.7%)	6 (46.2%)	0	3 (100%)	0	2 (33.3%)	0	2 (66.7%)	28 (41.2%)	89 (48.9%)
Fever													
Yes (>38.5 ^{0}C)	4 (18.2%)	4 (7.1%)	1 (25%)	1 (16.7%)	1 (7.7%)	0	1 (33.3%)	0	0	0	1 (33.3%)	9 (13.2%)	22 (12.1%)
No (≤ 38.5^{0}C)	18 (81.8%)	52 (92.9%)	3 (75%)	5 (83.3%)	12 (92.3%)	0	1 (33.3%)	1 (100%)	6 (100%)	0	2 (66.7%)	59 (87.8%)	159 (87.4%)
Unknown	0	0	0	0	0	0	1 (33.3%)	0	0	0	0	0	1 (0.5%)
BMI (n = 182)													
Underweight (< 18.5)	19 (86.4%)	48 (85.7%)	3 (75%)	6 (100%)	8 (61.5%)	0	3 (100%)	1 (100%)	4 (66.7%)	0	3 (100%)	50 (73.5%)	145 (79.7%)
Healthy weight (18.5 - 24.9)	1 (4.5%)	4 (7.1%)	1 (25%)	0	5 (38.5%)	0	0	0	0	0	0	11 (16.2%)	22 (12.1%)
Overweight (25 - 29.9)	0	0	0	0	0	0	0	0	0	0	0	1 (1.5%)	1 (0.5%)
Obese (> 30)	0	1 (1.8%)	0	0	0	0	0	0	1 (16.7%)	0	0	2 (2.9%)	4 (2.2%)
Unknown	2 (9.1%)	3 (5.4%)	0	0	0	0	0	0	1 (16.7%)	0	0	4 (5.9%)	10 (5.5%)
Duration of Diarrhoea (n = 182)													
1 – 3 days	9 (40.9%)	32 (57.1%)	3 (75%)	3 (50%)	6 (46.1%)	0	2 (66.7%)	0	3 (50%)	0	1 (33.3%)	33 (48.5%)	92 (50.5%)
4 – 5 days	10 (45.5%)	12 (21.4%)	1 (25%)	1 (16.7%)	4 (30.8%)	0	0	0	2 (33.3%)	0	1 (33.3%)	18 (26.5%)	49 (26.9%)
≥ 6 days	3 (13.6%)	10 (17.9%)	0	1 (16.7%)	3 (23.1%)	0	0	1 (100%)	0	0	0	16 (23.5%)	34 (18.7%)
Unknown	0	2 (3.6%)	0	1 (16.7%)	0	0	1 (33.3%)	0	1 (16.7%)	0	1 (33.3%)	1 (1.5%)	7 (3.8%)
HIV status (n = 182)													
Positive	3 (13.6%)	4 (7.1%)	1 (25%)	0	2 (15.4%)	0	0	0	1 (16.7%)	0	1 (33.3%)	12 (17.6%)	24 (13.2%)
Negative	13 (59.1%)	38 (67.9%)	3 (75%)	1 (16.7%)	5 (38.5%)	0	2 (66.7%)	1 (100%)	2 (33.3%)	0	0	36 (52.9%)	101 (55.5%)
Exposed	4 (18.2%)	7 (12.5%)	0	4 (66.7%)	2 (15.4%)	0	1 (33.3%)	0	3 (50%)	0	2 (66.7%)	12 (17.6%)	35 (19.2%)
Unknown	2 (9.1%)	7 (12.5%)	0	1 (16.7%)	4 (30.8%)	0	0	0	0	0	0	8 (11.8%)	22 (12.1%)
Mode of Feeding (n = 89)													
Breast feeding	4 (50%)	11 (37.9%)	1 (33.3%)	1 (33.3%)	2 (66.7%)	0	1 (50%)	0	1 (25%)	0	0	19 (55.9%)	40 (44.9%)
Mixed feeding	4 (50%)	18 (62.1%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	0	1 (50%)	1 (100%)	3 (75%)	0	2 (100%)	15 (44.1%)	49 (55.1%)

NoV = Norovirus; RoV = Rotavirus; Giardia = *Giardia lambliae;* Crypto = *Cryptosporidium parvum*; BMI = Body mass index

DISCUSSION

Diarrhoeal disease is a major contributor to child morbidity and mortality worldwide, especially in low and middle-income countries (Lanata *et al.,* 2013). Variable aetiologies of diarrhoea among children of five years and below have been recorded in different countries of the world (Shrivastava *et al*, 2017; Zhang *et al.,* 2016; Saeed *et al*., 2015). This study showed that 62.6% of the children were positive for at least one diarrhoea pathogen. As in other studies from South Africa (NHLS, 2013; Seheri *et al*., 2010), RoV was the most commonly diagnosed aetiology in this study. This is in keeping with a previous report from KZN where 55 % of diarrhoea cases were attributed to RoV (NHLS, 2013). Noteworthy is the fact that our study and the previously reported finding in KZN were conducted in the winter months of the year when RoV is most prevalent. The RoV vaccine was included in the national immunization program of South Africa about eight years ago and has been reported to effectively reduce the burden of RoV hospitalization (Madhi *et al*., 2010). Consequently, NoV may become the most commonly detected aetiology of diarrhoea among children less than 5 years old in the nearest future. This speculation was supported by our findings where NoV was the second most prevalent pathogen. In addition, all the NoV detected were of genogroup II (GII). This is surprising since previous studies in other part of South Africa has reported multiple genogroups of NoVs (Kabuea *et al*., 2016; Mans *et al*., 2016; Murray *et al*., 2013).

The most common co-infection detected was the norovirus-rotavirus combination which correlates with a previous report (Zhang *et al*., 2016). In contrast, rotavirus-*Shigella* co-infection was observed as the most frequent combination in a recent study in India (Shrivastava *et al*., 2017). However, our study was only focused on the non-bacterial aetiologies of diarrhoea. It will be important to conduct a study which considers the broad representation of the enteric pathogen community in children of 5 years and under to draw a firm conclusion regarding the prevalence of co-infections of diarrhoeal pathogens. Such a study should take seasonal differences into account. Although we did not analyse the influence of co-infection on severity of diarrhoea, but it was reported previously that co-infection might lead to a higher severity and complications of diarrhoea than infection with single pathogens (Shrivastava *et al*., 2017).

Few studies have highlighted the role of intestinal protozoa as an important aetiology of diarrhoeal disease in South Africa especially in KwaZulu-Natal (Iyaloo *et al*., 2015; Jarmey-Swan *et al*., 2001). In our study, intestinal protozoa were less frequently detected as compared to viruses. We observed that 3.3 % and 2.2 % of diarrhoeal cases were due to *C. parvum* and *G. lamblia* respectively. In contrast, a study among diarrhoeic children of 0 – 59 months old in rural Mozambique reported *Cryptosporidium* and *G. lamblia* as the most frequently detected diarrhoea aetiology after rotavirus (Nhampossa *et al.,* 2015). This disparity

may be attributed to the difference in socioeconomic conditions in the study areas. Therefore, a higher prevalence of diarrhoea pathogens is expected in the rural area of Mozambique in comparison to the urban area of KZN.

Most of the children enrolled in this study were less than 2 years old and diarrhoeal pathogen detection was significantly higher in this age group as previous reported in another study (Nhampossa *et al.*, 2015). This observation may be due to the poorly developed immune system of these children or due to waning natural immunity acquired transplacentally or via breast milk. The lower prevalence of diarrhoeal pathogen in the above 2 years age group is not surprising since it was previously reported that protection against some of these pathogens such as RoV is developed following subsequent infection in the earlier years (Clark and Desselberger, 2015; Desselberger & Huppertz, 2011). A study in Finland reported that 89.3% of diarrhoea patients had fever and that higher temperature shows that there is an increase in the severity of an infection (Hemming *et al*., 2014). They found a significantly higher RoV infection among children who had fever (Hemming *et al*., 2014). However, most of the children (87.4 %) in our study did not have fever at the time of specimen collection, and more than half of these patient had at least one infection.

The most widely used indicator for assessing undernutrition in children is weight measurement. Underweight is associated with undernutrition which leads to immune depression and increased risk of infections. We observed that about 80% of the children enrolled in our study were underweight and more than half of them had at least one diarrhoeal pathogen. This observation is consistent with a previous study in India where underweight children had a significantly higher prevalence of diarrhoea and infection in comparison to healthy weight children (Ramachandran and Gopalan, 2009). The statistically insignificant observation in our study regarding the prevalence of infection among underweight and healthy weight children shows that being underweight is not the only indictor for acquisition of infection among children with diarrhoea in KZN.

The effect of HIV on the immune system and the subsequent infection acquisition among HIV infected individuals has been widely studied. A study in Kenya revealed a higher prevalence of diarrhoea among HIV positive than HIV negative children. However, they reported that diarrhoeic HIV positive children were less likely to harbour bacterial pathogens than diarrhoeic negative children (Van-Eijk *et al.,* 2010). Similarly, in this study we observed that children who are HIV negative with HIV negative parents are more likely to have non-bacterial diarrhoeic infection than children who are HIV positive or HIV exposed. This could be the result of a higher prevalence of diarrhoea of non-infective origin such as increased malabsorption and metabolic enteritis (Van-Eijk *et al.,* 2010). In addition, a study in South Africa associated the use of trimethoprim-sulfamethoxazole prophylaxis to an increased risk of diarrhoea in both HIV infected and exposed infants (Coutsoudis *et al*., 2005). The advantage of exclusive breast feeding among

infants of HIV infected and uninfected mothers to diarrhoea morbidity and mortality in KZN has been previously documented (Rollins *et al.*, 2013). In this study, there was a tendency of higher infection rates among children who were mixed fed than the exclusively breastfed children irrespective of their HIV status. Although this observation was not significant, but it suggests that exclusive breast feeding potentially reduce diarrhoeal pathogen in the study population. A higher sample size may give us a better understanding of the effect of exclusive breastfeeding on the prevalence of diarrhoeal pathogen among infants and young children.

This hospital based study highlights the burden of major non-bacterial aetiologies of diarrhoea among children of five years and under in KZN. Single infection of viral origin as shown in more than half of our study population raises significant questions regarding the role of bacterial and parasitic pathogens in diarrhoeal disease. The potential synergistic effect during multiple infection in the overall pathogenesis of diarrhoeal diseases can only be understood when the overall burden of enteric pathogens is evaluated. The inability of laboratories in low income countries to detect some of these pathogens poses significant problems for treatment and infection control. Improved awareness about infection prevention and control, improve access to safe water, sanitation and hygiene, and the availability of zinc and vitamin A supplementation are measures to prevent and reduce the burden of diarrhoeal diseases in low and middle-income countries including South Africa.

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Ethics approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal in accordance with its ethical standards and with the 1964 Helsinki declaration.

Informed consent

Informed consent was obtained from the parents or guardian of all individual participants included in this study.

Author contributions

A Willem Sturm, Prashini Moodley and Osaretin E. Asowata designed the study. Osaretin E. Asowata, Nelisiwe V Nxele and Nakita Reddy participated in the sample collection and data management. Nelisiwe V Nxele, Osaretin E. Asowata, Nakita Reddy and Olubisi T. Ashiru participated in sample analysis. Nelisiwe V Nxele and Osaretin E. Asowata prepared the first draft of the manuscript. A. Willem Sturm and Prashini Moodley revised the manuscript. All authors participated in the preparation of manuscript and approved the final version for publication.

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APPENDICES

APPENDEX I: Consent Form

The study has been explained to me in detail and I grant permission for stool specimen to be collected from my child/ward. The stool specimen will be examined for germs causing diarrhoea and medicines that may kill these germs.

Yes..... No.....

I give permission for the doctor/nurse to record the HIV status of my child/ward on the information sheet and I understand that this information will not be linked to my child's name.

Yes..... No.....

I grant permission for a blood specimen to be collected from my child/ward and examined for HIV if this result is not available in his/her file

Yes..... No.....

I grant permission for a blood specimen to be collected from my child/ward and examined for antibody levels against some germs that cause diarrhoea.

Yes..... No.....

We hereby declare that we do not intend to export or sell the collected specimens for commercial purposes

Name of Parent/Guardian: Name of Doctor/Nurse

---------- ----------

Signature---------- Signature----------

Date: _____

Name of Translator (if applicable)

Signature----------

NB: This sheet is to be removed by the study coordinator upon completion of enrolment and delivered in a sealed envelope to the principal investigator. The forms will be stored in a separate, locked cabinet and destroyed when the study is officially closed.

APPENDEX II: Questionnaire

Patient no. _____

Date of birth (dd/mm/yy) _____

Date of admission _____

Date of specimen collection _____ No specimen ☐

Is the stool

Watery	☐
Bloody	☐
Unknown	☐

Age _____

Gender

M	F

Weight _____ kg _____ centile

Height _____ kg _____ centile

HIV status	P	N	Exposed	Unexposed	Unknown
On ART?	Y	N			

CD4 count/ percentage _____

How long does the patient have diarrhea for _____

Is the child underweight for age?	Y	N
Does the child have kwashiorkor?	Y	N

Has the child received immunizations to date?

Birth	BCG	
	OPV	
6 weeks	OPV	
	RV (1)	
	DTaP-IPV/Hib (1)	
	Hep B (1)	
	PCV (1)	
10 weeks	DTaP-IPV/Hib (2)	
	Hep B (2)	
14 weeks	RV (2)	
	DTaP-IPV/Hib (3)	
	Hep B (3)	
	PCV (2)	
9 months	Measles (1)	
	PCV (3)	
18 months	DTaP-IPV/Hib (4)	
	Measles (2)	

How many doses has of vitamin A has the child received _____

Has the child received Zinc supplementation?

☐ YES ☐ NO ☐ UNKNOWN

Has the child received Anti-helminthic?

_____

If child < 6 months, is the child

Exclusively breastfed	
Formula-fed	
Mixed fed	

What is the water supply at home?

Piped water in dwelling	
Piped water on site	
Neighbor's tap	
Communal tap	
Rainwater	
Borehole on site	
River /stream /flowing	
Dam /pool /stagnant water	
Well	
Spring	
Other	

Specify _____

Type of sanitation

Flush	
VIP	
Urine-diversion	
Other	

Specify _____

Availability of soap to wash hands

Always	
Sometimes	
Never	

Current treatment

_____

_____

_____

_____

Any treatment prior to coming to this clinic/hospital

_____

_____

_____

APPENDEX III: Ethics Certificates

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
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4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

08 July 2015

Prof P Moodley
719 Umbilo Road
Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley

PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and Outbreak Investigation. REF: BE222/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 06 August 2015
Expiration of Ethical Approval: 05 August 2016

I wish to advise you that your application for Recertification received on 03 July 2015 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The approval will be ratified by a full Committee at a meeting to be held on 11 August 2015.

Yours sincerely

Mrs A Marimuthu
Senior Administrator Biomedical Research Ethics Committee

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 March 2016

Ms N Nxele (209528542)
Discipline of Microbiology
School of Laboratory Medicine and Medical Sciences
209528542@stu.ukzn.ac.za

Protocol: Detection of Human Novoviruses among children 5 years and below in KwaZulu-Natal.
Degree: MMedSc
BREC reference number: BE350/15 *(sub-study of BE222/13)*

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 31 July 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated December 2015 to queries raised on 05 September 2015 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **17 March 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **12 April 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc: supervisor: moodleya@ukzn.ac.za
cc: postgrad: dudhrs'hp@ukzn.ac.za