EFFECTIVENESS OF A MONOVALENT HUMAN ROTAVIRUS VACCINE AMONG CHILDREN OF 5 YEARS AND UNDER IN KWAZULU-NATAL

OSARETIN EMMANUEL ASOWATA

SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (MEDICAL MICROBIOLOGY) IN THE SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES, UNIVERSITY OF KWAZULU-NATAL, SOUTH AFRICA

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PREFACE

Diarrhoeal disease is one of the leading cause of morbidity and mortality among children of 5 years and under in South Africa. Rotaviruses have been documented as causing one-third of all diarrhoea hospital admissions. Vaccination is the main public health intervention to prevent and control rotavirus infections. Two oral live-attenuated rotavirus vaccines has been licensed for global use and one of these vaccines has been included in the national immunization programme of South Africa since August 2009. However, rotavirus remains a major aetiology of diarrhoea in infants and young children in South Africa. Clinical trials in South Africa have reported a low effectiveness of this vaccine in comparison to that reported from high income countries. Reasons for the reduced effectiveness of the rotavirus vaccine seen locally are speculative. The ability of this vaccine to protect against emerging strains of rotavirus is not fully understood. This study was conducted to evaluate possible factors influencing the effectiveness of the rotavirus vaccine on Kwazulu-Natal, South Africa.
DECLARATION

This work was carried out by me, Osaretin Emmanuel Asowata. It has not been submitted in any form to the University of KwaZulu-Natal or any other tertiary institution for degree award purposes. All experiments was carried out in the department of Infection Prevention and Control, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal under the supervision of Professor Prashini Moodley and co-supervised by Dr. Olubisi Ashiru.

_________________                                                                                        ________________
Osaretin Emmanuel Asowata                                                                           Professor Prashini Moodley
(Candidate)                                                                                                        (Supervisor)

_________________                                                                                             ____________________
O.T Ashiru                                                                                                    Dr. Olubisi Ashiru
(Candidate)                                                                                                        (Co-Supervisor)
DEDICATION

This work is dedicated to my mother, Patience Asowata and the loving memory of my late father, Moses Asowata.
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I wish to thank the children and their parents who gave consent to be enrolled in our study. Many thanks to the staff of the paediatric unit of King Edward VIII hospital for their kindly support during the specimen collection phase of our study. Noteworthy is Dr. Moherndran Archary who gave us the needed support and assistance. I wish to thank the staff of the school of Laboratory Medicine and Medical Sciences especial the administrative secretary, Prathna Dudhrajh for her ever-ready attitude to help. I am indebted to the College of Health Science’s research team led by the Dean of Research, Professor Moses Chimbari for the financial support through the college research scholarship which made it possible for me to complete my project. I wish to thank the senior staff members of the KwaZulu-Natal’s Department of Health and provincial pharmaceutical store for their support.

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CONFERENCE PRESENTATIONS


LIST OF ABBREVIATIONS

ACIP: Advisory committee on immunization practices
CDC: Centre for Disease Control and Prevention
cDNA: Complementary Deoxyribonucleic Acid
DNA: Deoxyribonucleic Acid
dsRNA: Double Stranded Ribonucleic Acid
EIA: Enzyme Immunossay
ELISA: Enzyme Linked Immunosorbent Assay
EM: Electron Microscope
ENS: Enteric Nervous System
GSK: GlaxoSmithKline
NSP: Non-structural proteins
PAGE: Polyacrylamide Gel Electrophoresis
RCWG: Rotavirus classification working group
RNA: Ribonucleic Acid
RT-PCR: Reverse Transcription Polymerase Chain Reaction
UNICEF: United Nations Children Education Fund
VP: Virus protein
WHO: World Health Organization
ABSTRACT

Human rotavirus infection is the leading cause of gastroenteritis in infants and young children worldwide. In South Africa, gastroenteritis is a major cause of childhood morbidity and mortality in children less than 5 years, and rotavirus infection has been documented as causing one-third of all gastroenteritis related hospital admissions. Vaccination is the major public health intervention to control rotavirus disease. The Rotarix® is the only rotavirus vaccine included in the national immunization program of South Africa. The effectiveness of this vaccine is questionable due to the continual outbreaks of rotavirus infection in South Africa, including KwaZulu-Natal, regardless of the high vaccination coverage. This study focused on evaluating the factors influencing the effectiveness of the Rotarix® vaccine in children 5 years and under in KwaZulu-Natal, South Africa.

After obtaining written informed consent from parents or guardians, stool and blood specimens where collected from children 5 years and under presenting to King Edward VIII hospital (KEH VIII) in Durban, South Africa. The study was conducted between June 2014 and June 2015. Demographic and clinical information was collected using a well-structured questionnaire. Enzyme immunoassay (EIA) was performed to detect rotavirus antigen in the stool and rotavirus immunoglobulin G (IgG) in the serum. Selected EIA positive and negative samples were confirmed using G-types and P-types consensus primers in a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The RT-PCR positives were genotyped using genotypes specific primers. The avidity of the rotavirus specific IgG was determined using the urea elution technique. Rotarix® vaccines stored at optimum temperatures were collected from the provincial pharmaceutical store. The effect of sub-optimal temperatures on the potency of the Rotarix® vaccine were determined using the plaque assay.

Three hundred and sixty-five (365) stool specimens were collected. Rotavirus antigen was detected in 83 (22.7%) patients from stool specimens. The stratification of rotavirus cases by vaccination status was not significant (p=0.4). The distribution of rotavirus was not significantly associated with HIV status of the children (p=0.7). We observed that seasonality was a significant driving force influencing the prevalence of rotavirus infection in our setting (p<0.001). We recorded the highest rotavirus prevalence in the winter months of the year with 79 (45.9%) positive cases of rotavirus associated diarrhoea.

Blood specimens were only collected in 35 patients. From the corresponding stool specimens [21 (60%) EIA positives and 14 (40%) EIA negatives], 29 (82.9%) were positive for rotavirus using conventional RT-PCR. Genotyping revealed G9P[8] (20.7%) to be the most prevalent genotype followed by G9P[4] (13.8%), G12P[4] (10.3%), G9P[6] (6.9%) and a 3.4% prevalence was recorded for each of G4/G8P[6], G4P[6], G12P[6], G8P[10] and G9P[10]. We were unable to fully genotype some of the rotavirus strains.
(non-typeable) by the available primers. 2 (6.9%) and 4 (13.8%) were non-typeable for the G and P types respectively. However, all 35 serum samples were positive for rotavirus IgG. We observed that the rotavirus specific IgG had no significant effect on the prevalence of rotavirus detection in stool (p=0.8). There was no significant difference in the mean avidity of IgG in the 3 vaccination strata (p=0.3). Exposure of the Rotarix® vaccine to the seasonal temperatures and to extreme temperatures of 40°C for 3 to 72 hours as well as -20°C and -80°C for 12 hours did not affect the potency of the vaccine beyond its expected standard.

Our study highlighted the genetic diversity of rotaviruses and poor immunogenicity of the vaccine as key factors affecting the effectiveness of the rotavirus vaccine. Whether the vaccine is able to induce homotypic and heterotypic protection in immunized children is critical in predicting the long range effectiveness of this vaccine against uncommon regional rotavirus strains. Interventions targeted at improving socio-economic conditions in low income countries might be a starting point towards the control and prevention of rotavirus infection in these settings.
1. INTRODUCTION

Rotaviruses are group of viruses belonging to the family *Reoviridae* (Estes and Kapikian, 2007). This family has twelve genera (Schiff *et al*., 2007), of which rotavirus is the most clinically significant and is the leading cause of acute infantile diarrhoea worldwide (Estes and Kapikian, 2007). Diarrhoea is a condition of having three or more loose or liquid stools per day or an abnormal condition of excessive loose stools and is commonly caused by gastroenteritis (Navaneethan and Giannella, 2008). Diarrhoea is one of the second leading cause of death among under-5 children in Africa (UNICEF, 2009).

Approximately 114 million children are infected with rotavirus worldwide, leading to about 453,000 deaths annually (Tate *et al*., 2012). Rotavirus has been classified into eight groups (A to H) based on their genetic and antigenic properties (Matthijnssens *et al*., 2012). Rotaviruses belonging to group A, B and C are found in both humans and animals, while the other groups (D, E, F, G and H) have only been isolated from animals to date (Estes and Kapikian, 2007). Group A rotaviruses are the most common cause of diarrhoea in humans, especially in children under five years old (Matthijnssens *et al*., 2012). Group B rotavirus was identified as a cause of epidemic diarrhoea amongst Chinese adults (Hung *et al*., 1983; Su *et al*., 1986). Members of Group C rotavirus have been reported as causes of sporadic diarrhoeic cases in family outbreaks (Oseto *et al*., 1993). Subsequent rotavirus infections have resulted in variable levels of protections against future infections and the mechanism of this protection is not fully understood. Two oral rotavirus vaccines have been licensed for global use. The monovalent Rotarix® and the pentavalent RotaTeq®. These vaccines have high efficacies in high-income countries such as the United States of America and European countries viz. Finland, Austria and Israel (Vesikari *et al*., 2007a; Vesikari *et al*., 2006) where the severity of rotavirus associated diarrhoea is minimal. In contrast, reduced levels of vaccine protection has been recorded in low income countries in Africa viz: Malawi, South Africa, Ghana, Kenya and Mali (Armah *et al*., 2012; Sow *et al*., 2012; Tapia *et al*., 2012; Armah *et al*., 2010b). The rotavirus disease in Africa is characterized by a higher severity of diarrhoea. Seasonal variation of rotavirus infection has been described in high-income settings, whilst an all year detection of rotavirus infection is associated with the low-income environment (Patel *et al*., 2013). Increased diversity of circulating strains is also associated with the low-income settings and has been linked to the higher severity of rotavirus associated diarrhoea. Currently, 32 countries in Africa have included the rotavirus vaccines as part of their national immunization program (PATH, 2015). However, the lower effectiveness of these vaccines in Africa in comparison to high income countries (Bar-Zeev *et al*., 2015) is a problem given frequent rotavirus outbreaks despite high vaccination coverage (NICD, 2013). Determinants of protections induced by these vaccines and protection against diverse strains not covered by these vaccines raises significant questions (Clark and Desselberger, 2015).
The monovalent Rotarix®, which is given as a two-dose schedule in infancy contains an attenuated human G1P[8] rotavirus strain (Angel et al., 2007). In contrast, the pentavalent RotaTeq® given as a 3 dose schedule was propagated using five bovine-human reassortant strains. These strains contain genes which encodes the human G1, G2, G3, G4, P[8] antigens as well as genes encoding the bovine G6 and P(7) antigens (Angel et al., 2007). Despite the differences in the genetic components of these vaccines, studies show high similarities in their effectiveness in children up to 2 years of age. The exact mechanism of action by which these different vaccines exert similar effectiveness (Vesikari, 2012) is not fully understood. The efficacy for each of these vaccines was not the same under different socio-economic conditions. High efficacies of the Rotarix® vaccine were reported in high-income countries such as Hong Kong (95.6%) (Lau et al., 2013), Japan (91.6%) (Kawamura et al., 2011), Europe (90.4%) (Vesikari et al., 2007b; GlaxoSmithKline, 2013), Latin America and Finland (84.7%) (Ruiz-Palacios et al., 2006). Efficacy studies of the Rotarix® vaccine recorded low efficacies in the low-income settings of Malawi (49.2%) (Cunliffe et al., 2012; Steele et al., 2012; Mahdi et al., 2010) and South Africa (72.2%) (Steele et al., 2012; Mahdi et al., 2010) in the first year of life among the children tested. Much lower efficacy were recorded in the second year of life with a 34% and 32% efficacy attributed to Malawi and South Africa respectively. For the RotaTeq® vaccine, Europe, North America and Latin America had a combined efficacy of 98% and 88% in the first and second year of life respectively (Vesikari et al., 2007a; Vesikari et al., 2006). In Finland, a 94.0% efficacy was reported against rotavirus associated emergency department visit and hospitalization for up to 3.1 years after vaccination regardless of the circulating serotypes (Vesikari et al., 2010). In contrast, lower efficacies of immunization with RotaTeq® vaccine were reported in the low-income settings of Africa. A combined efficacy of 64.2% and 19.6% were reported in the first and second year of life respectively in Ghana, Kenya and Mali (Armah et al., 2012; Sow et al., 2012; Tapia et al., 2012; Armah et al., 2010b). Post-vaccination effectiveness of these vaccines against hospitalization and emergency department visits has been reported in the United States of America (86%) (Staat et al., 2011), Australia (89.3%) (Buttery et al., 2011), Israel (89%) (Muhsen et al., 2010), Nicaragua (88%) (Mast et al., 2011), Austria (92.8%) (Paulke-Korinek et al., 2010) and Brazil (95.7%) (Justino et al., 2011). These data were recorded in the first year after vaccination. Almost all rotavirus vaccine effectiveness studies have been conducted in high, middle and low income countries outside Africa. These studies may have reported high efficacies against rotavirus infections in the high income and in some middle income countries (Staat et al., 2011; Mast et al, 2011; Justino et al., 2011) but the ability of these vaccines to elicit total protection against emerging strains and strains not covered by the vaccines in low income setting is questionable.
Post-vaccination effectiveness study from Africa are scanty. Groome et al., (2014) reported a 57% and 40% Rotarix® vaccine effectiveness in children who were completely vaccinated and those who received a single dose respectively in South Africa. This study was conducted amongst children of 18 weeks to 23 months old. They concluded that the vaccines were able to protect children against acute rotavirus associated diarrhoea hospitalization in the first and second year of life. However, the reported vaccine effectiveness is much lower in comparison to that of high income countries. The reason(s) for this disparity in vaccine effectiveness is debatable. Understanding the effect of socio-economic conditions on rotavirus vaccine effectiveness will be of utmost importance in future vaccine development. In addition, the absence of a definite correlate of protection, has limited all rotavirus vaccine efficacy trials to clinical endpoints. A correlate of protection is needed to serve as a surrogate endpoint which will enable the prediction of vaccine efficacy for new vaccines, thereby contributing to the rapid development and licensure of new rotavirus vaccines. Furthermore, the incomplete knowledge of the variable levels of protection conferred by these rotavirus vaccines in different settings is also a hindrance towards maximizing their public health impact.
Approximately 114 million children are infected with rotavirus annually which result in approximately 453,000 deaths (Parashar et al., 2006a and Tate et al., 2011a). About 50% of morbidity and mortality occur in developing countries. In South Africa, diarrhoeal diseases are ranked the third major cause of childhood mortality in children less than 5 years, where the majority of deaths are among Black African children (Bradshaw et al., 2003). Rotaviruses have been documented as causing one-third of all diarrhoea admissions in the hospital (Steele et al., 2003). The Rotarix® (GlaxoSmithKline, Belgium) and the RotaTeq® (Merck, USA) are current globally licensed vaccines. However, only the Rotarix® have been included in the expanded immunization program of South Africa and it is administered orally at 6 and 14 weeks of age. The immunogenicity and potency of this vaccine is questionable not just in South Africa but in other sub-Saharan African countries where rotavirus remains the major aetiology of diarrhoea among infants and young children irrespective of their vaccination status. This may be due to factors such as the genetic diversity of rotaviruses, which may be as a result of point mutation, genetic reassortment, rearrangement and intragenic recombination. Incomplete information on the immunogenicity of rotavirus vaccine is also a big challenge in predicting the long-term effectiveness of these vaccines (Angel et al., 2007; Velazquez, 2009). The mechanism by which this rotavirus vaccine protect against future rotavirus infections caused by novel genotypes or uncommon regional strains is still not well understood. Reduced potency of these vaccines may be responsible for the continued occurrence of rotavirus infection among vaccinated children. This reduced potency may be a result of vaccine exposure to sub-optimal temperature while in storage or on transit. Timing of immunization may also be responsible for the observed reduction in efficacy of this vaccine. Rotavirus vaccine potency and post-vaccination vaccine effectiveness studies are few in South Africa (Groome et al., 2014). There is a need to evaluate possible factors influencing the potency and effectiveness of the Rotarix® vaccine to give a better understanding of the vaccine protective capability. We hope to better understand why rotavirus remains a major cause of diarrhoea among infants and young children irrespective of the high vaccination coverage in South Africa.

2.1. Aim and Objectives

This study focused on assessing factors influencing the effectiveness of rotavirus vaccine (Rotarix®) in children of 5 years and under in KwaZulu-Natal, South Africa.

The specific objectives of this project are:

1. To study demographic factors associated with rotavirus infection in the study population
2. To detect and characterize the circulating human rotaviruses in the study population

3. To determine the presence and avidity of rotavirus specific immunoglobulin G (IgG) elicited in this group of children

4. To determine the effect of sub-optimal temperature on the potency of the Rotarix® vaccine.
3. LITERATURE REVIEW

3.1. Child Mortality

Child mortality is also known as under-5 mortality and refers to death of infants and children under the age of five. The overall global decline in child mortality is a result of improved socioeconomic development and implementation of child survival interventions, however millions of children still die every year before their fifth birthday (You et al., 2010). According to the United Nations Children's Emergency Fund (UNICEF, 2010), 12.4 million under-5 children died in 1990 worldwide, 8.1 million in 2009 (UNICEF, 2010), 7.6 million in 2010 (UNICEF 2011), 6.9 million in 2011 (WHO, 2013) and 6.6 million in 2012 (WHO, 2013). The aim of the United Nations (UN) millennium development Goal 4 (MDG 4) was to reduce mortality of children younger than 5 years by two-thirds between 1990 and 2015. Many countries, especially in South-East Asia and Sub-Saharan Africa have not met this target, and approximately half of these deaths occur in these regions.

Globally, annual mortality in under-5 has decreased by 47% from an estimated rate of 90 deaths per 1000 live births in 1990 to 48 deaths per 1000 live births in 2012 (WHO, 2013). An accelerated rate of reduction of this mortality was recorded from 1.2% a year between 1990 and 1995 to 3.9% between 2005 and 2012, and this has not been significant in meeting MDG 4. This is due to the unequal distribution of this progress. The reduction in under-5 mortality rates between 1990 and 2012 were observed to be over 60% in 3 World Health Organization (WHO) regions; the Americas, European and the Western Pacific. Mortality in the WHO African Region remains high (WHO, 2013). About 4.7 million deaths are recorded annually in this region, followed by South East Asia with 3.1 million deaths (WHO, 2013). Almost 75% of all these deaths are attributed to six conditions: neonatal causes, pneumonia, diarrhoea, malaria, measles and HIV/AIDS. Achieving this MDG goal will require that key effective and affordable interventions are covered universally. These interventions include: improved care of newborns and their mothers; feeding of infants and young child; immunization; prevention and case management of pneumonia, diarrhoea and sepsis; malaria control; and prevention and care of HIV/AIDS. These interventions could reduce mortality by more than 50% in high incident countries. WHO (2013) further promotes 4 main strategies to assist affected countries deliver these interventions: appropriate and timely treatment of complications of newborns; integrated management of childhood illness for all under-5 children; expanded programme on immunization; appropriate feeding of infants and young children.
3.2 Diarrhoeal Diseases

Diarrhoea is a condition of having three or more loose or liquid stools per day or an abnormal condition of excessive loss. It is commonly caused by gastroenteritis (Navaneethan and Giannella, 2008). Rotavirus-associated gastroenteritis accounts for 40% of diarrhoea cases among under-5 children (Greenberg and Estes, 2009), while norovirus is the most common cause of viral diarrhoea in adults. Adenovirus (types 40 and 41) and astrovirus are also significant viral etiologies of diarrhoeal infections (Mitchell, 2002). Bacterial diarrhoeal infections are commonly caused by *Campylobacter spp* but infections with *Salmonella spp*, *Shigella spp*, and some strains of *Escherichia coli* are also frequent (Viswanathan et al., 2009). Toxins produced by *Clostridium difficile* causes severe diarrhoea in aged people, particularly those treated with antibiotics for unrelated infections (Rupnik et al., 2009). Parasitic protozoa such as *Giardia lamblia* and *Entamoeba histolytica* have been diagnosed as causative agents of diarrhoea (Dans and Martinez, 2006; and Gonzales et al., 2009). In hygienic conditions where there is adequate and safe food and water supply, a healthy individual recovers within a few days. But ill or malnourished individuals can suffer severe dehydration which may be life threatening (Alam and Ashraf, 2003).

In 2004, about 2.5 billion cases of diarrhoea occurred globally, and 1.5 million deaths was recorded amongst children under the age of 5 years. More than half of these were in Africa and South Asia (UNICEF, 2009). This is lower than the 4.5 million deaths recorded in 1980 (Snyder and Merson, 1982). WHO estimates that deaths in lower income countries account for approximately 85% of all deaths associated with diarrhoea (WHO, 2009a). In South Africa, a 10.2% under-5 mortality due to diarrhoea was reported (Seheri et al., 2010) and diarrhoea was among the top 3 causes of under-5 mortality.

3.3 Brief History of Rotavirus

In 1943, Jacob Light and Horace Hodes proved that a filterable agent found in the faeces of children with infectious diarrhoea was also responsible for the livestock diarrhoea (Scours) in cattle (Light and Hodes, 1943). Preserved samples of this agent were later processed and found to be rotavirus (Mebus et al., 1976). Ruth Bishop and colleagues in 1973 described related viruses found in children having gastroenteritis (Bishop et al., 1973). In 1974, Thomas Henry Flewett observed by means of electron microscopy, that the virus particle looked like a wheel and suggested the name rotavirus (Flewett and Woode, 1978; and Flewett et al., 1974). The name was officially adopted by the International Committee on taxonomy of viruses four years later (Mathews, 1979). In 1976, other animal species were found to harbour related viruses (Woode et al., 1976). These viruses were all found to cause acute gastroenteritis
and recognized to collectively affect humans and animals worldwide (Flewett and Woode, 1978). In 1980, rotavirus serotypes were first described (Wyatt et al., 1982; 1983) and in 1981, rotavirus from humans was first grown in monkey kidney cell line cultures (Urasawa et al., 1981). Rotavirus research was facilitated by its ability to grow in tissue culture, and this led to the development and evaluation of the first candidate vaccine by mid-1980s (Institute of Medicine, 1986; De Zoysa and Feachem, 1986).

A rotavirus vaccine called Rotashield was licensed in the United States of America (USA) in 1998 after clinical trials in the USA, Finland and Venezuela found it to be 80 to 100% effective at preventing severe diarrhoea caused by Rotavirus group A (ACIP, 1999; Kapikian, 2001). This vaccine was later withdrawn by the manufacturer in 1999 because it was discovered to contribute to an increased risk for intestinal intussusception, with a risk of one in every 12,000 vaccinated infants (Bines, 2005). In 2006, the Rotarix® and RotaTeq® vaccines against human rotavirus infection were approved to be safe and effective in children (Dennehy, 2008) and in June 2009, it was recommended by the WHO that Rotavirus vaccination should be included in all national immunization programmes.

### 3.4. Structure of Rotavirus

Rotavirus (RV) virion is a 100nm non-enveloped icosahedral particle harbouring a capsid made up of three concentric protein layers (Figure 1) (McClain et al., 2010; Settembre et al., 2011). The genome within this virion is surrounded by the capsid and this genome is composed of eleven segments of double-stranded (ds) RNA. The first 10 genome segment codes for one protein, except for the segment 11, which code for two (Estes and Kapikian, 2007), making up a total of 12 proteins, six structural proteins (VP1 - VP4 and VP6 - VP7) and six non-structural proteins (NSP1 – NSP6) (Figure 1). The non-structural proteins (NSP) enhance virus functions such as genome replication, particle assembly, host innate responses regulation (Feng et al., 2008), and viral gene expression stimulation. The innermost protein layer of the virion is formed by the VP2 which is also known as the core shell protein (McClain et al., 2010). Attached to the interior side of the VP2 layer are two minor proteins: the VP1, a viral RNA-dependent RNA polymerase and the VP3, an RNA capping enzyme (Liu et al., 1992; Lu et al., 2008). The combination of the VP1, VP2, VP3, and the dsRNA genome form the virion core. This virion core is surrounded by VP6, the intermediate protein layer component. The VP7 glycoprotein makes up the outer protein layer and projecting outward from the VP7 layer are spikes of the protease sensitive VP4 attachment protein. During infection, the VP4 spike cleaves by protease into two polypeptides, VP8* and VP5* (Arias et al., 1996). The head of the spike is represented by VP8*, while the VP5* forms the stalk.
and base (Settembre et al., 2011). During infection, VP4 is cleaved by trypsin-like proteases in the intestine, thereby stimulating virus interaction and susceptible enterocytes are penetrated.

The rotavirus VP7 and VP4 proteins (including the VP4 fragments VP8* and VP5*) contain neutralizing antibodies inducing multiple antigenic epitopes (Aoki et al., 2009; Dormitzer et al., 2002). The amino acids critical to the antigenic properties of rotavirus has been identified and located through studies into the atomic structures of VP7 and VP4 and the characterization of viral neutralization escape mutants. This information combined with nucleotide sequencing of the VP7 and VP4 genes will be of utmost importance in the monitoring of circulating human rotaviruses (HRVs) for antigenic variations that may affect the rotavirus vaccines effectiveness (Patton, 2012).

Figure 1: Rotavirus capsid structure and double stranded RNA genome (Patton J, 2012)
(A) Intact triple-layered virion with VP4 spikes projecting from the VP7 outer capsid shell. (B) Cut-away of virion revealing the three protein layers of the virion: VP2, VP6, and VP7. Note that the foot of the VP4 spikes extends into the VP6 layer. (C) VP4 centric plug perspective identifying the VP8* and VP5* regions of the VP4 spike protein. (D) Double stranded RNA segments of the RV genome resolved by gel electrophoresis. Segments are labeled as g1-g11 (g = gene) and their protein products listed. Associated functions or properties of the protein products are given (genotype name). The underlined letter identifies the segment in the gene constellation acronym: Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx.

3.5. Classification of Rotavirus

Rotavirus genus is a member of the *Reoviridae* family. It is classified into groups and subgroups, and into serotypes and/or genotypes based on antibody reactivity to VP6 and on the outer capsid proteins, VP4 and VP7 respectively. These rotaviruses are subdivided into groups A to H (Matthijnssens *et al.*, 2012). The group A rotaviruses account for the majority of human rotavirus infections that are associated with mortality and morbidity, making them the target of ongoing vaccine development programs (Patton, 2012). Some members of group B, C and H rotaviruses have been implicated as causes of diarrhoeal disease in humans (Attoui *et al.*, 2012). Unlike the group A Rotaviruses, which are predominately associated with diseases in children under 5 years old, adult diarrhoea rotavirus (ADRV) from group B (Fang *et al.*, 1989) have been associated with large outbreaks of severe diarrhoea in thousands of adults in China. Group C rotaviruses causes diarrhoea in children that are older (4 to 7 years) compare to the group A infections (Caul *et al.*, 1990; Matsumoto *et al.*, 1989). Outbreaks associated with group C rotaviruses tend to be sporadic and self-limiting in nature, and have been a major cause of food-born contamination in institutional settings. Groups D, E, and G rotaviruses have been shown to only infect avian species (Trojnar *et al.*, 2010).

Rotaviruses can also be classified based on their genetic constitution (genotypes) and the surface antigens of the virus (serotypes). This classification is based on the outer capsid proteins, VP7 (G-types) which is a glycoprotein and the VP4 (P-type), a protease sensitive protein (Matthijnssens *et al.*, 2011b; Coulson, 1996). The genotyping system is based on analysis of VP7 and VP4 genes by reverse transcription polymerase chain reaction (RT-PCR) or by cDNA sequencing (Gentsch *et al.*, 1992; Gouvea *et al.*, 1990). About 27 G genotypes (G1 - G27) and 37 P genotype (P1 – P (37), have thus far been described for rotaviruses (Matthijnssens *et al.*, 2011b). The limitation of the G and P type classification system is that it adopted only the VP7 and VP4 genes and ignored the other nine genes. Thus, necessary information required to fully evaluate the genetic diversity and evolutionary dynamics and relationships of co-
circulating rotavirus strains were omitted by this classification system. This limitation was partially overcome by the advancement of high throughput sequencing technologies that allows routine full genome sequencing of rotavirus strains and the creation of a complete sequence-based classification system that allows each genome of the virus to be assigned a particular genotype (Matthijnssens et al., 2008a). In this classification system, the genome segments for VP7 – VP4 – VP6 – VP1 – VP2 – VP3 – NSP1 – NSP2 – NSP3 – NSP4 – NSP5/6 are represented by the acronym Gx – P[x] – Ix – Rx – Cx – Mx – Ax – Nx – Tx – Ex – Hx, and x represents an Arabic numeral greater or equal to 1. Currently, the following genotypes have been identified among the group A Rotaviruses: VP7: 27G, VP4: 35P, VP6: I16, VP1: R9, VP2: C9, VP3: M8, NSP1: A16, NSP2: N9, NSP3: T12, NSP4: E14, and NSP5/NSP6: H11 genotypes (Matthijnssens et al., 2011a).

Full genome sequencing has shown that the internal genes of human G1P[8], G3P[8], G4P[8] and G9P[8] rotaviruses almost invariably belong to genotype 1 (Heiman et al., 2008; Jere et al., 2011a; Matthijnssens et al., 2008a; McDonald et al., 2009). In exceptional case, only 1 or 2 of the internal genes are usually involved in these genome sequences (Esona et al., 2011). These viruses having internal genomic constellations that are predominantly of genotype 1 are referred to as genogroup 1 viruses. The human G2P[4] viruses belong to genotype 2 and this was revealed through sequencing of the internal genes. These viruses are called genogroup 2 viruses. It has also been revealed through sequencing studies that Rotavirus strains with genotype 1 or 2 internal constellations of genes are rarely found in animals, other than humans (Patton, 2012). Phylogenetic studies has indicated that an evolutionary relationship exist between human Genogroup 1 rotaviruses and porcine rotaviruses, and between human genogroup 2 rotaviruses and bovine rotaviruses (Matthijnssens et al., 2008a). This evolutionary relationship account for the many genotype 1 genes that the human G1P[8], G3P[8], G4P[8] and G9P[8] viruses share with some porcine rotaviruses, and for the genotype 2 genes that the human G2P[4] viruses share with some bovine rotaviruses (Patton, 2012).

3.6. Rotavirus Epidemiology

The molecular epidemiology of different strains of rotavirus is complex. The circulating strains in each year are highly unpredictable. Thus, there is a year to year, season to season and region to region variation (Steele et al., 1995). These attributes could be a direct result of selective pressure of serotype specific antibodies (Franco et al., 2006) and the segmented nature of the rotavirus genome, and these could enhance reassortment during mixed infections by different strains. A natural reassortment between animal
and humans strains have reportedly led to the emergence of unusual strains which are not solely animal and solely human strains (Gouvea et al., 1994; Esona et al., 2004).

### 3.6.1. Global Rotavirus strains

The introduction of the rotavirus vaccines has to some extent determined circulating strains in areas where these vaccines are in use due to vaccine selection pressure. Common human rotaviruses associated with diarrhoeal disease worldwide are of the genotype combinations G1P[8] (52%), G2P[4] (11%), G3P[8] (9%), G4P[8] (8%) and G9P[8] (11.4%) (Santos and Hoshino, 2005; WHO, 2011). These common global strains may be responsible for almost 100% of infections in highly developed countries during some rotavirus seasons (Iturriza-Gomara et al., 2011; Iturriza-Gomara and Gray, 2011; Payne et al., 2011). However, there is a high diversity of circulating genotypes in developing countries especially in Africa; and the emergence of uncommon strains have been observed in recent studies independent of vaccine use (Mwenda et al., 2014; Seheri et al., 2014; Santos and Hoshino, 2005). It has been observed that many of these common human rotavirus strains may co-circulate within a season and thus create an environment for the formation of unusual strains through mechanisms such as genetic reassortment and point mutation (Payne et al., 2009; WHO, 2011). The rotavirus genotypes G1P[8] is the primary cause of human diarrhoeal disease among the global strains. However, other strains may become primarily responsible for this disease at different location and from one season to the next especially in developing countries (O’ Ryan, 2009; Payne et al., 2009; Zuridah et al., 2010). The cyclical distribution of genotypes in different seasons of rotavirus is unclear to epidemiologists, making the design of highly effective vaccines challenging (Patton, 2012).

In developing countries, uncommon binary G/P combination of human rotavirus strains arising due to reassortment with animal rotaviruses have been found to cause disease in young children (Armah et al., 2010a; Binka et al., 2011; Jere et al., 2011b; WHO, 2011). Noteworthy, is that, these uncommon strains of G/P types vary across regions. An example is shown by the WHO directed surveillance program in 2010, where they found the G12P[8] and G12P[6] viruses as uncommon strains in Southeast Asia; then the G2P[6], G3P[6] and G1P[6] viruses in sub-Saharan Africa; the uncommon strains in the Western Pacific were the G1P[4] and the G2P[8] viruses; and the G9P[4] in the Americas (WHO, 2011). Therefore, it is difficult to predict which of this uncommon strain will spread worldwide to become a common strain. The G9P[8] strain is an example of a previously uncommon genotype that later became a global strain (Clark et al., 2004; Cunliffe et al., 2001; Matthijnssens et al., 2010a). In Africa, the G8 and P[6] epitopes have been detected in a large population of circulating strains of Rotaviruses, making them
epidemiologically important in the region (Mwenda et al., 2010; Steele and Ivanoff, 2003; Steele et al., 1999; Adah et al., 1997; Cunliffe et al., 1999). The G12 strains were first detected in South Africa in 2004 and since then, have been detected increasingly in Zambia, Zimbabwe, Kenya, Ethiopia, Uganda, Cameroon, Democratic Republic of Congo (DRC), Togo and Burkina Faso (Mwenda et al., 2010; Page et al., 2009).

3.6.1.1. Rotavirus strains in Africa

Rotavirus genotypes in Africa are highly diverse with reports of several unusual strains circulating. This is in contrast to Europe, North America and Australia where the G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are responsible for more than 90% of the circulating strains (Banyai et al., 2012; Gentsch et al., 2005; Santos and Hoshino, 2005). The G1P[8] (14% to 31%) strain has consistently remained the most prevalent strain in Africa. Unusual strains such as the G1P[6] (3.9% to 6.5%), G2P[6] (5.0% to 9.5%), G3P[6] (4.6% to 7.4%), G1P[4] (0.1% to 0.6%), G9P[4] (0.3% to 3.1%), G12P[6] (2.5% to 5.4%) and G12P[8] (2.5% to 11.7%) have been detected and these account for between 27% to 40% of circulating strains every year (Mwenda et al., 2014). Seheri et al. (2014) reported a high variability in the circulating rotavirus strains in Africa. The predominant genotypes observed from analyzing 3412 rotavirus enzyme immunoassay positive and 1223 negative stool samples collected between 2007 and 2011 from 16 African countries (viz: Zambia, Zimbabwe, Cameroon, Democratic republic of Congo, Ethiopia, Kenya, Mauritius, Tanzania, Uganda, Burkina-Faso, Cote d’Ivoire, The Gambia, Guinea-Bissau, Nigeria, Senegal and Togo) were: G1P[8] (18.4%), G9P[8] (11.7%), G2P[4] (8.6%), G2P[6] (6.2%), G1P[6] (4.9%), G3P[6] (4.3%), G8P[6] (3.8%) and G12P[8] (3.1%). They further reported an uneven distribution amongst these strains and that there was year-to-year variation in the circulating genotypes. Mixed genotypic infection and partially typed rotaviruses were frequently detected. Genotypes G2P[8] (0.5%), G9P[4] (0.5%), G8P[4] (2.7%) and G12P[4] (0.1%) are examples of the non-typical strains that were detected in the study (Seheri et al., 2014). It has been suggested that a possible re-assortment between human and animal strains has occurred in Mauritius, Tanzania, Kenya, Guinea-Bissau, Senegal and Nigeria where G8P[14] (0.1%), G6P[6] (0.6%), G4P[6] (0.1%), G9P[14] (0.1%) and G10P[6] (0.2%) were detected, as these strains were previously detected either in cattle or porcine.
3.6.1.2. Rotavirus strains in South Africa

In South Africa, a pre-vaccination burden of disease and strain surveillance study found that G1P[8] (24%), G2P[4] (12%), G3P[8] (9%), G9P[8] (4%) were the most prevalent G and P genotype combinations. Uncommon types, as well as mixed and untypeable genotypes were also detected (Seheri et al., 2012). Post-vaccination surveillance is ongoing to monitor the circulating strains of rotaviruses in view of the continual rotavirus infections in this region. However, few post-vaccination data in South Africa has made it difficult to monitor these circulating strains and determine strain specific vaccine effectiveness. Groome et al., 2014 reported that the most prevalent post-vaccination strain in their study was G12P[8] (43%) followed by G2P[4] (14%), G1P[8] (12%), G9P[8] (8%), G8P[4] (7%), G2P[6] (5%), uncommon strains (8%) and mixed strains (3%). They also reported that the strain specific effectiveness for the two doses of vaccine were 71% against the dominant G12P[8] strains; 62% against any homotypic or partially heterotypic genotypes with G or P proteins of same type as the vaccine strains; and 52% against fully heterotypic G/P binary in which the G and P proteins were totally different from those of the vaccine strains. They implicated waning immunity as a possible reason why the vaccine is unable to protect against several strains especially the non-vaccine G/P strains. Although this study was only powered to access the Rotarix® vaccine effectiveness in the first year of life (Groome et al., 2014), they reported that the vaccine will effectively protect children from rotavirus associated diarrhoea hospitalization in the first and second year of life. In addition, the predominance of G12P[8] (43%) (Groome et al., 2014) strain recorded in this post-vaccination data was in contrast to G1P[8] (Seheri et al., 2012) recorded in the pre-vaccination era. A higher prevalence of G9P[8] (8%) post-vaccination was also recorded as opposed to the pre-vaccination prevalence of 4%. It will be important to conduct a comprehensive study to identify mechanisms for these changes in rotavirus gene specific prevalence in South Africa.

3.6.2. Rotavirus Transmission

Rotaviruses are primary transmitted via the faecal-oral route. Respiratory route of transmission has also been suggested (Estes and Kapikian, 2007). Overcrowding and poor hygiene have been cited as factors that support the transmission of rotaviruses. The virus may spread through contaminated water or food, direct contact with contaminated surfaces and person to person contact (Ward et al., 1991; Dennehy, 2000). Children’s day care centers or homes can facilitate the transmission of rotavirus easily through frequent exposure to susceptible hosts. Nosocomial rotavirus infections in paediatric wards and hospital nurseries have been frequently reported (Steele and Alexander, 1987; Bishop, 1984). It is difficult to
control infections caused by rotaviruses because of their stability on surfaces and high concentrations in faeces of infected individuals (Ward et al., 1991). These viruses are highly infectious and are resistant to chemical disinfectants and antiseptics making transmission more difficult to prevent (Murray et al., 2005). This is because rotavirus is non-enveloped virus and it has been reported previously that non-envelop viruses are highly resistant to chemical disinfection (Howie et al., 2008).

### 3.6.3. Clinical Features

Group A Rotavirus infection presents as asymptomatic, mild to severe and sometimes progresses to life threatening dehydrating diarrhoea in infants and children under 5 years old (Bernstein, 2009). A number of asymptomatic children shed rotavirus in their stool and are carriers within the community (Ramani et al., 2010). Also, older children and adults who are infected by rotavirus may also have asymptomatic or symptomatic infection which may facilitate rapid global spread (Anderson and Weber, 2004). The incubation period in young children is 24 to 48 hours post infection (Bishop, 1984). Acute gastroenteritis associated with this virus is characterized by vomiting lasting 1 to 2 days accompanied by fever. Following this, is the onset of watery diarrhoea which last for about 3 to 8 days. The clinical symptoms associated with these viruses may vary from child to child. However, severe diarrhoea associated with dehydration and up to 10 to 20 bowel movements per day is characteristic of acute rotavirus infection. Abdominal cramps is also observed and in some cases, cough and runny nose are experienced (Christensen, 1989). If left untreated, rotavirus infection can be fatal and severe dehydration with associated electrolyte imbalances are the most frequent cause of death. Non-bloody diarrhoea, vomiting and life threatening complications were found to be more frequent in rotavirus infected children than those with bacterial pathogens (Binka et al., 2003; Nokes et al., 2008). Neonates, infants and older children with prior exposure to rotavirus have been found to exhibit asymptomatic infections (Bishop, 1984).

Four mechanisms by which rotavirus causes diarrhoea has been reported by Ramig (2004). They are malabsorption, secretion, villus ischemia and intestinal motility. Rotavirus diarrhoea exhibit a malabsorptive component that appears to be related to the primary rotavirus infection. Infection of the villus enterocytes leads to a cascade of events associated with calcium ion (Ca^{2+}). It appears that the disruption of Ca^{2+} homeostasis is mediated by the synthesis of viral proteins (Del Castillo et al., 1991; Michelangeli et al., 1991). Increased Ca^{2+} permeability at both the plasma membrane and the endoplasmic reticulum leads to an increase in Ca^{2+}, triggering a chain of events that leads to cell lysis. Rotavirus associated diarrhoea exhibit a secretory component that appears to be secondary to virus-induced
functional changes at the villus epithelium. The central players in secretion appear to be non-structural protein 4 (NSP4) and the enteric nervous system. The actual role and targets of secreted NSP4 are unknown. The effects of infection in the enterocyte epithelium may be amplified by NSP4. In rotavirus infected mice, villus ischemia was observed in their intestinal epithelium with minimal damage (Osborne et al., 1988; Starkey et al., 1986). It was proposed that virus induced release of an unknown vasoactive agent from infected epithelium could result in diarrhoea causing a local villus ischemia and subsequent functional damage to enterocytes (Osborne et al., 1991). However, other animal models has failed to exhibit villus ischemia, therefore making this observation less significant. Intestinal motility is significantly increased in some diarrhoeal infections. The intestinal transit time is decreased in rotavirus infection, implying increased motility (Michelangeli and Ruiz, 2003). The enteric nervous system generally controls motility, but the molecular stimulator of motility is unknown.

It is clear that diarrhoea is a multicomponent disease. Malabsorptive and secretory components have good evidence as mechanisms of diarrhoea. The mediators of diarrhoea disease components range from primary cellular damage to secreted viral enterotoxic peptide and a virus induced interaction with the enteric nervous system.

3.6.4. Age distribution of Children with Rotavirus Infection

The burden of disease study conducted in South Africa showed that about 90% of children less than 2 years old hospitalized or visiting the out-patient department were infected with rotavirus. These infections occur as early as 2 months of age (Seheri et al., 2010). Rotavirus infection was frequently reported in children of 3 to 17 months in Africa (Seheri et al., 2010; Steele et al., 1986) as opposed to 6 to 23 months of age in Europe (Giaquinto et al., 2010).

3.6.5 Seasonal distribution of Rotavirus Infection

Rotavirus infection is also influenced by climatic conditions and this is mostly observed in regions with seasonal changes. In temperate countries, peak season for rotavirus infection is during the cooler, drier months. This infection occurs predominantly between December and March in the Northern Hemisphere and between May and September in the Southern Hemisphere (Koopmans and Browns, 1999; Kim et al., 2005; Nakagomi et al., 2005; Gleizes et al., 2006). Winter seasonality of rotavirus infection has been shown in many studies; however, few cases may occur during warmer months. A peak in rotavirus associated hospitalization is observed in the United States, Europe, India, Asia, Republic of Korea and
In tropical countries, rotavirus infections are less affected by seasonal changes. Infections occur throughout the year with a slight increase during the drier months (Cook et al., 1990). In Africa, rotavirus incidences are mostly observed during the drier months and less infection rate in the wet or rainy season of the year (Mwenda et al., 2010).

In South Africa, epidemic peaks of rotavirus infection occurs during the cooler and drier months of the year (Steele et al., 1986; Cunliffe et al., 1998). A consistent annual increase in rotavirus infection leading to gastroenteritis is observed in the winter months (March/April to September/October). Research data indicates that the incidence of rotavirus infection could be less than 5% during the summer months and more than 50% in the winter season. These seasonal variations have been attributed to many factors such as virus survival in the environment, relative humidity, increasing airborne transmission, host physiology and overcrowding during the winter months (Bishop, 1996).

In Egypt, a year round detection of rotavirus was observed, with September to November being associated with a higher number of cases of rotavirus diarrhoea (Ahmed et al. 2014). Another study in Egypt reported higher prevalence of rotavirus from July to November (hot season) (Naficy et al., 1999) and this is dissimilar to an earlier finding where the peak of rotavirus infection was in November to April (cold season) (Zaki et al., 1986). These seasonal changes may be ascribed to various factors such as economic conditions, population growth and urbanization. Seasonality may be a challenge in identifying appropriate vaccine administration time. In addition, socio-economic condition has been reported as a stronger predictor of the magnitude of rotavirus seasonality as opposed to climate or geographical location (Patel et al., 2013b). Hence, high and middle income countries are more likely to exhibit rotavirus seasonality than low-income countries where an all year round rotavirus infection is expected. In order to understand the factors that drive the global seasonality of rotavirus disease, it will be important to evaluate the effect of vaccination on seasonality in different socio-economic settings (Patel et al., 2013b).

### 3.7. Current Rotavirus vaccines

Globally, two live attenuated vaccines (Rotarix® - GlaxoSmithKline Biologicals, Rixensart, Belguim and RotaTeq® - Merck and Co., New Jersey, USA) have been licensed since 2006. The monovalent Rotarix® (RV1), which is given as a two-dose schedule in infancy contains an attenuated human G1P[8] rotavirus strain (Angel et al., 2007). In contrast, the pentavalent RotaTeq® given as a 3 dose schedule was
propagated using five bovine-human reassortant strains. These strains contain genes which encodes the human G1, G2, G3, G4, P[8] antigens as well as genes encoding the bovine G6 and P[7] antigens (Angel et al., 2007). Despite the differences in the genetic components of these vaccines, studies show huge similarities in their efficacy in children up to 2 years of age. The exact mechanism of action by which these different vaccines exert similar efficacy (Vesikari, 2012) is not fully understood. In 2009, the strategic advisory group of experts on immunization at the World Health Organization (WHO) recommended that rotavirus vaccine be included in the national immunization schedule of member states. The reinforcement of these recommendations in January 2013 have led to the increasing use of these vaccines worldwide with financial support from the Global Alliance for Vaccines and Immunizations (GAVI) (Babji and Kang, 2012; Lopman et al., 2012a; WHO, 2013; WHO, 2009a). At least one of these global rotavirus vaccines has been included in the national immunization programme of about 77 countries worldwide with GAVI alliance supporting 35 of these countries (PATH, 2015).

High efficacies of the Rotarix® vaccine were reported in high-income countries such as Hong Kong (95.6%) (Lau et al., 2013), Japan (91.6%) (Kawamura et al., 2011), Europe (90.4%) (Vesikari et al., 2007b; GlaxoSmithKline, 2013), Latin America and Finland (84.7%) (Ruiz-Palacios et al., 2006). Studies on the efficacy of the Rotarix® vaccine were lower in the low-income settings of Malawi (49.2%) (Cunliffe et al., 2012; Steele et al., 2012; Mahdi et al., 2010) and South Africa (72.2%) (Steele et al., 2012; Mahdi et al., 2010) in the first year of life among the children tested. Much lower efficacy were recorded in the second year of life with a 34% and 32% efficacy in Malawi and South Africa respectively. For the RotaTeq® vaccine, Europe, North America and Latin America had a combined efficacy of 98% and 88% in the first and second year of life respectively (Vesikari et al., 2007a; Vesikari et al., 2006). In Finland, a 94.0% efficacy was reported against rotavirus associated emergency department visit and hospitalization for up to 3.1 years after vaccination regardless of the circulating serotypes (Vesikari et al., 2010). In contrast, lower efficacies of RotaTeq® were reported in the low-income settings of Africa. A combined efficacy of 64.2% and 19.6% were reported in the first and second year of life in Ghana, Kenya and Mali (Armah et al., 2012; Sow et al., 2012; Tapia et al., 2012; Armah et al., 2010b). Post-vaccination effectiveness of these vaccines against hospitalization and emergency department visit has been reported in the United States of America (86%) (Staat et al., 2011), Australia (89.3%) (Buttery et al., 2011), Israel (89%) (Muhsen et al., 2010), Nicaragua (88%) (Mast et al., 2011), Austria (92.8%) (Paulke-Korinek et al., 2010) and Brazil (95.7%) (Justino et al., 2011). These data were recorded in the first year after vaccination.
3.7.1 Rotavirus Vaccine in South Africa

South Africa was the first African country to include rotavirus vaccines (Rotarix®) into its National immunization programme, starting in the Eastern Cape region in August 2009 with a spread to other part of the country (Neuzil et al., 2010). This introduction was based on data emanating from studies on the burden of disease of rotavirus (Steele et al., 2003; Seheri et al., 2010) and the vaccines safety and efficacy trials in South Africa (Steele et al., 2010a). This vaccine is of two dose schedule, administered orally at 6 and 14 weeks of age, simultaneously with other expanded programme on immunization (EPI) vaccines. More than 3 million doses of Rotarix® has been administered nationally with variations from province to province (Seheri et al., 2012). The vaccine coverage increased rapidly since 2009 and by 2010, about 67% of children under a year old had received a complete schedule of Rotarix® immunization. The vaccine coverage rate has since increased to more than 90%. However, outbreaks in some areas within South Africa have made rotavirus a continuous burden and the effectiveness of these vaccines remain questionable. Post-vaccination effectiveness study in South Africa is scanty. Groome et al., (2014) reported a 57% and 40% Rotarix® vaccine effectiveness among children who received the complete dose and a single dose respectively in South Africa. This study was conducted among children of 18 weeks to 23 months old. They concluded that the vaccines were able to protect children against acute rotavirus associated diarrhoea hospitalization in the first and second year of life. However, these vaccine effectiveness is low in comparison to what is obtained in the high income countries. Socio-economic conditions has been reported as the main reason for this regional differences in vaccine effectiveness. The effect of socio-economic conditions on rotavirus vaccine effectiveness needs further exploration, as this will be of utmost importance in future vaccine development. The observed high effectiveness of the rotavirus vaccine in the high income countries may be a direct result of the vaccine virus isolation in the United States of America which is a high income country. Vaccine development using a virus strain isolated in a low income country may be the key to the finding a more effective vaccine in this region.

3.8. Immunity to Rotavirus

Protective correlates of immunity and cellular immune response against rotavirus in humans remain incompletely understood even though rotavirus immune responses has been studied extensively in mice and gnotobiotic piglets (Franco et al., 2006; Desselberger and Huppertz, 2011). Rotavirus specific antibodies of the immunoglobulin G (IgG) and immunoglobulin A (IgA) classes are recognized to be important (Moon et al., 2015; Patel et al., 2013b). However, there is an inter-species variation in the efficiency of antibody transfer into the gut. Also, the age of an animal is another factor influencing this antibody transfer. For example, mice IgA is actively transported in bile unlike in humans (Franco et al.,
Similarly, it is difficult to evaluate the mechanism of immunity in mice because they are only susceptible to rotavirus disease in the first 15 days of life (Burns et al., 1995; Franco and Greenberg, 1999). The observed differences in the actions of human and animal rotavirus strains has hindered the drawing of a firm conclusions regarding protection in human based on animal data. Partial protection by anti-VP6 antibodies have been observed in mice (Burns et al., 1996) but not protective in piglets (Yuan et al., 2000). Although current understanding of rotavirus disease and immunity has been enhanced by the use of animal models (Clark and Desselberger, 2015), it is important to develop effective techniques to study rotavirus immunity in human infants and young children.

3.8.1. Protection induced by natural Rotavirus Infection

Primary rotavirus infection usually results in acute gastroenteritis, but protection is developed following subsequent infection with lower risk of disease (Desselberger and Huppertz, 2011). This protection is also elicited in asymptomatic primary infection (Clark and Desselberger, 2015). This is an important finding since up to three quarters of infections in children between 6 and 24 months of age are asymptomatic. (Bernstein et al., 1991; Velazquez et al., 1996; Fischer et al., 2002). A study in Mexico showed that children were completely protected from moderate to severe diarrhea after two rotavirus infection (Velazquez et al., 1996). In contrast, in India, about 80% protection was conferred by three rotavirus infections of similar severity as in the Mexican study (Gladstone et al., 2011). Furthermore, a single infection was reported to protect two-thirds of infants from reinfection during an epidemic in Guinea-Bissau but in a subsequent epidemic, only about one-third remained protected (Fischer et al., 2002).

Natural protection has been found to be serotype specific and related to the levels of neutralizing antibodies against the virus strain. Repeat exposure to restricted serotypes or genotypes induces heterotypic protection against diverse genotypes (Desselberger and Huppertz, 2011; O’Ryan et al., 1994). This heterotypic protection suggests the presence of cross-reactive neutralizing epitopes (Arias et al., 1994). Reasons for this observed variability in protection preceding natural infections in different setting are yet to be determined. It has been suggested that this variability may result from differences in the inoculum size of the subsequent rotavirus challenge in different settings or variations in the level of generated immunity conferred by natural infection (Clark and Desselberger, 2015). Maternal antibodies acquired transplacentally may elicit a weak protection against rotavirus gastroenteritis during the first month of life (Ramachandran et al., 1998; Ray et al., 2007; Jayashree et al., 1988a). Breast milk contains IgA and has been reported to provide passive protection to infants (Hjelt et al., 1985; Jayashree et al., 1988b).
Rotavirus specific B-cells circulating in the blood of children express mainly the gut-specific homing receptor α4β7, and this is suggestive of a local protective action (Gonzalez et al., 2003; Jaimes et al., 2004). The induction of cytokines-secreting virus specific CD8+ cells by rotavirus infection is relatively poor (Jaimes et al., 2002) even though these cells are found in the peripheral blood in most adults (Makela et al., 2004). Circulating rotavirus specific T-helper (Th) cells were found in infant’s blood samples during convalescence phase (Offit et al., 1993; Offit et al., 1992). In-vitro infection of dendritic cells with rotavirus can stimulate rotavirus specific T cells to secrete Th1 cytokines (Narvaez et al., 2005; Mesa et al., 2007). Interferon gamma (IFN-γ) is also produced after infection of these cells with rhesus rotavirus. However, these dendritic cells are less efficient in presenting antigens in infants and young children than in adults (Jaimes et al., 2002). Cell mediated immune responses in rotavirus infected children is not well understood and needs to be further researched (Desselberger and Huppertz, 2011).

3.8.2. Protection induced by Rotavirus vaccines

The correlates and determinants of protection induced by the rotavirus vaccines remain questionable since the vaccine efficacies appear to partly depend on the socio-economic conditions of the countries where efficacy has been evaluated (Patel et al., 2013a; Lopman et al., 2012b; Zaman et al., 2010; Madhi et al., 2010). Clinical trials have reported low efficacies of these vaccines in low income countries of sub-Saharan Africa (Mahdi et al., 2010; Tregnaghi et al., 2011; Cunliffe et al., 2012; Mahdi et al., 2012; Steele et al., 2012; Steele et al., 2010b; Armah et al., 2012; Armah et al., 2010b; Sow et al., 2012; Tapia et al., 2012). In contract, high efficacies has been reported in high income countries of Europe, America and Asia (Vesikari et al., 2007b; Kawamura et al., 2011; Linhares et al., 2008; Ruiz-Palacios et al., 2006; Phua et al., 2009; Lau et al., 2013).

Heterogenic immunity has been observed in gnotobiotic calves resistant to human rotavirus strains after vaccination with a calf rotavirus strain (Wyatt et al., 1979). This finding, with data from gnotobiotic piglets (Zissis et al., 1983) led to the use of bovine Rotavirus G6P6 strain as the first human rotavirus vaccine candidate (Vesikari et al., 1984). This vaccine induced heterotypic immunity even though it did not share neutralizing epitopes with the most frequently circulating human rotavirus strains (Ruuska et al., 1990). When it was discovered that neutralizing antibodies against rotavirus is not the only representation of correlate of protection, different concepts of vaccine development emerged (Rennels et al., 1996; Joensuu et al., 1997; Perez-Schael et al., 1997; Ruiz-Palacios et al., 2006; Vesikari et al., 2006; Vesikari et al., 2007b). Strong neutralizing antibody response can only be elicited by multivalent vaccines and the RotaTeq® was produced based on this principle. In contrast, the monovalent Rotarix® vaccine, isolated
in 1989 from a human clinical isolate was developed on the basis that cross protection occurs during successive natural infection (Velazquez et al., 1996). Both the monovalent (Rotarix®) and pentavalent (RotaTeq®) vaccines induces homotypic and heterotypic immune response (De Vos et al., 2004; Vesikari et al., 2004) and protection usually but not always correlated with levels of rotavirus specific IgG or IgA antibodies (Rennels et al., 1996; Clark et al., 1990; Lanata et al., 1996; Madore et al., 1992; Ward and Bernstein, 1995; Ward et al., 1997). However, protection correlated less with the levels of neutralizing antibodies specific for rotavirus after these vaccines were received (Ward et al., 1992; Block et al., 2007; Ward et al., 2004). The degree of protection achieved with the RotaTeq® was not proportional to the G type’s specific neutralizing antibody response (Block et al., 2007). Individuals with IgA deficiency may be protected from severe rotavirus disease by Rotavirus specific IgG responses that are higher than those in IgA competent persons (Istrate et al., 2008). It has also been documented that the levels of rotavirus specific plasma IgA and B cells carrying the gut homing receptor may be a possible but weak correlate of protection in vaccinated children (Rojas et al., 2007).

Information regarding cell mediated immunity after rotavirus vaccination in human is very little. Acute gastroenteritis has been observed in vaccinated children with combined severe immunodeficiency (Patel et al., 2010).

3.8.3. Factors influencing Rotavirus vaccine induced protection

The variable levels of protection associated with the rotavirus vaccines in different settings has also been reported for other oral vaccines. Reduced efficacy of the oral polio and oral cholera vaccines in low income or resource limited settings was reported by Serazin et al in 2010. Different reasons have been proposed for these observations and they include environmental, viral and host factors (Clark and Desselberger, 2015). However, it is important to identify the key factors in order to design new strategies towards the development of a more effective vaccine. Some of the factors under consideration have been found to be interrelated (Serazin et al., 2010) and not fully understood. Further exploration is however needed towards identifying key hindrances to vaccine induced protection in resource limited settings.

Environmental enteropathy, is a sub-clinical condition affecting the small intestine in children living in low income countries has been implicated as an important determinant of vaccine-induced protection (Campbell et al., 2003). This condition is characterized by chronic exposure to faecal pathogens due to poor sanitation, leading to chronic inflammation of the intestine (Fagundes et al., 1994). It is believed that Gram negative bacterial components such as lipopolysaccharides mediates this inflammation by stimulating innate pattern-recognition receptors, and this is reflected by an observed increase in the
release of pro-inflammatory cytokines and by inflammatory cell infiltration of the gut, subsequently leading to T-cell stimulation and a cell-mediated enteropathy (Campbell et al., 2003; Humphrey, 2009; Korpe and Petri, 2012). This condition has been studied extensively in Gambian infants (Lunn and Northrop-Clewes, 1992; Lunn and Northrop-Clewes, 1991). Furthermore, a several fold rise of activated lymphocytes in the gut of Gambian infants has been reported in comparison to UK controls (Campbell et al., 2003). Although the immunological effects of environmental enteropathy and its relationship with under-nutrition and poor growth is well described, its role in reducing rotavirus virus vaccine efficacy and other oral vaccines in low-income settings is not fully understood. Therefore, there is urgent need for further research in this area.

Differences in the colonizing microbiota of the gut of infants in high and middle income countries with those born in low income settings has also been found to affect Rotavirus vaccine efficacy. Great diversity has been observed in the intestinal microbial composition of children living in low income countries when compared with the microbiota of children in high income countries (Lin et al., 2013; Adlerbert et al., 1991; Nowrouzian et al., 2003). Intestinal colonization of 4 week old infants with Escherichia coli or Bifidobacteria has been associated with a significant difference in the number of memory B-cells when the infants get to 18 month of age (Lundell et al., 2012). This documented differences in colonizing organisms in early life between low and high income countries could subsequently influence the immune responses generated by rotavirus vaccines and other oral vaccines through the modification of both innate and adaptive immune development and function. It was discovered that treatment of Ascaris lumbricoides, an intestinal helminth, with albendazole prior to the administration of an oral cholera vaccine resulted in enhanced seroresponse rates to this cholera vaccine (Cooper et al., 2000).

Nutritional deficiencies of zinc, vitamin A and vitamin D have been reported to negatively affect the immune system and consequently influence the rotavirus vaccine protection potential. Furthermore, maternal IgG and IgA which is transferred through the placenta and via breast milk respectively may offer protection from rotavirus gastroenteritis, however, these antibodies have been documented to compromise the effectiveness of Rotavirus vaccine and other vaccines given early in life (Moon et al., 2010). Recently, Moon and colleagues (2015) reported that high levels of pre-existing serum IgG, including transplacentally acquired maternal IgG appeared to inhibit the immunogenicity of the Rotarix® vaccine among infants in South Africa. They added that this may partly be responsible for the observed low efficacy of rotavirus vaccines in South Africa and other low income countries (Moon et al., 2015).
Vaccine storage and distribution from manufacturer to the vaccine recipient in cold chain is of utmost importance to maintain vaccine stability and potency. World Health Organization (WHO) has recommended in guidelines that all vaccines except oral polio vaccines be kept at 2°C to 8°C during in-country distribution (WHO, 2006). However, a poor cold chain system may expose vaccines to sub-optimal temperatures ranging from hot to freezing temperatures. Damage from exposure to these sub-optimal temperatures result in loss of potency of these heat and freeze sensitive vaccines such as rotavirus vaccine, diphtheria, tetanus, pertussis, liquid *Haemophilus influenzae* type b (Hib), hepatits B and inactivated polio virus vaccines (WHO, 2006; Boros et al., 2001; Ho et al., 2002; Bolgiano et al., 2001; Diminsky et al., 1999). Considering the complexities and challenges of maintaining the vaccine cold-chain during storage and distribution, it will be reasonable to imagine that a higher rate of vaccine exposure to sub-optimal temperature will be recorded in low income settings in comparison to high or middle income settings. Reasons for this will be the inadequate or erratic electricity supplied in low income countries, education level of inventory or store managers, and longer hours of vaccine transport due to inaccessible road networks in these low income settings. Consequently, loss of potency may be responsible for the reduced vaccine efficacy in the low income settings. There is an urgent need for research in this area, to ascertain the degree to which the vaccine cold chain is maintained locally.

A number of other factors have been proposed to influence the efficacy of rotavirus vaccine in low-income countries and they require further exploration (Serazin et al., 2010).

### 3.9. Treatment of Rotavirus disease

Currently, supportive care is the only option for management of patients with rotavirus infection as there is no specific treatment. Oral rehydration solution (ORS) or fluid replacement intravenously is offered to patients to restore lost body fluids and electrolytes. Lack of electrolytes can be life threatening if not replaced quickly (Murray et al., 2005). Chronic or immunosuppressed individuals infected with rotavirus can be treated with anti-rotavirus immunoglobulin of bovine colostrum origin (Sarker et al., 1998). This is practiced largely in developed countries. However, it is likely to be needed more in developing countries where rotavirus morbidity and mortality is higher.

### 3.10. Laboratory detection of Rotavirus

Human rotaviruses may be detected in stool samples or from rectal swabs in the laboratory using different techniques including electron microscopy (EM), cell culture, enzyme immunoassay (EIA) and latex
agglutination assay (rapid diagnostic test), polyacrylamide gel electrophoresis (PAGE), reverse transcription polymerase chain reaction (RT-PCR), real time PCR and gene sequencing.

3.10.1. Cell Culture

African green monkey kidney cells (MA104 cell line) are commonly employed for the isolation of rotaviruses (Estes and Kapikian, 2007). However, rotaviruses are very difficult to grow in cell culture (Murray et al., 2005), making this method unreliable for diagnostic analysis of rotavirus infections.

3.10.2. Electron Microscopy

Electron microscopy (EM) is a reliable and rapid detection method for rotaviruses which has a characteristic morphology. This detection method is less sensitive compare to enzyme immunoassay since about $10^6$ to $10^8$ virion per milliliter is required in the sample for detection (White and Fenner, 1994; Arguelles et al., 2000; Logan et al., 2006; Farkas and Jiang, 2007). While the enzyme immunoassay requires $10^5$ to $10^6$ virion particles per milliliter for detection. EM can detect virion that are negatively stained, although, most laboratories do not have this detection technology (Fischer and Gentsch, 2004).

3.10.3. Enzyme Immunoassay (EIA) and Latex agglutination assay

Detection of human rotavirus antigen in faecal or stool specimen using EIA is the standard diagnostic test and this is due to its sensitivity and ease of use (Beards et al., 1984). Labeled antibodies are used to detect antigen-antibody reactions (Atlas, 1997). EIA consists of plate coated with a capture antibody and the diluted stool sample is added to it to detect the presence of a specific antigen. The sensitivity of EIA is in the range of $10^5$ to $10^6$ viral particles per milliliter, and it has 100% specificity and 98.4% sensitivity in comparison to RT-PCR (Arguelles et al., 2000).

Latex agglutination assay for detecting rotaviruses utilizes latex beads coated with a virus specific immunoglobulin. Agglutination occurs when the latter reacts with test sample antigens (Murray et al., 2005). This assay is generally less sensitive compared to EIA (Arguelles et al., 2000; Farkas and Jiang, 2007). A low virion quantity in the test samples can lead to a false negative result using this assay, and this result in low specificity and sensitivity (White and Fenner, 1994). This assay has 93% specificity and
57% sensitivity in comparison to EIA. However, this assay is still used for routine diagnosis by some pathology laboratories.

3.10.4. Polyacrylamide Gel Electrophoresis (PAGE)

This technique has been used in the molecular characterization of the dsRNA of rotavirus by determining the genomic diversity of these viruses through studies of the unique migration patterns of the 11 segments of the dsRNA (Seheri et al., 2012). This method allows negatively charged macromolecules to separate through the gel according to their size. Visualization of these migration patterns are done by staining the gel with silver nitrate (Herring et al., 1982; Laird et al., 2003; Desselberger et al., 2005; WHO, 2009b). The sensitivity of PAGE is in the range of $10^8$ to $10^9$ viral particles per milliliter (Arguelles et al., 2000). PAGE negative samples that are EIA positive could be a result of virus particles degradation (Fischer and Gentsch, 2004).

3.10.5. Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

In RT-PCR, viral RNA is converted through the action of enzymes into complementary deoxyribonucleic acid (cDNA), which is then amplified by PCR, to detectable level (Atlas, 1997). Human rotaviruses are typed into the G and P genotype using specific primers which target specific regions of the VP7 and VP4 genes (WHO, 2009b). Non-typeable rotavirus genotypes are minimal due to the use of RT-PCR and this method has improved rotavirus detection compared to EIA or EM (Logan et al., 2006). This method is the preferred choice for genotyping (Fischer and Gentsch, 2004).

3.10.6. Real-Time RT-PCR

Real-time RT-PCR is a detection method that uses an instrument to amplify and detect rotaviruses simultaneously using a fluorescent marker. Real time RT-PCR product is plotted in an amplification curve that shows a log phase indicating the presence of the organism, an amplification phase and the endpoint (WHO, 2009b). Real-time RT-PCR is a more sensitive and faster method of detecting and quantifying rotavirus when compared to the conventional RT-PCR method (Zeng et al., 2008).
4. METHODOLOGY

4.1. Ethics Permission
The project was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), Durban, South Africa (REF: BE222/13).

4.2. Study Site and Patient Population
Written informed consent (Appendix I) was obtained from parents or guardian of symptomatic (diarrhoea, vomiting and/or fever) children of 5 years and under presenting to King Edward VIII Hospital (KEH VIII) in Durban, South Africa, between June 2014 and June 2015. KEH VIII Hospital is a teaching hospital attached to the UKZN Medical School (Nelson Mandela School of Medicine). It is a 922 bedded hospital with an annual out-patient attendance of about 360,000 patients. It is situated in the eThekwini district of the KwaZulu-Natal (KZN) Province. It provides regional and tertiary services to the whole of KZN and Eastern Cape Provinces.

Demographic and clinical data were collected using a well-structured questionnaire (Appendix II). Some of the data, such as the patient’s age, gender, HIV status, receipt of zinc supplements and anti-helminthic agents, vaccination status and mode of feeding were confirmed from the patient’s hospital folders and vaccination cards.

4.3. Specimen Collection
Stool specimens were obtained from soiled diapers of the children recruited to the study. The stool was placed into sterile, leak proof containers. Venous blood was also collected into a yellow-cap blood collection tubes (BD® microtainer tubes).

The specimens were transported to the Infection Prevention and Control (IPC) Laboratory in the School of Laboratory Medicine and Medical Sciences (LMMS) of UKZN in a cooler box with ice and maintained at a temperature below 8°C.

In the laboratory, stool specimens were stored in the -80°C prior to analysis. Blood specimen were centrifuged at 1000g for 10 minutes and sera were stored at -20°C in micro-tubes prior to analysis.
The stool samples were tested for the presence of rotavirus antigen using the enzyme immunoassay (EIA) techniques. Some of the EIA positive and negative specimens were further confirmed and genotyped using conventional reverse transcriptase polymerase chain reaction (RT-PCR).

The serum samples were tested for the presence of rotavirus specific immunoglobulin G (IgG). The avidity of these IgG were determined using the urea elution technique as previously described by Hedman and Rousseau (1989).

Rotavirus vaccines (Rotarix®) were donated by the provincial pharmaceutical store of the KwaZulu-Natal Department of Health (DOH). The vaccines were stored between 2°C to 8°C as recommended by the manufacturer. The potency of the vaccines were determined after vaccine exposure to different suboptimal temperatures using the plaque assay technique. These potency values were compared to that of vaccines stored at optimal temperatures.

4.4. Enzyme immunoassay (EIA)

In this study, we used the ProSpecT™ Rotavirus antigen detection kit (Oxoid, United Kingdom) as the EIA platform as recommended by WHO (2009b). This assay utilizes a polyclonal antibody to detect group specific proteins, including the major inner capsid protein (VP6), present in Group A rotaviruses. Ten percent (10%) suspensions of stool samples were prepared for each stool specimen by mixing approximately 0.1 g (pea size) or 100μl of liquid stool with 1 ml of the sample diluent provided. Corresponding wells for each sample was labeled on the EIA worksheet (Appendix III). 100μl of the positive control supplied with the EIA kit was added to the first well. 100μl of negative control was added to the second well. An aliquot of 100μl of the stool suspensions for each sample was added to each of the remaining 94 wells. 100μl of the conjugate (an enzyme linked capture antibody) was added to each well. The plate was covered, sealed and incubated at 25°C for 60 minutes. After incubation, the contents of the wells were aspirated and the wells were washed for a total of 5 times with diluted wash buffer (350μl per well) using an automated microplate washer (Thermo Fisher scientific, Vantaa, Finland). Thereafter, 100μl of the enzyme substrate was added to each well, and the plate was covered and incubated for 10 minutes at 25°C. Then, 100μl of the stop solution was added to each well and mixed thoroughly to stop the substrate reaction. The color changes were observed visually and the result were read within 30 minutes in a microplate reader (Thermo Fisher scientific, Shanghai) set at 450nm and reported on the EIA worksheet. The validity of the test was determined by the positive and negative controls that were supplied with the EIA kit and included in the first and second wells respectively of each of the EIA runs performed.
4.5. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to detect Rotavirus

Purified double stranded RNA (dsRNA) from stool samples were used to detect human rotaviruses (HRV) using the conventional RT-PCR. This RT-PCR of Rotavirus dsRNA has 3 steps: Denaturation of the dsRNA, reverse transcription of the dsRNA (cDNA synthesis) and amplification of cDNA (WHO, 2009b) using specific primers targeting regions of the genes encoding the VP7 (G-type) and VP4 (P-type). Selected EIA rotavirus positive and negative stool samples were confirmed using RT-PCR.

4.5.1. Purification of genomic ribonucleic acid (RNA)

Genomic RNA purification was done using the QIAamp viral mini-kit (Qiagen, Hilden, Germany). Ten percent (10%) of the stool specimen suspension was prepared using freshly prepared phosphate buffered saline (PBS) (PH 7.2) in a 1.5ml eppendorf tube by mixing approximately 0.1g (pea size) of semi-formed or 100µl of liquid stool with 1ml of the PBS, vortexed and centrifuged at 14000g for 1 minutes. In a sterile eppendorf tube containing 560µl of lysis buffer (AVL) mixed with carrier RNA, 140µl of the stool suspension was added. The mixture was incubated at room temperature for 10 minutes, centrifuged at full speed (14000g) for 30 seconds to remove drops in the lid. Then, 560µl of 100% ethanol was added and the mixture was vortexed for 15 seconds and centrifuged at full speed for 30 seconds. 630µl of this mixture was transferred to the QIAamp mini column in a 2ml eppendorf tube and centrifuged at 6000g for 1 minute. The QIAamp was transferred into a new collection tube and the filtrate was discarded. The remaining 630µl mixture was also transferred to the QIAamp mini column and centrifuged as previously done. Thereafter, 500µl of the AW1 buffer was transferred into the QIAamp mini column and centrifuged at 6000g for 1 minute. The QIAamp mini column was transferred into a new 2ml eppendorf tube and the filtrate discarded. Then, 500µl of AW2 buffer was transferred into the QIAamp mini column and centrifuged at 14000g for 3 minutes and the filtrate was discarded. The mini column was transferred into a new 2ml tube and centrifuge at 14000g for 1 minute and the filtrate was discarded if any. The mini column was transferred into a labeled 1.5ml tube and 60µl of elution (AVE) buffer was added. It was incubated for 1 minute at room temperature and centrifuged at 6000g for 1 minute to elute the purified viral RNA. The quality and purity of the RNA was determined using the Nanodrop 2000c (Thermo Fisher scientific, California, USA). RNA was stored at -80°C prior to use.
4.5.2. Complementary DNA (cDNA) synthesis

The cDNA was synthesized using the Gene Amp PCR system 9700 (Life technologies, Singapore) and the high-capacity cDNA reverse transcription kit with RNase inhibitors (Thermo Fisher Scientific, California, USA) based on manufacturers protocol. The cDNA master-mix was prepared in a 1.5ml eppendorf tube. The master mix component were, 10x RT buffer, 25x dNTP mix (100mM), 10x Random primers, Multiscribe reverse transcriptase, RNase inhibitor and nuclease free water. 10µl of the extracted RNA was added to 10µl of the master-mix in a 200µl PCR tube (Thermo Fisher Scientific, California, USA) to give a 20µl cDNA reaction. The tubes were vortexed quickly and centrifuged at full speed for 30 seconds. The reaction was subjected to a thermal cycling condition of 25°C for 10 minutes, then 37°C for 120 minutes and 85°C for 5 minutes. This cDNA was used for the PCR reaction immediately or stored at -20°C awaiting use.

4.5.3. RT-PCR for VP7 (G-types) and VP4 (P-types)

The cDNA was used as a template for the VP7 and VP4 specific PCR using the platinum Taq DNA polymerase kit (Thermo Fisher Scientific, California, USA). The PCR master-mix for a 25µl reaction was prepared in two different 1.5ml eppendorf tube on ice for each VP types based on the number of sample. A 2.5µl aliquot of cDNA was used in a total reaction volume of 25µl containing nuclease free distilled water, 1x PCR buffer, 1.8mM of Magnesium chloride, 0.2mM dNTP mix, 0.2µM each of forward and reverse primers, Beg 9 and End 9 for VP7 (Gouvea et al., 1990; Iturriza-Gomara et al., 2004); Con3 and Con2 for VP4 (Gentsch et al., 1992; Iturriza-Gomara et al., 2000) and 2U of Taq polymerase. The VP7 reaction mixture in a 200µl PCR tubes was subsequently subjected to an initial denaturation step of 95°C for 5 minutes, followed by a 35 cycle amplification at 95°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute and final elongation step at 72°C for 5 minutes. While the VP4 reaction mixture was subjected to an initial denaturation step of 95°C for 5 minutes, followed by a 35 cycle amplification at 95°C for 1 minute, 50°C for 2 minutes, 72°C for 1 minute and final elongation step at 72°C for 5 minutes. Negative controls with RNase free distilled water and positive control cDNA templates were included in both reaction. The PCR products were subjected to electrophoresis in agarose gels with a 100bp DNA ladder (Sigma Aldrich, Missouri, USA) as a molecular marker.
4.5.4. VP7 and VP4 genotype specific PCR

Positive PCR samples were assigned genotypes using genotype specific primers. The genotype specific primers (20 µM) for the VP7 were those for G1, G2, G3, G4, G8, G9, G10 and G12 (Gouvea et al., 1990; Iturriza-Gomara et al., 2004), used with a reverse primer (RVG), combined as a single primer mix (multiplex) in a 1.5ml eppendorf tube. While that for the VP4 were those of P[4], P[6], P[8], P[9], P[10], P[11] and P[14] (Gentsch et al., 1992; Iturriza-Gomara et al., 2000) used with Con3 as the forward primer combined as a single primer mix in a 1.5ml eppendorf tube. Master-mix for both genotypic groups were prepared in 2 different 1.5ml eppendorf tubes on ice based on the number of samples. A 2.5µl aliquot of cDNA was used in a total reaction volume of 25µl containing nuclease free distilled water, 1x PCR buffer, 1.8mM of Magnesium chloride, 0.2mM dNTP mix, 0.2µM of the genotyping primers and 2U of Taq polymerase. The VP7 genotypes reaction mixture was subsequently subjected to an initial denaturation step of 95°C for 5 minutes, followed by a 30 cycle amplification at 95°C for 1 minute, 42°C for 2 minute, 72°C for 1 minute and final elongation step at 72°C for 5 minutes. While the VP4 genotypes reaction mixture was subjected to an initial denaturation step of 95°C for 5 minutes, followed by a 30 cycle amplification at 95°C for 1 minute, 45°C for 2 minute, 72°C for 1 minute and final elongation step at 72°C for 5 minutes. RNase free distilled water and positive cDNA templates were included in the reaction as negative and positive control respectively. The PCR products were subjected to electrophoresis in agarose gels with a 100bp DNA ladder (Sigma-Aldrich, Missouri, USA) as a molecular marker.

4.5.5. Electrophoresis of RT-PCR products

The RT-PCR products were detected using 1.5% (w/v) agarose gel in TAE buffer subjected to electrophoresis. Gel trays were set-up with comb ready for the gel mixture and the electrophoresis tank was filled with TAE buffer. The agarose was dissolved in a microwave oven and swirled to mix until clear. Then, it was cooled to about 55°C and a known volume of gel red stain was added. The gel was then be poured into the tray and allowed to set before being submerged in the TAE buffer. 8µl of PCR product and 2µl of gel loading buffer were loaded into the sample wells. Also, 2µl of the 100bp DNA ladder (Sigma-Aldrich, Missouri, USA) was combined with 2µl of gel loading buffer and it was loaded into one of the sample well. The gel was ran at 100volts for about one hour and viewed using an ultraviolet (UV) light source. The PCR band sizes were compared with the molecular weight marker and the expected sizes of each genotype were recorded. The expected band sizes for the used primers are shown below;
Table 1: Primers for detecting rotavirus G-types (Consensus primers) and the different Genotypes

<table>
<thead>
<tr>
<th>Primers</th>
<th>Genotypes</th>
<th>Amplicons sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beg 9 and End 9</td>
<td>G-types</td>
<td>1062</td>
</tr>
<tr>
<td>aBT1</td>
<td>G1</td>
<td>749</td>
</tr>
<tr>
<td>aCT2</td>
<td>G2</td>
<td>652</td>
</tr>
<tr>
<td>mG3</td>
<td>G3</td>
<td>812</td>
</tr>
<tr>
<td>aDT4</td>
<td>G4</td>
<td>583</td>
</tr>
<tr>
<td>aAT8v</td>
<td>G8</td>
<td>885</td>
</tr>
<tr>
<td>mG9</td>
<td>G9</td>
<td>306</td>
</tr>
<tr>
<td>mG10</td>
<td>G10</td>
<td>396</td>
</tr>
<tr>
<td>mG12b</td>
<td>G12</td>
<td>558</td>
</tr>
</tbody>
</table>

Table 2: Primers for detecting rotavirus P-types (Consensus primers) and the different genotypes

<table>
<thead>
<tr>
<th>Primers</th>
<th>Genotypes</th>
<th>Amplicons sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con3 and Con2</td>
<td>P-types</td>
<td>876</td>
</tr>
<tr>
<td>2T-1</td>
<td>P4</td>
<td>483</td>
</tr>
<tr>
<td>3T-1</td>
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<td>1T-1v</td>
<td>P8</td>
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<tr>
<td>4T-1</td>
<td>P9</td>
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</tr>
<tr>
<td>5T-1</td>
<td>P10</td>
<td>594</td>
</tr>
<tr>
<td>mP(11)</td>
<td>P11</td>
<td>312</td>
</tr>
<tr>
<td>P4943</td>
<td>P14</td>
<td>546</td>
</tr>
</tbody>
</table>
4.6. Rotavirus specific IgG avidity study

Serology was used to determine the presence of rotavirus specific IgG in serum samples of the study population. Thereafter, we evaluated the avidity of these IgG. We adopted the enzyme linked immunosorbent assay (ELISA)-IgG avidity test using the urea elution technique as previously described by Hedman and Rousseau (1989). This technique is able to determine the avidity indices of the antibodies (IgG) elicited due to the Rotarix® vaccine or due to natural infection. A commercially available serum rotavirus specific IgG kit (MyBiosource, California, USA) was adopted for this assay. We prepared a urea solution (6 molar) using commercially available urea powder (Sigma-Aldrich, Missouri, USA). The microtitre plate was divided into equal halves of 48 wells each, labeled urea and non-urea respectively. A 100μl of positive and negative control and 100μl of a 1 in 10 dilution of serum samples in the sample diluent provided were dispensed into wells in each half of the rotavirus specific antigen coated microtitre plate. The plate was sealed with an adhesive strip and incubated for 30 minutes at 37°C. Each well was aspirated and the urea labeled-half of the wells was soaked or incubated in the wash buffer containing 6 molar urea for 10 minutes and the other half (non-urea labeled) in wash buffer without urea. Thereafter, both halves were washed with 200μl of wash buffer (without urea) four times in an automated microplate washer (Thermo Fisher scientific, Vantaa, Finland). After the wash, a horseradish peroxidase (HRP)-conjugated antihuman IgG was added to each well and incubated at 37°C for 20 minutes. Then, the wells were washed for a total of five times. When the wash was over, 50μl each of two chromogenic substrates were added to the wells, and incubated at 37°C for 10 minutes in the dark. The enzymatic reaction was stopped with 50μl of stop solution. The plates were tapped gently to ensure thorough mixing and the absorbance or optical density (OD) of each well were taken within 10 minutes using a microplate reader (Thermo Fisher scientific, Shanghai) set at a wavelength of 450nm. The results (ΔOD) were expressed as the OD value of the virus antigen in each sample divided by the OD value of the negative control. The avidity index (AI) was expressed as a percentage and calculated as the ΔOD of samples with urea divide by that of samples without urea, multiplied by 100 (ΔOD with urea/ΔOD without urea x 100).

4.7. Rotavirus vaccine (Rotarix®) potency study

In this study, we quantitatively evaluated the effect of sub-optimal temperature on the potency of the Rotarix® vaccine. This sub-optimal temperature environment was created in our laboratory using a digital oven and freezers set at the required temperatures. To quantify this vaccine virus after exposure to the different sub-optimal temperature, we adopted the plaque assay technique previously used by Smith et al., 1979, with some modifications. Vaccines were exposed to the average temperatures experienced over the different seasons in KwaZulu-Natal for 1 hour to 6 hours. Thereafter, there potency was evaluated. These
average seasonal temperatures were: summer (27.3°C), winter (23.3°C), spring (24°C) and autumn (25.7°C). These average seasonal temperatures were obtained online from the South African weather station (2015) and they represent the average seasonal temperatures in the last ten years. The vaccines were also exposed to freezing temperatures of -20°C and -80°C for 12 hours and a high temperature of 40°C for up to 72 hours. The potencies of these vaccines were evaluated after these exposures. The potency of vaccines stored optimally was used as our reference standard.

Vero cells (ATCC CCL-81) were cultured in T75 flasks using Eagle’s minimum essential medium (EMEM) with HEPES culture medium supplemented with 1% glutamate and 10% heat-inactivated fetal bovine serum (FBS) and maintained in a 37°C incubator with 5% CO₂.

These cells were plated into each well of a 24-well tissue culture plates at a density of 1 x 10⁶ cells/ml using a volume of 500µl. The plates were maintained under the same growth conditions as described above until cells were confluent, usually at about 24 hours to 36 hours. The vaccine reference standard and test samples were diluted in PBS containing trypsin (1:60) and a control dilution in PBS without trypsin. This trypsinized vaccine reference standard and the test samples were diluted in EMEM with HEPES medium supplemented with 1% glutamate. This was a serial dilution in 8 tubes (10⁻¹ to 10⁻⁸) with each tube containing 810µl of diluent with 90µl of the reference standard and samples. Diluted reference standard and test samples were vortexed for 15 seconds and incubated at room temperature (25°C) for 30 minutes prior to infection. All dilutions of the reference standard and test samples were prepared to ensure that the Vero cells monolayer is exposed to the same concentrations of medium components at all dilution levels.

The culture media were aspirated from the confluent Vero cells monolayer in the 24 well tissue culture plate and washed twice with PBS. Vaccine virus infection was performed by inoculating 200µl of the diluted reference standard or test sample into the wells in triplicate and the infected 24 well plates were placed in a 37°C incubator containing 5% CO₂ for 1 hour. An agar overlay (0.1%) was prepared by mixing 3ml of a 3% agarose with 27ml of media, containing EMEM, 2% FBS and 1% glutamate in a 40°C water bath to prevent the agar overlay from solidifying. After the 1 hour infection, media were gently aspirated from the infected Vero cells monolayer and 1ml of the agar overlay was gently dispensed into the wells. The agar over layer solidified within 15 minutes at room temperature and the plates were transferred into a 37°C incubator with 5% CO₂ until plaques were formed. The formed plaques became visible after about 4 days and they were fixed by dispensing 1ml of 3.6% formalin on the agar overlay at room temperature for 1 hour. Thereafter, the formalin and agarose were discarded under running water. The plaques were stained for 3 minutes with crystal violet, washed with running water and plates were
allowed to air dry. The plaques were counted under an inverted light microscope (Olympus, Tokyo, Japan) using the x40 objective lens. The concentration of the initial viral suspension in plaque forming unit per milliliter (PFU/ml) was calculated. PFU/ml = number of plaques ÷ dilution factor x volume of inoculum per plate.

According to the manufacturers of the Rotarix® vaccine (GSK), the vaccine is potent when it is greater than or equal to $10^6$ of 50% cell culture infective dose (Potency $\geq 10^6$ CCID$_{50}$). To convert the CCID$_{50}$ to plaque forming unit per milliliter (PFU/ml), we applied the Poisson distribution ($P(k) = e^{-m} x m^k / k!$) and in summary PFU/ml = $0.7 x$ CCID$_{50}$. Therefore, $10^6$ CCID$_{50} = 0.7 x 10^6$ PFU/ml = $7 x 10^5$ PFU/ml. Therefore, the Rotarix vaccine is potent if it forms plaque greater than or equal to $7 x 10^5$ PFU/ml (Potency $\geq 7 x 10^5$ PFU/ml). We compared the potency of each of the vaccines stored at these sub-optimal temperatures with that of our reference standard and based on the acceptable potency by in vaccine manufacturer report (Potency $\geq 7 x 10^5$ PFU/ml).

4.8. Statistical analysis
The sample size was calculated using the prevalence formulae by Hajian-Tilaki (2014). Assuming rotavirus prevalence of 25% and an ELISA sensitivity of 90%. An estimated 282 stool sample or higher was needed for the rotavirus prevalence study at 95% confidence interval. The clinical and socio-demographic data collected were analyzed based on its relationship with rotavirus cases. IBM SPSS (version 22) was the statistical analysis package used. Categorical variables were compared using statistical techniques such as fisher’s exact test or Chi-square based on the samples size. Nominal variables were analyzed using the Wilcoxon signed rank test. While the population means were compared using the student t-test. P<0.05 was considered to be statistically significant. Odds ratios and 95% confidence limits were used to measure the strength of the association. A multivariable logistic model was used to identify independent risk factors associated with rotavirus. All variables were included in the model with the exception of breastfeeding due to the missing values.
5. RESULTS

5.1. Clinical and Demographic Characteristics of Study Population
In total 365 infants and young children of 5 years and under were enrolled in this study. Of these, 83 (22.7%) were positive on stool specimens for rotavirus using EIA. Of the specimens collected, 316 (86.6%) were from patients younger than 2 years. Two hundred and seven (56.7%) of the recruited patients were male. Rotavirus was detected in the stool specimens of 46 male patients and 37 female patients (p = 0.8). Table 3 shows comparison of demographic characteristics between rotavirus associated cases and non-rotavirus associated cases. In the age group stratified analysis, the prevalence of rotavirus infection was significantly different between the various age groups (p=0.02). The highest prevalence was observed in the 12-23 months age group (33.7%).

The stratification of rotavirus cases by vaccination status was not significant (p=0.4). However, a higher rotavirus prevalence was observed in the fully vaccinated children compared to the non-vaccinated children [49(24.1%) vs. 19(18.1%)]. The distribution of rotavirus was not significantly associated with HIV status of the children (p=0.7). Thirty-nine children (10.7%) were HIV positive, 54 (14.8%) were HIV exposed and 69 (18.9%) were termed as unknown because records of their HIV status in their hospital folder and immunization cards could not be found.

There was a higher rotavirus prevalence among children who had received zinc supplementation in comparison to those who did not [52 (28.1%) vs. 19 (13.6%), p=0.04].

Mode of feeding data were collected for children who were ≤ 6 months of age [n=166 (45.5%)]. There was no significant association between the different mode of feeding and the rotavirus cases (p=0.9). Similarly, the prevalence of rotavirus among children who had received an anti-helminthic drug (23.3%) was not significantly different from those who did receive the drug (22%) (p=0.6). In contrast, seasonality was a significant driving force influencing the prevalence of rotavirus infection in our setting (p<0.0001). The highest rotavirus prevalence was recorded in the winter months of the year with 79 (45.9%) positive cases of rotavirus associated diarrhoea.
Table 3: Clinical and Demographic Characteristics of Study Participants (n=365)

<table>
<thead>
<tr>
<th></th>
<th>Rotavirus Positive (n=83)</th>
<th>Rotavirus Negative (n=282)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Male</td>
<td>46 (55.4%)</td>
<td>161 (57.1%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>37 (44.6%)</td>
<td>121 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>Mean age in months (SD)</td>
<td>10.3 (8.1)</td>
<td>11.7 (11.3)</td>
<td>0.6</td>
</tr>
<tr>
<td>Age groups (months)</td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>(0-5)</td>
<td>27 (32.5%)</td>
<td>105 (37.2%)</td>
<td></td>
</tr>
<tr>
<td>(6-11)</td>
<td>22 (26.5%)</td>
<td>70 (24.8%)</td>
<td></td>
</tr>
<tr>
<td>(12-23)</td>
<td>28 (33.7%)</td>
<td>64 (22.7%)</td>
<td></td>
</tr>
<tr>
<td>(24-35)</td>
<td>5 (6%)</td>
<td>26 (9.2%)</td>
<td></td>
</tr>
<tr>
<td>(36-60)</td>
<td>1 (1.2%)</td>
<td>17 (6%)</td>
<td></td>
</tr>
<tr>
<td>Vaccination status</td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Completely vaccinated</td>
<td>49 (59%)</td>
<td>154 (54.6%)</td>
<td></td>
</tr>
<tr>
<td>Incompletely vaccinated</td>
<td>15 (18.1%)</td>
<td>42 (14.9%)</td>
<td></td>
</tr>
<tr>
<td>Not vaccinated</td>
<td>19 (22.9%)</td>
<td>86 (30.5%)</td>
<td></td>
</tr>
<tr>
<td>HIV infection status</td>
<td></td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>HIV positive</td>
<td>9 (10.8%)</td>
<td>30 (10.6%)</td>
<td></td>
</tr>
<tr>
<td>HIV negative</td>
<td>50 (60.2%)</td>
<td>153 (54.2%)</td>
<td></td>
</tr>
<tr>
<td>HIV exposed</td>
<td>12 (14.5%)</td>
<td>42 (14.9%)</td>
<td></td>
</tr>
<tr>
<td>Status unknown</td>
<td>12 (14.5%)</td>
<td>57 (20.2%)</td>
<td></td>
</tr>
<tr>
<td>Zinc administration</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Given</td>
<td>52 (62.7%)</td>
<td>133 (47.2%)</td>
<td></td>
</tr>
<tr>
<td>Not Given</td>
<td>19 (22.9%)</td>
<td>121 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>12 (14.5%)</td>
<td>28 (9.9%)</td>
<td></td>
</tr>
<tr>
<td>Mode of feeding (n=166)</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Exclusively breast fed</td>
<td>13/43 (34.9%)</td>
<td>38/123 (30.9%)</td>
<td></td>
</tr>
<tr>
<td>Formula fed</td>
<td>26/43 (60.5%)</td>
<td>72/123 (58.5%)</td>
<td></td>
</tr>
<tr>
<td>Mixed feeding</td>
<td>4/43 (9.3%)</td>
<td>13/123 (10.6%)</td>
<td></td>
</tr>
<tr>
<td>Anti-helminthic administration</td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Given</td>
<td>16 (19.3%)</td>
<td>53 (18.8%)</td>
<td></td>
</tr>
<tr>
<td>Not Given</td>
<td>61 (73.5%)</td>
<td>216 (76.6%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (7.2%)</td>
<td>13 (4.6%)</td>
<td></td>
</tr>
<tr>
<td>Seasonality</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Winter</td>
<td>79 (95.2%)</td>
<td>93 (33%)</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>3 (3.6%)</td>
<td>82 (29.1%)</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>1 (1.2%)</td>
<td>102 (36.2%)</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>0</td>
<td>5 (1.8%)</td>
<td></td>
</tr>
</tbody>
</table>
Corresponding serum specimens were obtained from only 35 children on whom stool specimens were collected. Further investigations was restricted to these 35 patients with matched serum and stool specimens. Of the 35 stool specimens [21 (60%) EIA positives and 14 (40%) EIA negatives], 29 (82.9%) were positive for rotavirus using conventional RT-PCR. Genotyping revealed G9P[8] (20.7%) to be the most prevalent genotype followed by G9P[4] (13.8%), G12P[4] (10.3%), G9P[6] (6.9%) and a 3.4% prevalence was recorded for each of G4/8P[6], G4P[6], G12P[6], G8P[10] and G9P[10]. Some of the rotavirus strains were non-typeable by the available primers (table 4). Figure 2 and Figure 3 shows the gel image of the rotavirus genotypes for VP7 (G-types) and VP4 (P-types) respectively.

Table 4: Genotype Distribution of Rotavirus Strains (n=29) in Study Participants

<table>
<thead>
<tr>
<th>G-types</th>
<th>P-types</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G9</td>
<td>P[8]</td>
<td>6 (20.7)</td>
</tr>
<tr>
<td>G9</td>
<td>NT</td>
<td>4 (13.8)</td>
</tr>
<tr>
<td>G12</td>
<td>P[4]</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td>G9</td>
<td>P[6]</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>NT</td>
<td>P[4]</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>G4/8</td>
<td>P[6]</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G12</td>
<td>NT</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G8</td>
<td>NT</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G4</td>
<td>P[6]</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G12</td>
<td>P[6]</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G8</td>
<td>P[10]</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G1</td>
<td>NT</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>G9</td>
<td>P[10]</td>
<td>1 (3.4)</td>
</tr>
</tbody>
</table>
Figure 2: Gel image of Rotavirus VP7 genotypes (G-types)

-VE: Negative rotavirus cases; LD: Molecular weight Ladder; NT: Non-typeable genotypes

Figure 3: Gel image of rotavirus VP4 genotypes (P-types)

-VE: Negative rotavirus cases; LD: Molecular weight Ladder; NT: Non-typeable genotypes
5.2. Detection of rotavirus specific immunoglobulin G (IgG) or antibodies in serum

Rotavirus specific IgG was detected in all the 35 serum samples (100%). Twenty-nine (82.9%) of the stool samples corresponding to the serum samples had rotavirus specific antigen (table 5). The rotavirus specific IgG had no significant effect on the prevalence of rotavirus detection in stool (100% vs. 82.9%, p=0.8). There was no significant difference in the mean avidity of IgG in the 3 vaccination strata (table 6; p=0.3).

<table>
<thead>
<tr>
<th>Vaccination status</th>
<th>Antibody positive (%)</th>
<th>Antigen positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely vaccinated (n= 21)</td>
<td>21 (60%)</td>
<td>15 (51.7%)</td>
</tr>
<tr>
<td>Incompletely vaccinated (n= 3)</td>
<td>3 (8.6%)</td>
<td>3 (10.3%)</td>
</tr>
<tr>
<td>Not vaccinated (n= 11)</td>
<td>11 (31.4%)</td>
<td>11 (37.9%)</td>
</tr>
<tr>
<td></td>
<td>35 (100%)</td>
<td>29 (82.9%)</td>
</tr>
</tbody>
</table>

p=0.8

<table>
<thead>
<tr>
<th>Vaccination status</th>
<th>Mean (range)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely vaccinated (n=21)</td>
<td>58.5 (12.7 - 94.1)</td>
<td>22</td>
</tr>
<tr>
<td>Incompletely vaccinated (n=3)</td>
<td>40.6 (29.4 - 51.6)</td>
<td>11.1</td>
</tr>
<tr>
<td>Not vaccinated (n=11)</td>
<td>58.4 (11.9 - 98.6)</td>
<td>25.5</td>
</tr>
</tbody>
</table>

p=0.3
5.3. To determine the effect of sub-optimal temperature on the potency of the rotavirus vaccine

Experiments showed that vaccines stored at the optimum temperature (2°C to 8°C) had an average potency of $5.8 \times 10^{10}$ PFU/ml. The potency of the vaccines after exposure to the average seasonal temperatures in KZN is shown in table 7. This is well within the standard expected potency as per manufacturers guidelines ie: potency $\geq 7 \times 10^5$ PFU/ml. Figure 4 and figure 5 show the PFU of viable vaccine virus and the negative control respectively. Exposure to extreme temperatures of 40°C for 3 to 72 hours (table 8) as well as -20°C and -80°C for 12 hours (table 9) also did not affect the potency of the vaccine beyond its expected standard.

**Table 7: Rotavirus vaccine potency (PFU/ml) after exposure to average seasonal temperatures in KZN**

<table>
<thead>
<tr>
<th>Exposure (hrs)</th>
<th>Winter (23.3°C)</th>
<th>Spring (24°C)</th>
<th>Summer (27.3°C)</th>
<th>Autumn (25.7°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.5 \times 10^{10}$</td>
<td>$3.5 \times 10^{10}$</td>
<td>$3.4 \times 10^{10}$</td>
<td>$10.7 \times 10^{10}$</td>
</tr>
<tr>
<td>2</td>
<td>$2.8 \times 10^{10}$</td>
<td>$2.7 \times 10^{10}$</td>
<td>$2.7 \times 10^{10}$</td>
<td>$3.6 \times 10^{10}$</td>
</tr>
<tr>
<td>3</td>
<td>$2.9 \times 10^{10}$</td>
<td>$3.5 \times 10^{10}$</td>
<td>$3.4 \times 10^{10}$</td>
<td>$4.6 \times 10^{10}$</td>
</tr>
<tr>
<td>4</td>
<td>$2.7 \times 10^{10}$</td>
<td>$3.7 \times 10^{10}$</td>
<td>$4.0 \times 10^{10}$</td>
<td>$7.5 \times 10^{10}$</td>
</tr>
<tr>
<td>5</td>
<td>$2.6 \times 10^{10}$</td>
<td>$5.8 \times 10^{10}$</td>
<td>$1.9 \times 10^{10}$</td>
<td>$5.9 \times 10^{10}$</td>
</tr>
<tr>
<td>6</td>
<td>$2.5 \times 10^{10}$</td>
<td>$3.6 \times 10^{10}$</td>
<td>$3.6 \times 10^{10}$</td>
<td>$5.5 \times 10^{10}$</td>
</tr>
</tbody>
</table>
Figure 4: Vaccine virus Infected Vero cells showing plaques (denoted by arrows)

Figure 5: Uninfected Vero cells (no plaque)
Figure 8: Rotavirus vaccine potency (PFU/ml) after exposure to 40\(^\circ\)C

<table>
<thead>
<tr>
<th>Exposure (hrs)</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.2 \times 10^{10}</td>
</tr>
<tr>
<td>6</td>
<td>2.3 \times 10^{10}</td>
</tr>
<tr>
<td>12</td>
<td>2.9 \times 10^{10}</td>
</tr>
<tr>
<td>18</td>
<td>2.7 \times 10^{10}</td>
</tr>
<tr>
<td>24</td>
<td>3.0 \times 10^{10}</td>
</tr>
<tr>
<td>36</td>
<td>2.1 \times 10^{10}</td>
</tr>
<tr>
<td>48</td>
<td>1.5 \times 10^{10}</td>
</tr>
<tr>
<td>72</td>
<td>0.8 \times 10^{10}</td>
</tr>
</tbody>
</table>
### Table 9: Rotavirus Vaccine Potency (PFU/ml) after exposure to Freezing

<table>
<thead>
<tr>
<th>Exposure (hrs)</th>
<th>Temperature °C</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>-20</td>
<td>$3.5 \times 10^{10}$</td>
</tr>
<tr>
<td>12</td>
<td>-80</td>
<td>$3.0 \times 10^{10}$</td>
</tr>
</tbody>
</table>
6. DISCUSSION

Reasons for the low level of rotavirus vaccine protection in low income countries (Groome et al., 2014), in contrast to the high level of protection in the high income countries (Staat et al., 2011; Buttery et al., 2011; Muhsen et al., 2010; Mast et al., 2011; Paulke-Korinek et al., 2010; Justino et al., 2011) have thus far been speculative. This observation is not unique to the rotavirus vaccine since it has also been reported for oral polio and cholera vaccines (Serazin et al., 2010). Although a multitude of factors have been documented as likely causes for the observed low effectiveness (Clark and Desselberger, 2015), socio-economic status has been implicated as the most influential factor underpinning the global effectiveness of the rotavirus vaccines. Viral, environmental and host factors have also been identified as possible determinants of protection conferred by these vaccines (Clark and Desselberger, 2015). Protection from non-vaccine virus genotype has been documented as a major characteristic of these vaccines. However, the mechanism by which the vaccines protect against diverse and emerging rotavirus strains is still not well understood. In mid-2013, an investigation into a diarrhoea outbreak that occurred in KZN, revealed that in 55% of cases, rotavirus was detected in the stool of patients (NHLS, 2013). Surprisingly, most of the strains detected were of the G1P[8] genotype, which is the same as the genotype combination of the currently used vaccine (Rotarix®) in KZN.

In this study, possible factors influencing the effectiveness of the Rotarix® vaccine in KZN were evaluated amidst the continued routine vaccination programme. Children presenting with diarrhoea regardless of vaccination status were enrolled. This is in contrast to infants enrolled in efficacy studies (Mahdi et al., 2010; Tregnaghi et al., 2011; Cunliffe et al., 2012; Mahdi et al., 2012; Steele et al., 2012; Steele et al., 2010b; Armah et al., 2012; Armah et al., 2010b; Sow et al., 2012; Tapia et al., 2012 Vesikari et al., 2007b; Kawamura et al., 2011; Linhares et al., 2008; Ruiz-Palacios et al., 2006; Phua et al., 2009; Lau et al., 2013) where they were vaccinated before the onset of the rotavirus season.

The prevalence of rotavirus in the stools of these patients were assessed and possible associations of this disease with a range of clinical and socio-demographic characteristics were identified. Findings in this study indicates that rotavirus is still a major aetiology of diarrhoea among infants and young children in South Africa with a 23% prevalence. This is concordant with an earlier finding in which a 31% prevalence was reported by Bar-Zeev and colleagues (2015) in Malawi. The prevalence of rotavirus was not significantly associated with gender and HIV status in our study. This supports a previous finding from Tanzania (Moyo et al., 2014). In contrast, Moyo et al (2014) reported a higher prevalence of rotavirus among non-diarrhoeic HIV infected children than HIV uninfected children (Moyo et al., 2014). However, further studies focusing on the safety, immunogenicity and reactogenicity of the rotavirus
vaccines among HIV infected population are urgently required as previously emphasized by Steele et al., (2009).

Globally, all children become infected with rotavirus before their third year of life (Seheri et al., 2012) and this infection occurs at earlier age among children in low income countries than those in high income countries. In this study, a significantly higher prevalence of rotavirus among infants and young children less than 2 years old was observed. An increasing rotavirus detection rate from birth until the end of the second year of life was also observed. The highest rotavirus prevalence was recorded in the age group of 12 to 23 months (30.4%). Above this age, there was a tendency for the prevalence of rotavirus to decline. The higher prevalence in this age group may be attributed to waning immunity elicited by natural or vaccine induced protection since the last dose of the Rotarix® vaccine is administered at 14 weeks of age in South Africa. A booster dose is recommended for older children, before the rotavirus season commences (Jagai et al., 2012). This study demonstrated a high seasonality of rotavirus infection in KZN. A significantly high rotavirus associated diarrhoea cases in the winter season when compared to other seasons in KZN was recorded. This variation in seasonality is further supported by findings in previous studies where the highest prevalence of rotavirus were in the cool winter months (Moyo et al., 2007; Koopmans and Browns, 1999; Kim et al., 2005; Nakagomi et al., 2005; Gleizes et al., 2006; Sam et al., 1992; Steele et al., 1986; Cunliffe et al., 1998; Mhalu et al., 1988). This finding is useful when making recommendations to the Department of Health regarding the appropriate timing of booster doses in the above 2 year age groups.

Nutritional deficiency is one of the many factors that has been proposed to affect the protection induced by the rotavirus vaccine in low income settings. Noteworthy is zinc deficiency which has been reported to affect the immune system (Clark and Desselberger, 2015). This study revealed that children who received zinc supplement were more likely to be infected with rotavirus when compared to those who did not. However, the highest prevalence of rotavirus was recorded among children whose status of zinc supplement receipt were unknown. In the latter, there was no information regarding zinc supplementation in the children’s folders and immunization cards, and their parent/s or guardian/s were not sure if they had received zinc supplements. Assuming that most of the children in the ‘unknown category’ did not receive zinc supplements, then this finding will be concordant to previous findings (Clark and Desselberger, 2015), where a higher prevalence of rotavirus was detected among children who did not receive zinc supplementation. Nonetheless, the effect of zinc on rotavirus vaccine immunogenicity needs further exploration.
Intestinal colonization with bacteria and helminthes as early as 4 weeks of life have been found to significantly alter the numbers of memory B-cells when the infants reaches 18 months of age (Lundell et al., 2012). The difference in intestinal organisms between populations of low and high income countries, directly relates to the difference in rotavirus vaccine effectiveness in these settings. Treatment of the intestinal helminth, *Ascaris lumbricoides*, with albendazole before the administration of oral cholera vaccine has been shown to enhance the rate of seroconversion by this vaccine (Cooper et al., 2000). Therefore, rotavirus associated diarrhoea cases was stratified based on children who had received an anti-helminthic drug before admission, those who did not and those whose status of receipt are unknown. There was no significant relationship between rotavirus associated diarrhoea cases and the receipt of an anti-helminthic drug. This study was not powered to ascertain if anti-helminthic drug was given before vaccination or afterwards. In addition, the presence or absence of helminthes in the intestine of our study population was not ascertained. It is therefore difficult to draw conclusions about intestinal colonization with helminthes and there effect on the immunogenicity of the rotavirus vaccine in our population. In addition, the highest rotavirus prevalence was discovered among the group that did not have information on the receipt of anti-helminthic drugs. Assuming that most of the children in the latter group did not receive anti-helminthic agents, then our findings will corroborate previous report where the use of anti-helminthic drugs enhances the seroconversion elicited by the vaccine (Cooper et al., 2000). However, it will be important to conduct further studies on the effect of intestinal bacteria and parasites on rotavirus vaccine.

Maternal antibodies acquired transplacentally and via breast feeding has been documented to protect infants and young children from diseases (Moon et al., 2010). In this study, the mode of feeding of children who were 6 months old and younger was recorded. This study revealed that exclusive breast feeding had no effect on the rate of rotavirus infection in our setting. However, it has been reported that the effectiveness of rotavirus vaccine may be compromised by maternal antibodies from breast milk (Moon et al., 2010).

The Rotarix® vaccine schedule in South Africa is 6 weeks of age for the first dose and 14 weeks of age for the second dose. This is contrary to the schedule that was used in the efficacy trial of this vaccine in Africa, which was 10 and 14 weeks of age for the first and second dose respectively (Madhi et al., 2010). The degree of protection offered by the 6 and 14 weeks schedule was only established in the post vaccination effectiveness study (Groome et al., 2014). Noteworthy is the fact that the immunogenicity of the Rotarix® vaccine in completely vaccinated children at 6 and 10 weeks of age was lower than that of children vaccinated at 10 and 14 weeks of age with a 36% and 60% seroconversion rate respectively (Steele et al., 2010b). In this study, the study population was grouped based on vaccination status. These
included, children who received two doses of the Rotarix® vaccine within the vaccination timeline (completely vaccinated); those who received one dose but were not old enough for the second dose, or those who were old enough for the second dose but had not received it (incompletely vaccinated); and those who had not receive any does because they were younger than 6 weeks old or those who were old enough for vaccination but did not receive any (not vaccinated). The findings from this study suggested that vaccination had no significant effect on the prevalence of rotavirus associated diarrhoea among these children. KZN has a high (97.8%) immunization coverage (Van den Heever, 2012) and it is therefore difficult to imagine that a failure in the immunization programme could explain the above mentioned findings. Consequently, the utility of this rotavirus vaccine in developing countries need to be further explored. In addition, the observed variations in the immunogenicity of the Rotarix® vaccine at different vaccination schedules need to be further evaluated to maximize the potential benefit that this vaccine may offer.

The global vaccines has been reported to be relatively effective and recommended for use worldwide. It is surprising to note that country specific vaccines are currently being licensed and introduced based on the circulating genotypes in these countries. Examples of these vaccines are, the Lanzhou lamb rotavirus (LLR) vaccine which has been in use in China since 2000 (Fu et al., 2012), Rotavac vaccine which has been in use in India since 2014 (Bhandari et al., 2014) and the RV3-BB human neonatal rotavirus vaccine which has been in use in Australia and New Zealand since 2015 (Bines et al., 2015). These vaccines were designed using the most prevalent genotypes in these countries and it is envisaged that they will be more effective than the current global vaccines in these countries. However, the year to year, season to season and region to region variations in the circulating genotypes of rotaviruses in low income countries creates an obstacle to the design of an effective vaccine in these settings, including South Africa. The characteristic diversity of circulating rotavirus genotypes within confined geographical locations was highlighted in this study. Selected EIA positive samples were characterized using RT-PCR. 5 G-types, 4 P-types and some non-typeable strains were identified. The most prevalent G/P combination in our setting was G9P[8] (20.7%), followed by G9P[4] (13.8%), G12P[4] (10.3%), G9P[6] (6.9%) and a 3.4% prevalence was recorded for each of G4/8P[6], G4P[6], G12P[6], G8P[10] and G9P[10]. This correlated with previous finding in other Southern African countries viz: Zimbabwe and Zambia where G9P[8] accounted for 20% of the circulating genotypes (Seheri et al., 2014). The genotypes G1P[8] remains the most prevalent G/P combination worldwide. The rotavirus outbreak of 2013 in KZN (NHLS, 2013) revealed that G1P[8] was the most prevalent genotype. However, it is likely that the G1P[8] has been replaced by the G9P[8] in KZN through mechanisms such as vaccine selection pressure. Continuous surveillance of circulating strains is crucial, to better understand the effect of vaccination on rotavirus
strain distribution. G1 in combination with a non-typeable P type in only one patient was detected. The high diversity of the circulating strains, and the detection of non-typeable strains in this study, further calls to question the ability of the current vaccine to protect against diverse and novel strains of rotavirus.

The detection of rotavirus specific IgG in all serum samples may be due to natural immunity acquired from mothers or from previous infection or due to vaccine induced immunity. In this study, children under the age of 6 weeks (when first dose of Rotarix® vaccine is given) were serum IgG positive for rotavirus. In addition, rotavirus antigen was found in the stool of some of this children under the age of 6 weeks. Consequently, the children may have had their first or second rotavirus infection before receiving their first dose of vaccine. In a recent study from South Africa, it was reported that pre-existing serum IgG in infants and young children, including those acquired transplacentally, may have an inhibitory effect on the immunogenicity of rotavirus vaccine (Moon et al., 2015). This may have contributed to the lower effectiveness of the rotavirus vaccines in South Africa and other low income settings.

Furthermore, the avidities of the detected IgG were evaluated using the urea elution technique (Hedman and Rousseau, 1989). In this assay, low avidity antibodies were disallowed from binding to corresponding antigens by urea leaving only high avidity antibodies to bind. The avidity index of each antibody was measured and they were stratified into the 3 vaccination groups. No significant difference between the mean avidity of IgG in the vaccinated and those in the non-vaccinated group was observed. This finding suggests that IgG acquired via natural infection may confer equal protection as those acquired through vaccination. The absence of a correlate of rotavirus vaccine induced protection has made it difficult to fully understand the level of protection conferred by this vaccine.

To ensure that children are receiving fully potent and effective vaccines, the vaccine has to be kept at the optimum temperature recommended by the manufacturer. The optimum storage temperature for the Rotarix® is 2°C to 8°C. However, accidental or inevitable exposure of this vaccine to heat or freezing temperature while in storage or in transit can result in loss of potency as reported previously by Matthias et al (2007). The possibility of vaccine exposure to sub-optimal temperatures was considered as a factor responsible for the reduced effectiveness of the rotavirus vaccine in low income countries. Low income countries are often characterized by poor road networks and inadequate electricity supply. Poor road networks can expose vaccines to sub-optimal temperatures in transit while inadequate electricity supply can also expose the vaccines to temperature fluctuations during storage. A laboratory based potency assessment of the Rotarix® vaccine after exposure to sub-optimal temperatures was conducted. Digital oven and freezers were used to simulate vaccine exposure to heat and freezing temperatures respectively. Vaccines were exposed to the average temperatures of the different season in KZN and to extreme
temperatures such as 40°C, -20°C and -80°C. There potencies were compared to the expected vaccine potency recommended by the manufacturer and the potency of the control sample kept at optimum temperatures. Surprisingly, the vaccines remain potent after exposure to the seasonal temperatures for 1 – 6 hours, 40°C for 3 – 72 hours, -20°C and -80°C for 12 hours. These findings reveal that the Rotarix® vaccine is highly potent and fit for use in low income settings including KZN. However, reasons for the maintained potency after vaccines exposure to sub-optimal temperatures are still unknown. However this study only explored the effect of a constant ‘adverse’ temperature on vaccine potency and not the effect temperature fluctuations on vaccine potency. Further exploration is needed in this area.

Clinical and socio-demographic data suggest that this potent vaccine may be unable to elicit adequate protection in the mucosal layer of the gastrointestinal tract due to many factors. This study highlighted the genetic diversity of rotaviruses and poor immunogenicity of the vaccine as key factors affecting the effectiveness of the rotavirus vaccine. Whether the vaccine is able to induce homotypic and heterotypic protection in immunized children is critical in predicting the long range effectiveness of this vaccine against uncommon regional rotavirus strains. Interventions targeted at improving socio-economic conditions in low income countries may be a starting point towards the control and prevention of rotavirus infection in these settings.
7. REFERENCES


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8. APPENDICES

Appendix 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.
Appendix II: Questionnaire

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>__________________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of birth (dd/mm/yy)</td>
<td>__________________________</td>
</tr>
<tr>
<td>Date of admission</td>
<td>__________________________</td>
</tr>
<tr>
<td>Date of specimen collection</td>
<td>__________________________</td>
</tr>
<tr>
<td>No specimen</td>
<td>X</td>
</tr>
<tr>
<td>Is the stool</td>
<td>X</td>
</tr>
<tr>
<td>Watery</td>
<td>X</td>
</tr>
<tr>
<td>Bloody</td>
<td>X</td>
</tr>
<tr>
<td>Unknown</td>
<td>X</td>
</tr>
<tr>
<td>Age</td>
<td>__________________________</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
</tr>
<tr>
<td>Weight</td>
<td>kg</td>
</tr>
<tr>
<td>Height</td>
<td>cm</td>
</tr>
<tr>
<td>Temp</td>
<td>C</td>
</tr>
<tr>
<td>HIV status</td>
<td>P</td>
</tr>
<tr>
<td>On ART?</td>
<td>Y</td>
</tr>
<tr>
<td>CD4 count/ percentage</td>
<td>__________________________</td>
</tr>
<tr>
<td>How long does the patient have diarrhea for?</td>
<td>__________________________</td>
</tr>
<tr>
<td>Is the child underweight for age?</td>
<td>Y</td>
</tr>
<tr>
<td>Does the child have kwashiorkor?</td>
<td>Y</td>
</tr>
<tr>
<td>Has the child received immunizations to date?</td>
<td>__________________________</td>
</tr>
<tr>
<td>Vaccine</td>
<td>Date received</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Birth</strong></td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td></td>
</tr>
<tr>
<td>OPV</td>
<td></td>
</tr>
<tr>
<td><strong>6 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>OPV</td>
<td></td>
</tr>
<tr>
<td>RV (1)</td>
<td></td>
</tr>
<tr>
<td>DTaP-IPV/Hib (1)</td>
<td></td>
</tr>
<tr>
<td>Hep B (1)</td>
<td></td>
</tr>
<tr>
<td>PCV (1)</td>
<td></td>
</tr>
<tr>
<td><strong>10 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>DTaP-IPV/Hib (2)</td>
<td></td>
</tr>
<tr>
<td>Hep B (2)</td>
<td></td>
</tr>
<tr>
<td><strong>14 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>RV (2)</td>
<td></td>
</tr>
<tr>
<td>DTaP-IPV/Hib (3)</td>
<td></td>
</tr>
<tr>
<td>Hep B (3)</td>
<td></td>
</tr>
<tr>
<td>PCV (2)</td>
<td></td>
</tr>
<tr>
<td><strong>9 months</strong></td>
<td></td>
</tr>
<tr>
<td>Measles (1)</td>
<td></td>
</tr>
<tr>
<td>PCV (3)</td>
<td></td>
</tr>
<tr>
<td><strong>18 months</strong></td>
<td></td>
</tr>
<tr>
<td>DTaP-IPV/Hib (4)</td>
<td></td>
</tr>
<tr>
<td>Measles (2)</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Exclusively breastfed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula-fed</td>
<td></td>
</tr>
<tr>
<td>Mixed fed</td>
<td></td>
</tr>
</tbody>
</table>

What is the water supply at home?

<table>
<thead>
<tr>
<th>Piped water in dwelling</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Piped water on site</td>
<td></td>
</tr>
<tr>
<td>Neighbor’s tap</td>
<td></td>
</tr>
<tr>
<td>Communal tap</td>
<td></td>
</tr>
<tr>
<td>Rainwater</td>
<td></td>
</tr>
<tr>
<td>Borehole on site</td>
<td></td>
</tr>
<tr>
<td>Borehole off site</td>
<td></td>
</tr>
<tr>
<td>River /stream /flowing</td>
<td></td>
</tr>
<tr>
<td>Dam /pool /stagnant water</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Specify</td>
</tr>
</tbody>
</table>

Type of sanitation

<table>
<thead>
<tr>
<th>Flush</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td></td>
</tr>
<tr>
<td>Urine-diversion</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Specify</td>
</tr>
</tbody>
</table>
Availability of soap to wash hands

<table>
<thead>
<tr>
<th>Always</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sometimes</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td></td>
</tr>
</tbody>
</table>

Current treatment

________________________________________

________________________________________

________________________________________

Any treatment prior to coming to this clinic/hospital

________________________________________

________________________________________

________________________________________
Appendix III: Enzyme Immunoassay (EIA) worksheet

<table>
<thead>
<tr>
<th>College: Health Sciences</th>
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<tbody>
<tr>
<td><strong>School:</strong> Laboratory Medicine and Medical Sciences</td>
</tr>
<tr>
<td><strong>Department:</strong> Medical Microbiology, Infection Prevention and Control</td>
</tr>
</tbody>
</table>

Kit____________________    Expiry Date____________ Test Date____________

Name___________________     Signature______________ Cut off Value_________

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</table>
Appendix IV: RT-PCR protocol for Rotavirus detection and genotyping

Total RNA were extracted from stool specimen (10% stool suspension) using the QIAamp viral RNA mini-kit (Qiagen, Hilden, Germany)

Conversion of RNA to cDNA using the high capacity cDNA reverse transcription kit with RNase inhibitor (Thermo Fisher Scientific, California, USA). The cDNA master-mix component are in the table below;

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25x dNTP mix (100mM)</td>
<td>0.8</td>
</tr>
<tr>
<td>10x RT Random primers</td>
<td>2.0</td>
</tr>
<tr>
<td>Multiscribe reverse transcriptase</td>
<td>1.0</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>3.2</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

cDNA reverse transcription reactions were prepared by pipetting 10µl of the above master-mix into a PCR tube and adding 10µl of RNA. The thermal cycling condition was as below:

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25°C</td>
<td>37°C</td>
<td>85°C</td>
</tr>
<tr>
<td>Time</td>
<td>10 minutes</td>
<td>120 minutes</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
### PCR Reaction set-up

<table>
<thead>
<tr>
<th>Component</th>
<th>25µl reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>To 25µl</td>
<td>-</td>
</tr>
<tr>
<td>10x PCR buffer, -Mg</td>
<td>2.5µl</td>
<td>1x</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.9µl</td>
<td>1.8mM</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>0.5µl</td>
<td>0.2mM</td>
</tr>
<tr>
<td>10µl Forward primer</td>
<td>0.5µl</td>
<td>0.2mM</td>
</tr>
<tr>
<td>10µl Reverse primer</td>
<td>0.5µl</td>
<td>0.2mM</td>
</tr>
<tr>
<td>cDNA</td>
<td>2.5µl</td>
<td>&lt;500ng</td>
</tr>
<tr>
<td>Platinum Taq DNA polymerase</td>
<td>0.1µl</td>
<td>2U/rxn</td>
</tr>
</tbody>
</table>