PREVALENCE AND GENETIC DIVERSITYOF ROTAVIRUS STRAINS AMONG CHILDREN WITH GASTROENTERITIS AT THE GERTRUDE'S CHILDREN'S HOSPITAL, NAIROBI, KENYA

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Prevalence and Genetic diversity of rotavirus strains among children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

This work is dedicated to my parents Dr. William B. O. Oduol and the late Mrs Victoria A. Odinga and my daughter late Joy Everlyn Aoko.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

AKUH	Aga Khan University Hospital
CDC	United States Centres for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CVR	Centre for Virus Research
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
GGCH	Gertrude's Garden Children's Hospital
ITROMID	Institute of Tropical Medicine and Infectious Diseases.
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
NACOSTI	National Commission for Science, Technology and Innovation
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
RT	reverse transcriptase

RVG rotavirus gastroenteritis

SDS Sodium dodecyl sulphate

SSC Scientific Steering Committee

TAE Tris-Acetic Acid- EDTA

VP Viral proteins

ABSTRACT

Rotavirus is the leading cause of severe diarrhoea among infants and young children. Each year more than 527,000 children die from rotavirus gastroenteritis, and two million are hospitalized. The licensing and introduction of two new live oral rotavirus vaccines was expected to substantially reduce child morbidity and mortality due to rotavirus gastroenteritis. This study was aimed at identifying and characterizing rotavirus strains isolated from children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi. It was a hospital based cross-sectional study done between January to July 2012 in which 331 patients were randomly selected among those who presented with gastroenteritis and whose parents consented to participate in the study. A structured questionnaire was used to establish their clinical, vaccination status and demographic characteristics. Enzyme-linked immunosorbent assay (ELISA) - based kit was used to detect Group A rotavirus antigens in 298 stool specimen giving a prevalence of 31.5%. Rotavirus dsRNA was extracted from virus particles, separated by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining. Fifty seven (60.1%) of the specimens gave visible RNA profiles. Thirty eight strains (40.4%) were assigned long electropherotypes while 19 (20.2%) showed short patterns electropherotypes depending on the migration of the 11 genome segments. Eighty three rotavirus antigen positive faecal specimen were selected and dsRNA extracted was reverse-transcribed and amplified using semi-nested RT-PCR in the presence of original consensus primers and genotyped using a mixture of serotype specific primers for the rotavirus genes specifying G (gene 9) and P (gene 4) classification. Forty three VP 7

(G serotype) and twenty six VP 4 (P serotype) were genotyped. Six single genotypes were demonstrated G1, G3, G9, G12, P [4], and P [6] while genotypes G3P [4], G3P [6], G9P [6], G12P [6], were found to be combined. Mixed infections observed included G9/3P [4], G1/3P [4] and G3/9P [6]. G3 were predominant at 27.9% of the G-genotypes detected while P [4] were detected in (16) 61.5 % of all P-genotype detected. Data was entered and analysed by SPSS version 20.0. The data generated from this study adds crucial information on the burden of the rotavirus disease and genotype distribution in Kenya country. Such information not only aids in seeking advocacy for introduction of unusual genotypes into the rotavirus vaccine but also helps in the evaluation of the efficacy of these vaccines in relation to the rotavirus genotypes in circulation. The heterogeneity and ever-changing epidemiology of rotavirus observed in this and other related studies highlight the need for continued surveillance of rotavirus strains throughout Kenya to ensure that vaccination provides optimal protection.

CHAPTER ONE

INTRODUCTION

1.1Background Information

1.1.1 Rotavirus

Rotavirus is the most common etiological agent associated with severe gastroenteritis leading to dehydration and death in young infants worldwide (Parashar *et al*., 2006) and is responsible for an estimated 527,000 deaths (range, 475,000–580,000 deaths) annually (WHO, 2007) and another two million are hospitalized (Simpson *et al*., 2007).

Epidemiological studies in infants have identified 5 common genotypes including G1, G2, G3, G4 and G9, which tend to predominate globally. However, in developing countries, additional genotypes may circulate and even predominate in some settings including G5, G8, G10, and G12 (Desselberger *et al*., 2001; Santos *et al.,* 2005). Current mortality figures for sub-Saharan Africa amount to 145,000 annually (Molbak *et al*., 2000). Studies in Nigeria (Aminu *et al*., 2010), Tunisia (Trabelsi *et al*., 2000) and Kenya have demonstrated that close to 90% of all children are infected with rotavirus by 2 years in these settings (Nyangao *et al*., 2010). It is estimated that, in Kenya, 68 deaths, 132 hospitalizations, and 21,800 clinic visits per 100,000 children aged less than 5 years annually are attributable to rotavirus diarrhoea. (Tate *et al.,* 2009). It has been established that the general age at infection is between 6 to 23 months. (Mutanda *et al*., 1984, 1986)

1.1.2 Transmission and Pathogenesis of Rotavirus

Rotavirus is transmitted by the faecal-oral route, via contact with contaminated hands, surfaces and objects (Butz *et al*., 1993) and possibly by the respiratory route (Dennehy, 2000). The faeces of an infected person can contain more than 10 trillion infectious particles per gram; (Bishop, 1996) only 10 to 100 of these are required to

transmit infection to another person (Graham *et al*., 1987). The virus replicates in the mucosal cells of the small intestines causing excess secretion of fluids and electrolytes into the bowel lumen and consequently loss of salt, glucose and water leading to diarrhoea. The diarrhoea is primarily due to stimulation of the enteric nervous system since no inflammation occurs. The incubation period for rotavirus disease is approximately 2 days. The disease is characterized by vomiting and watery diarrhoea for 3 to 8 days, with fever and abdominal pain, which occur frequently. Rotavirus gastroenteritis is a self-limiting illness, lasting for only a few days. Treatment is nonspecific and consists of oral rehydration therapy to prevent dehydration.

Although the severity of rotavirus infections differs between children living in developed and developing countries, the rates of infection is similar in both settings (Dennehy, 2008). Clean water supplies and good hygiene have little effect on the transmission of infection, and further improvements are unlikely to prevent disease (Dennehy, 2008). Safe and effective vaccines are needed, especially in poorer countries where most deaths from the disease occur (Parashar *et al*., 2009). The diversity of rotavirus genotypes may have significant implications for vaccine development and successful implementation of vaccination programmes, especially if it emerges that prevalent strains are not targeted by current vaccines (Kirkwood, 2010).

1.1.3 Gertrude's Children's Hospital

Gertrude's Children's Hospital was established in 1947. It is the most established paediatric hospital in Eastern and Central Africa, providing healthcare to children in Kenya. The hospital attends to over 300,000 outpatients annually through a network of 15 facilities in and around Nairobi and admits over 9,000 patients annually at its 100 bed facility located at Muthaiga, Nairobi (https://www.gerties.org). Two vaccines Rotarix and Rotateq were introduced in the country from 2007 and were administered privately at the hospital.

1.2 Problem Statement

Genetically and antigenically, diverse rotavirus strains co-circulate in humans. In Kenya rotavirus diarrhoea occurs throughout the year with seasonal peaks observed during dry seasons of January to March and June to September. Morbidity and mortality rates in developing countries remain high despite efforts to improve sanitary conditions, water quality, and healthcare infrastructure. These high rates have driven efforts to develop safe and effective rotavirus vaccines. Two vaccines a multivalent directed against G1, G2, G3, G4 and P. [8] and a monovalent vaccine to G1P [8] have been developed. These vaccines demonstrate homotypic protection but the degree to which they cross-protect against less common G-type and P-type combinations is yet to be established. It is apparent that as well as the major global genotypes G1-G4 there is occurrence of divergent rotavirus strains such as G9, G8 and G8/G9 mixed infections in Kenya. Additional data is needed to detect the unusual and new strains that may be circulating after introduction of these rotavirus vaccines. Limited information is available on the prevalent strains after introduction of vaccines from 2007 in Nairobi. This study was intended to identify and characterize circulating genotypes among children attending GGH.

1.3 Justification

Rotavirus gastroenteritis leads to considerable resource utilization in health care settings and the society (Osano *et al*., 2011). Vaccination does not confer lifelong immunity to children but reduces morbidity and mortality. Some studies (Kiulia *et al*., 2008, Nyangao *et al*.,2010), have demonstrated presence of both usual and unusual genotypes including possible reassortants and even animal strains being responsible for 30.5% and 15.7% of infections respectively and another 9.2% of mixed infections. These results suggest a relatively high degree of natural reassortment in the viral genome in the Kenyan communities. The diversity of rotavirus genotypes will have significant implications for successful implementation of the vaccination programme, especially if it emerges that current vaccines do not target prevalent strains. This study sought to identify and characterize the diversity of

strains that are prevalent and therefore provide information useful in choice of appropriate vaccine.

1.4 Research Questions

a) What are the demographic and clinical characteristics of the children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi?

b) What are the prevalent rotavirus strains in children presenting with acute rotavirus gastroenteritis at the Gertrude's Children's Hospital, Nairobi?

c) What are the rotavirus strains isolated from children with RVG who have been vaccinated against rotavirus infection?

1.5 Hypothesis

Children who have been vaccinated against the virus will not invariably be infected by the vaccine strains.

1.6 Objectives

1.6.1 General Objective

To determine prevalence and identify rotavirus strains among children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi.

1.6.2 Specific Objectives

1. To assess the demographic and clinical characteristics of children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi
2. To determine prevalence of rotavirus infections among children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi

3. To characterize the rotavirus strains isolated from children who have been vaccinated against rotavirus infection at the Gertrude's Children's Hospital, Nairobi

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of Rotavirus

Rotavirus is a member of the *Reoviridae* family (Kapikan *et al*., 2001). It is approximately 76.5nm in diameter and has a characteristic wheel-like appearance when viewed by electron microscopy (the name rotavirus is derived from the Latin *rota*, meaning "wheel").

Rotaviruses are classified into groups, subgroups, genotypes and on the basis of electrophoretic migration of gene segments. The group and subgroup specificity are present on the inner capsid VP6 , thus far only groups A, B and C have been identified as human pathogens, however the same groups are found in animals. Groups D, E, F, and G have only been identified in animals and birds (Amimo *et al*., 2013). Majority of infections are caused by group A rotaviruses. Group B rotavirus infection are uncommon but have recently been associated with outbreaks in China and India (Shariff *et al*., 2003). Group C rotavirus has been isolated in Kenyan children (Mwenda *et al*., 2003). The classification of rotaviruses according to genotypes is based on both VP4 (protease sensitive) and VP7 (glycoprotein) antigenic specificity. Both VP4 and VP7 proteins induce neutralizing antibodies and therefore protective immunity.

The genes encoding the outer capsid VP7 and VP4 form the basis of classification of group A rotaviruses into G and P genotypes, respectively (Estes *et al*., 2007). There are twenty P genotypes; Genotypes P [4], P [6], P [8] are most frequently associated with human infections and fourteen G genotypes with G-types 1, 2, 3 and 4, accounting for 80% of human infections. G8 and G9 are emerging as common genotypes with G9 described in Kenya (Cunliffe *et al*., 2001).

2.2 Rotavirus Structure

The genome of rotavirus consists of 11 unique double helix molecules of RNA which are 18,555 nucleotides in total. Each helix, or segment, is a gene, numbered 1 to 11 by decreasing size. Each gene codes for one protein, except genes 9 and 11, which each code for two proteins (Pesavento *et al*., 2006). Viral particles are approximately 76.5 nm in diameter (Prasad and Chiu, 1994, Patton, 1995) and are not enveloped. There are six structural viral proteins (VPs) that form the virus particle (VP1, VP2, VP3, VP4, VP6 and VP7). In addition, there are six non-structural proteins (NSPs), only produced in cells infected by rotavirus. These are named NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6 (Santos and Hoshino, 2005). These VPs and NSPs are encased within three icosahedral protein shells making it triple-layered. (Fig 2-1)

Each of these proteins is encoded in a unique genome segment except NSP5 and NSP6, which are encoded in overlapping reading frames of a single segment. VP1, 2, and 3 forms the core of the virion and are involved in genome replication and packaging. VP4, present as spikes on the outer capsid is cleaved by intestinal proteolytic enzymes to VP5 and VP8 subunits to initiate the process of viral penetration into host cells. VP7 is the outer capsid glycoprotein and may be involved in viral attachment as well as inducing antibody response (Kapikan *et al*., 2001)

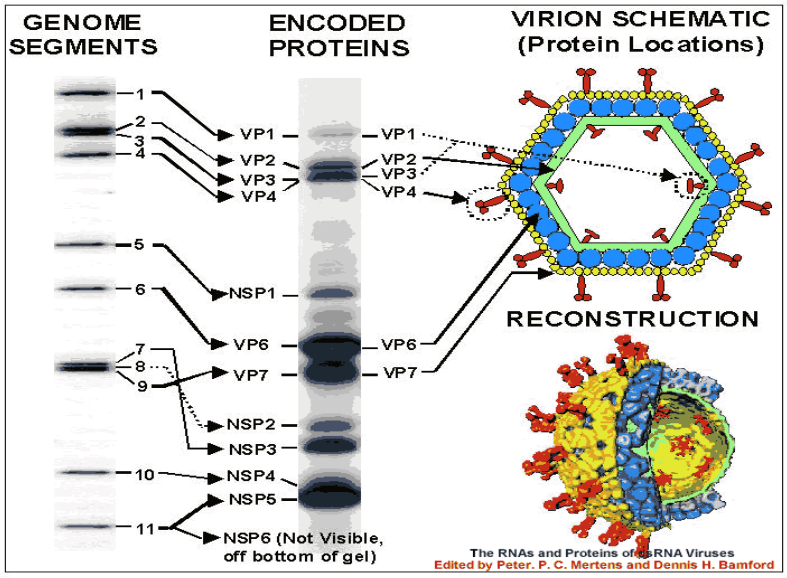

Figure 2.1: Coding assignments and virion locations of rotavirus proteins and 3D structure of rotavirus particle. (Mossel et al., 2015)

Source: The 10th Rotavirus Surveillance workshop laboratory manual, University of Limpopo-Medunsa Campus, 2009.

2.3 Rotavirus Genome organization

VP1 is located in the core of the virus particle and is an RNA polymerase enzyme (Vásquez-del *et al.,* 2006). In an infected cell this enzyme produces mRNA transcripts for the synthesis of viral proteins and produces copies of the rotavirus genome RNA segments for newly produced virus particles. VP2 forms the core layer of the virion and binds the RNA genome (Arnoldi *et al.*, 2007). VP3 is part of the inner core of the virion an enzyme named guanylyl transferase. It is a capping enzyme that catalyses the formation of the 5' cap in the post-transcriptional modification of mRNA (Fresco and Buratowski, 1994). The cap stabilises viral mRNA by protecting it from nucleic acid degrading enzymes. VP4 is on the surface of the virion that protrudes as a spike (Gardet *et al.,* 2006). It binds to molecules on

the surface of cells' receptors and initiates the entry of the virus into the cell (Arias *et al.,* 2002). Preceding infection, VP4 has to be modified by a protease enzyme (found in the gut) into VP5 and VP8 (Konno *et al.,* 1993). It determines the virulence of the virus and determines the P-type of the virus (Hoshino *et al.,* 2002). VP6 forms the bulk of the capsid. Being highly antigenic it can be used to identify rotavirus species (Bishop, 1996). This protein is used in laboratory tests to identify rotavirus A species in infections (Beards *et al.,* 1984). VP7 is a glycoprotein which forms the outer surface of the virion. Its structural functions are used in determination of the G-type of the strain and, along with VP4, are involved in immunity to infection (Pesavento *et al.,* 2006).

NSP1, the product of gene 5, is a non-structural RNA-binding protein (Hua *et al*., 1993). NSP2 is an RNA-binding protein that accumulates in cytoplasmic inclusions and is required for genome replication (Kattoura *et al*., 1994 and Taraporewala and Patton, 2004). NSP3 is bound to viral mRNAs in infected cells and is responsible for the shutdown of cellular protein synthesis (Poncet *et al.,* 1993). NSP4 is a viral enterotoxin that induces diarrhoea and was the first viral enterotoxin discovered (Dong *et al.,* 1997). NSP5 is encoded by genome segment 11 of rotavirus A and in virus-infected cells it accumulates in the viroplasm (Afrikanova *et al*., 1996). NSP6 is a nucleic acid binding protein (Rainsford *et al.*, 2007), encoded by gene 11 from an out of phase open reading frame (Mohan *et al*., 2001).

The 11 segments of double-stranded RNA (dsRNA) of the rotavirus genome are easily separated by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining. Rotavirus can be classified into electropherotypes on the basis of migration pattern of their RNA segments on polyacrylamide gel electrophoresis (PAGE) (Kapikian *et al.,* 2001). The relative migration of RNA segments are further used to differentiate strains into long, short, super short, and abnormal RNA profiles (Desselberger *et al*., 2001).Group A rotaviruses exhibit a characteristic migration pattern of 4:2:3:2. (Niture *et al*., 2011) as shown in Fig 2-2 below. Nevertheless, electropherotypes are useful epidemiological indicators on the evolution and spread

of rotavirus strains, and the observation of changes in electropherotypes helps to monitor the patterns of disease outbreaks and transmissions (Matthijnssens *et al.,* 2008).

The most prominent variation is observed between 'short', 'long' and 'super-short' RNA patterns, based on the difference in the mobility of gene segments 10 and 11(Kalica *et al.,* 1981; Albert *et al.,* 1981; Estes, 1996). The short electropherotypes results from a partial duplication in gene 11, which causes it to migrate more slowly than gene segment 10. The standard-sized gene 11 of long electropherotypes strains migrates faster than segment 10. Each distinct dsRNA profile defines a different viral electropherotype or strain (Fischer and Gentsch, 2004). Moreover, a strong association of short and long RNA electropherotypes has been found with subgroups I and II respectively as demonstrated in some studies (Kalica *et al.,* 1981)

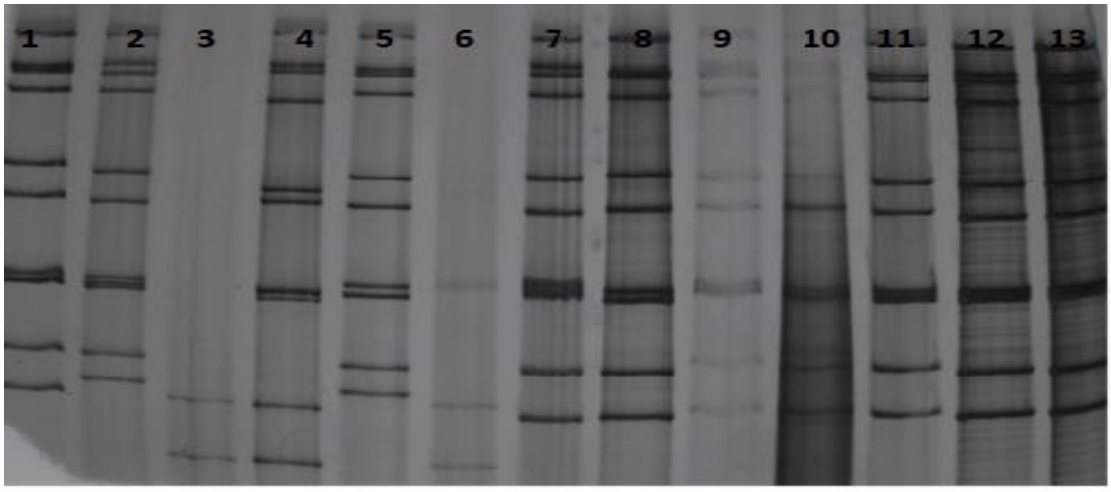

Figure 2.2: Short Electrophoretic Patterns of Nigerian Rotavirus Strains Determined By Page.

All strains except for lanes 3, 4, and 6 exhibit short (S) pattern. I, II, III, and IV indicate clusters of migrating gene segments typical of human Group A rotavirus

Source: Electropherotypes and G-Types of Group A Rotaviruses Detected in Children with Diarrhea in Lagos, Nigeria (Ayolabi *et al,* 2013)

The rotavirus RNA segments are divided into four size classes where class I has four large segments (1-4) , class II are medium- sized segments (5 and 6), class III of small segments (7,8 and 9) and class IV having the two smallest segments (10 and 11).

2.4 Laboratory Diagnosis of Rotavirus

Electron microscopy (EM) is highly specific for detection of rotavirus. However, the method is too labor intensive for routine detection of rotavirus in large numbers of stool specimens. In addition, EM requires an expensive instrument and highly trained personnel and cannot distinguish between rotaviruses. Antigen detection is the most widely used method for rotavirus diagnosis based on detection of protein antigens on rotavirus particles in stool specimens. These include an EIA that uses rotavirus specific antibodies to capture antigen onto wells of plastic plates, a latex agglutination utilizing latex particles coated with anti-rotavirus antibodies and lateral-flow immunoassays using immunochromatographic methods.

However, in multiple studies both techniques have shown variable sensitivity and specificity with a risk of misdiagnosis (Al-Yousif *et al*., 2001; Eing *et al*., 2001). Rotavirus dsRNA can be extracted from clinical specimen and analyzed by electrophoresis on polyacrylamide gel where patterns are visualized after silver staining. Human rotavirus Groups A, B, and C have distinct patterns of gene-segment distribution that are designated electropherotypes (Steele *et al*., 2004). The stained gels can be dried and stored (WHO, 2009).

A variety of sensitive conventional or real-time reverse-transcription polymerase chain reaction (RT-PCR) methods have been developed based on primers specific for several different rotavirus genes (Gouvea *et al*., 1991, 2004 Wilde *et al*., 1991) and Kang *et al*., 2004). These methods are particularly useful in detecting rotavirus in extra-intestinal tissues, in studies of the duration of viral shedding in stool and the correlation between disease severity and virus load (Kang *et al*., 2004, and Richardson *et al*., 1998). Polymerase Chain Reaction (PCR) has become the

preferred method for human rotaviruses detection as well as genotype characterization in epidemiologic studies (Noppornpanth and Poovorawan, 1999; De Oliveira *et al*., 2008). RT-PCR is also useful for verifying that RNA extracts contain intact rotavirus RNA.

2.5 Epidemiology of Rotavirus Gastroenteritis

The diversity of rotavirus strains in Kenya was demonstrated as early as 1983 with ELISA and serotype-specific monoclonal antibodies (Kiulia *et al*., 2008). A study in Nairobi, Nanyuki, and Narok between 1989 and 1991 identified serotype GI strains in majority of specimen analyzed (Gatheru *et al*., 1993). In another study in Kenya conducted in Nanyuki and Kitui in 1991-1993 and in Nairobi in 1991-1994 (Nakata *et al*.,1999) found serotype G4 as predominant, followed by G1 and G2 strains. Serotype G3 strains were rarely isolated, and G8 strains were identified for the first time in Africa. Additional studies in Nairobi between 1999 and 2000 indicated that G3 strains were predominant, with G4, G8, and G9 circulating at lower levels (Kiulia *et al*., 2008). A study in Nairobi by Nyangao and colleagues between 2000 and 2002 from 2 hospitals in Nairobi and Kisumu revealed serotype G1 strains were predominant in the specimen analyzed, followed by G2, G8, and G9 strains (Nyangao *et al*., 2010). According to the same study the Kenyan G2 strains, which have previously been shown to display diversity, and which cluster separately during phylogenetic analyses (Page *et al*., 2004) were detected in both Nairobi and Kisumu at low levels (Nyangao *et al*., 2010). Genotypes G8 and G9 were associated with a variety of VP4 P types: P[8], P[4], and P[6], which the study shows that are continuously circulating in the Kenyan population. Genotypes G1P [8], G2P [4], G3P [8], and G9P [8] are thought to be an important cause of diarrhoea in infants and young children worldwide (Gentsch *et al*., 2005).

2.6 Clinical presentation of Rotavirus Gastroenteritis

Clinically rotavirus gastroenteritis (RVG) ranges from asymptomatic infection to mild short-lived watery diarrhoea to severe gastroenteritis with dehydration. The

stools are pale and watery, with milky odour. Vomiting may occur with the diarrhoea. Other extra intestinal manifestations include acute myositis, polio like paralysis and encephalitis (Gilger, *et al*., 1992). Hepatic involvement in rotavirus infection is suggested by the associated elevation of the liver enzymes and biliary atresia seen more in group C rotavirus infections (Riepenhoff-Talty, *et al*., 1996). Rotavirus has also been demonstrated to replicate in the liver and kidneys of an immunodeficient child (Gilger, *et al*., 1992). Neurological involvement is suggested by several reports of children with concomitant convulsions and rotavirus diarrhoea which is only attributable to rotavirus infection when all other causes of convulsions have been ruled out (Lynch, *et al*., 2001). Rotavirus antigens have been detected in the myocardium of patients who died unexpectedly without any other detected pathology (Cioc and Nuoro, 2002). In vitro studies demonstrate an ability of rotavirus to replicate in the primary islet cells; an observation that correlates with the temporal association of infection with development of pancreatic islet cells auto antibodies (Coulson, *et al*., 2002).

In hospitalized patients, the duration of diarrhoea is usually 2-23 days with a median of 6 days. The disease leads to loss of fluids and electrolytes through diarrhoea and vomiting which results in dehydration and electrolyte derangements. Electrolyte imbalance complicates care especially if there is no laboratory support (Miguel *et al*., 2005). RVG is usually self-limiting in children with a normal, healthy immune system and an adequate level of nutrition (De wit, et al., 2003). Severe disease and death are seen more often in malnourished children (Steel *et al.*, 1984).

2.7 Rotavirus vaccination

In the country the two vaccines available and in use are Rotarix® (Glaxo Smith Kline) and Rotateq®(Merck). Rotarix® is derived from the attenuated human G1P [8] rotavirus group A strain 89–12 [8], while Rotateq® contains five human-bovine reassortant rotavirus group A strains, WI79-9 (G1),SC2-9 (G2), WI78-9 (G3), BrB-9 (G4), and WI79-4 (P [8]) (Donato *et al*., 2012)

The principle behind rotavirus protection is that primary rotavirus infection of young children does not confer immunity against reinfection, but it does protect against the development of clinically severe disease during rotavirus reinfection (Bishop *et al*., 1983; Velazquez *et al*., 1996). This means the children are not totally protected, they could still get infected with the same serotype. Only that the infection will not be severe as compared to those children who had no previous infection. In longitudinal studies, 88% of children were protected against severe diarrhea on reinfection after a single rotavirus infection (Velazquez *et al*., 1996). This observation became the strategic basis for administration of rotavirus oral vaccines. The first rotavirus infection elicits a predominantly serotype-specific (homotypic) neutralizing antibody response, and subsequent infections, even with the same rotavirus serotype, generally elicit a broader serotype cross-reactive (heterotypic) response (Bishop *et al*., 1983; Velazquez *et al*., 1996; Gorrell and Bishop, 1999).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The research was conducted at the Gertrude's Children's Hospital (GCH). The main hospital is located in Muthaiga area of Nairobi along Muthaiga road. GCH is a leading privately owned Paediatric Hospital in East Africa with excellent facilities and a network of outpatient clinics in Nairobi. It offers a variety of healthcare services to infants, school-age children, and teenagers. The laboratory work was done at the Rotavirus laboratory in the Centre for Virus Research (CVR) of Kenya Medical Research Institute (KEMRI).

3.2 Study Design

This was a hospital based cross-sectional study done from January to July 2012.

3.3 Study Population

The study population were children less than 5 years old who presented with gastroenteritis symptoms attending the Gertrude's Children's Hospital.

3.3.1 Inclusion criteria

i. A child aged less than 5 years who presented with gastroenteritis symptoms at the hospital on admission or during the first 48 hours of hospitalization.
ii. Those whose parent or guardian gave consent.

3.3.2 Exclusion criteria

i. Onset of severe gastroenteritis > 48 hours after admission in children below 5 years of age (nosocomial infections).

ii. Patients with bloody stool.

iii. Children less than 5 years who presented with gastroenteritis at GCH and their parents or guardians did not consent to participate in the study.

3.4 Sampling procedure

All children with gastroenteritis who presented at the GCH were randomly selected using systematic random sampling method. A structured questionnaire (Appendix III) was used to gather clinical and demographic information of the patient both from the hospital records and from the consenting parent/guardian.

3.5 Sample size determination

The sample size was calculated based on a previous prevalence study of rotavirus gastroenteritis (RVG) in an urban hospital in Nairobi, which had been established to be 28.4% (Nakata *et al.*, 1999) as there was no current published data stating otherwise.

The sample size was calculated using statistical Fischer *et al* 1998 formula as shown below;

$$n = \frac{Z^2_{1-\alpha/2}\, P\,(1-P)}{d^2}$$

Where; n = Sample size

$Z_{1-\alpha/2} = 1.64$

P = Estimated Prevalence from a previous study (28.4%; Nakata *et al., 1999*)

d = degree of accuracy (0.1)

Therefore:

$$n = \frac{1.64^2 \times 0.284\ (1-0.284)}{0.1^2} = \textbf{55 samples}$$

With an additional 10% (6) to adjust for improperly kept records giving a total of **61 samples** used for the study calculated at 90% confidence interval.

3.6 Specimen Collection, Transportation, Storage and Disposal

Faecal specimen was collected in a sterile polypot from a patient with symptoms of gastroenteritis. Each patient was requested to provide a stool sample only once during the study. The samples were stored in a -20°C freezer awaiting transportation in cool boxes to KEMRI. All procedures for working with Biosafety level 2 (BSL2) agents such as rotavirus were observed in accordance with the WHO Biosafety manual. All the biohazardous materials were disposed off into the KEMRI incinerator.

3.7 Specimen Processing

One millilitre of 10% (w/v) faecal suspension was prepared in phosphate-buffered saline (PBS), vortexed and clarified by centrifugation. The clarified supernatant about 1-1.5ml was collected and stored for short term at 4-8◦C.

3.8 Sample storage, export and further studies

The clarified supernatant not used up was stored at -70 degrees centigrade for up to a period of two years in the KEMRI laboratory. During this period samples may be

sent to Regional Rotavirus Laboratories for confirmation testing or more advanced characterization.

3.9 Rotavirus Enzyme-linked Immunosorbent Assay

Rotavirus kit (ProSpecT^{TM} Rotavirus Microplate Assay from Oxoid Ltd) was used to reconfirm Group A rotavirus antigens on rotavirus particles in the stool specimens as the hospital used rapid tests to detect presence of rotavirus antigens. This is a qualitative enzyme immunoassay for detection of Group A rotavirus. Rotavirus specific antibodies are pre-coated onto wells of plastic plates. The antigen is detected in a colorimetric reaction using a second rotavirus-specific antibody coupled to a detector enzyme

Feacal suspensions that were prepared and preserved in PBS were further diluted in ProSpecT Rotavirus sample diluent to prepare 10% suspension of feaces. 2 drops (100 µl) of each diluted feacal suspension and at least one negative and positive controls were added to separate micro wells. Then 2 drops of Conjugate was added to each micro well. The plate was covered and incubated at 20-30 ºC for 60 minutes. The contents of the wells were aspirated and wells washed by filling each well with 400 µl diluted Wash Buffer. Fluid from each well was aspirated out 5 times. After the last wash, the contents of the wells were removed then the plate was struck on a clean paper towel. When automatic washers were used they were programmed to complete 5 wash cycles. After the final wash, the plate was inverted and tapped on absorbent paper to remove the last traces of wash buffer. Two (2 drops) of substrate was added to each micro well. The plate was covered again and incubated at 20-30 ºC for 10 minutes. Substrate reaction was stopped by adding 2 drops of Stop Solution to each micro well. The fluid in the micro wells was thoroughly mixed before reading the results. The coloured product remained stable for 30 minutes after addition of the Stop Solution. The results were read spectrophotometrically at 450 nm. The Negative Control value was less than 0.150 absorbance units. The Positive Control value was greater than 0.500 absorbance units.

3.10 Molecular Analysis of ELISA-positive samples

Phenol-chloroform extraction of RNA from stool for Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done as stipulated in the Manual of Rotavirus detection and characterization methods (WHO, 2009).

A stool suspension was made by mixing a pea sized amount of faecal material to 5ml distilled water. Sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS) was placed in 37 ºC water bath to prevent the SDS from precipitating out. To 450 µl of stool suspension in an eppendorf tube 50 µl of pre-warmed 1M NaAc containing SDS (pH 5.0) was added. The mixture was incubated in a 37 ºC water bath for 15 minutes. Then, 500 µl phenol-chloroform (1:1) was added to the eppendorf and vortexed for 1 minute. These were further incubated for 15 minutes in 56 ºC water bath. To reduce the air pressure within the tube, it was opened and immediately resealed prior to vortexing. Vortexing was done for 1 minute and centrifuged for 3 minutes at 12000 rpm. Carefully the upper aqueous phase which contains the dsRNA was removed and placed in a clean eppendorf tube. The interface material was carefully avoided as it contains protein and DNA that would contaminate the sample and potentially degrade the RNA. To the sample 40 µl 1/10 volume 3M NaAc and 1 ml ice-cold absolute ethanol was added and mixed gently by turning the tube over 4-6 times. This was incubated at -20 ºC overnight to allow the dsRNA to precipitate. To pellet the dsRNA the sample mix was centrifuged at 4 ºC for 15 minutes at 12000 rpm. Then the supernatant was poured off and the sample allowed to air-dry. The pellet was then resuspended in 30 µl PAGE sample dye before loading it on to the PAGE gel. The rotavirus dsRNA was run on 10% polyacrylamide resolving gels using a large-format gel electrophoresis system(Hoefer SE600) and 3% spacer gel was used to enhance resolution of the dsRNA segments. Electrophoresis was conducted at 100V for 16-20h at room temperature. The gels were stained by silver staining to group the rotavirus in electropherotypes as described in the Laboratory Manual developed by the African Rotavirus Workshop in South Africa, 2002.

Further, Group A rotavirus genotyping was done after RNA extraction using Trizol from the 10% stool suspension. The previously prepared stool suspensions were centrifuged at 5000 rpm for 5 minutes at room temperature. About 200 µl of the stool supernatant was transferred to a clean eppendorf tube and 500 µl of Trizol added to it. The tubes were vortexed and incubated at room temperature for 5 minutes. A measure of 100 µl chloroform was added and vortexed for 30 seconds and incubated for 3 minutes. This is followed by centrifugation at 4 ºC at 12000 rpm for 15 minutes to separate the phases. Approximately 450 µl of the clear aqueous phase was carefully transferred to a clean eppendorf tube. An additional 700 µl of ice-cold isopropyl alcohol was added and mixed gently by turning the tubes 4-6 times then incubated at room temperature for 20 minutes. The dsRNA was pelleted by centrifugation at 4 ºC at 12000 rpm. The supernatant was discarded while the pellets allowed to air dry. The dsRNA pellet was resuspended in 10µl sterile deionised water and used for RT-PCR genotyping separately for VP7 and VP4 genes. Rotavirus genotyping was based on semi-nested RT-PCR, in which viral RNA from faecal specimen was reverse-transcribed using heat stable DNA polymerase enzyme according to RT-PCR protocol as described by the South and West African Regional Rotavirus Laboratories and amplified by PCR in the presence of consensus primers for the rotavirus genes specifying G (gene 9) and P (gene 4) genotypes.

3.10.1 RT-PCR for G Genotyping

For G genotyping, RT master mix (2.0µl of 5xAMV buffer; 0.25µl each of 10 mM dATP, dCTP, dGTP, dTTP and 0.2µl AMV RT) was prepared. VP7 consensus primers (beg9 or sbeg9 and end 9 or RVG9) diluted to 20 pmol and 1 µl of each primer added to all sample tubes. To the primer add 5µl of dsRNA. Added to the primer mixture is ddH_2O to a final volume of 10 µl and mixed by pipetting. The samples were denatured for 5 minutes at 94 ºC and transferred immediately to an ice-bath. To each tube 3.2 µl of RT master mix was added and mixed well. The mixture was spinned for 10 seconds and incubated at 42 ºC for 30 minutes. PCR amplification master mix (1.0µl each of 10mM dATP, dCTP, dGTP, dTTP; 4µl

10xTaq buffer, 2.4µl 25mM $MgCl_2$, 29.3µl deionised water) was prepared and placed on ice followed by addition of 0.3µl Taq polymerase before use. Sample tubes were centrifuged for 10 seconds after incubation at 42 ºC and placed on a sample rack in ice. 36.8µl of the amplification master mix was added to each RT sample tube and mixed. The samples were placed in the thermocycler and incubated using 30 cycles of 95ºC for 30 seconds, 42ºC for 30 seconds, 72ºC for 1 minute. PCR fragments are analysed on 1% TAE agarose gels to determine the sizes using 100 bp molecular weight markers compared by VP& cDNA products. Genotypying was done using RVG9 and a cocktail of type-specific primers aBT1 (G1), aCT2 (G2), aDT4 (G4), aET3 (G3) to determine G1 to G4 while aAT8 (G8) and aFT9 (G9) to determine G8 and G9 rotavirus VP7 genotypes (Table 3-1). Each genotyping and consensus end primer was diluted to contain 20 pmol. To labelled tubes 1-2 µl first-round RT-PCR products were added. 38µl of genotype master mix (1.0µl each of 10 mM dATP, dCTP, dGTP, and dTTP; 7 x 1.0µl 20pmol for each genotyping primer and conserved end primer; 4.0µl 10x Taq buffer; 2.4 µl 25mM $MgCl_2$; 0.3µl Taq polymerase) was added to 20.3 µl deionised water and mixed with 1-2µl of first round RT-PCR products in each tube. Amplification was done for 30 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 1 minute. PCR fragments/bands are run on 2% TAE agarose at 80 -90 volts with appropriate molecular weight markers to determine the genotype of the rotavirus strain.

Table 3.1: Oligonucleotide primers for G serotyping as designed by Gouvea *et al.*, 1990 and Gault *et al*., 1999.

Primer	Sequence (5'-3')	Position(nt)	Strain (genotype)
SBeg	GGCTTTAAAAGAGAGAATTTC	1-21	Group A
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Group A
End9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	Group A
EndA	ATAGTATAAAATACTTGCCACCA	922-944	Group A
aAT8	GTCACACCATTTGTAAATTCG	178-198	69M (G8)
aBT1	CAAGTACTCAAATCAATGATGG	314-335	Wa (G1)
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	DS-1 (G2)
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	ST-3 (G4)
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	P (G3)
aFT9	CTAGATGTAACTACAACTAC	757-776	WI61 (G9)
G12	CCGATGGACGTAACGTTGTA	548-567	G12
RVG9	GGTCACATCATACAATTCT	1062-1044	Group A

Table 3.2: The expected sizes of the VP7 genotyping PCR products using Gouvea et al., 1990 and Gault et al., 1999 primers

Primer	Genotype	expected product size (bp)
beg9 and end9	VP7 genotypes	1062
9FT9	G9	306
9ET3	G3	374
G12	G12	513
9DT4	G4	583
9CT1	G2	625
9BT1	G1	749
9AT8	G8	885

3.10.2 RT-PCR for P Genotyping

Thin-walled tubes for each amplification reaction were labelled on the top with sample number, primers used and date including a positive and negative control. For the first round amplification for P genotyping (VP8 subunit of VP4 gene), 3.2µl RT master mix (2.0µl 5xAMV buffer; 0.25µl each of 10mM dATP, dCTP, dGTP, dTTP and 0.2µl AMV RT) was prepared and placed on ice. Each of the VP4 consensus primers (con2 and con3) in were diluted to 20 pmol and 1 µl of each primer added to all sample tubes. 5µl of dsRNA was added to the primers and water mixed up to a final volume of 10µl by pipetting. The samples were denatured for 5 minutes at 94 ^{o}C. and transferred to all sample tubes on an ice bath. To each tube 3.2 µl RT master mix was added. The mixture in tubes was spinned for 10 seconds and incubated at 42 ^{o}C for 30 minutes. The RT mix was added in 5 minutes. 36.8 µl PCR amplification master mix (1.0µl each 10mM dATP, dCTP, dGTP, and dTTP; 4.0µl 10x Taq buffer; 2.4µl 25mM $MgCl_2$; 29.7µl deionised water was prepared and 0.3µl Taq polymerase added to RT sample tube. The sample tubes were centrifuged for 10

seconds after incubation at 42ºC which were then placed in a sample rack on ice. Amplification was done for 30 cycles of 95 ºC for 30 seconds, 42ºC for 30 seconds, and at 72ºC for 1 min. The PCR fragments were analysed on 1% TAE agarose gels to determine the sizes using 100 bp molecular weight markers for VP4 cDNA products. The second step RT-PCR was done using consensus primer con3 and a cocktail of specific primers (Table 3-2); 1T-1, 2T-1, 3T-1, 4T-1 and 5T-1 to determine rotavirus VP4 genotypes (Gentsch et al, 1992). Each genotyping and consensus end primer was diluted to contain 20pmol. To the labelled tubes 2 µl of first –round RT-PCR products are placed. 38µl Genotype master mix containing 1µl each of 10mM of dATP, dCTP, dGTP , dTTP , 4µl of 10x Taq buffer, 2.4µl of 25mM $MgCl_2$, 0.3µl Taq polymerase and 22.3µl deionised water was added to each labelled tube containing RT-PCR products and mixed well. Amplification was done for 30 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and at 72ºC for one minute. The PCR fragments were run on 2% TAE agarose gel at 80-90 volts with appropriate molecular weight markers to determine the genotype of the rotavirus strains.

Table 3.3: Oligonucleotide primers for P serotype PCR typing as designed by Gentsch *et al.*, 1992.

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
1T-1	ACTTGGATAACGTGC	339-356	KU [P8]
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5 [P4]
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076 [P6]
4T-1	TGAGACATGCAATTGGAC	385-402	K8 [P9]
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M [P10]
Con3	TGGCTTCGCCATTTTATAGACA	11-32	Group A
Con2	ATTTCGGACCATTTATAACC	868-887	Group A

Table 3.4: The expected sizes of the VP4 genotyping PCR products using Gentsch *et al.*, 1992 primers

Primer	**Genotypes**	**Expected product size (bp)**
Con2 and Con3	Group A	876
3T-1	P[6]	267
1T-1	P[8]	345
4T-1	P[9]	391
2T-1	P[4]	483
5T-1	P[10]	594

3.11 Data Management

All collected data was recorded in a laboratory record book and then entered in Microsoft excel worksheet. Data was backed up in a CD, flash disc, an external disk and hard drive disks. All data collected was kept secure and confidentiality ensured

by use of passwords. Data coding and analysis was done using the SPSS Version 20 software. Pearson's chi-square was used to determine associations between those infected and those not infected by the virus. Level of significance was fixed at 0.1 (p=0.1). Results are presented in frequency tables, and percentages.

3.12 Ethical Considerations

Approvals to conduct the study were obtained from GCH and KEMRI's Scientific Steering Committee (SSC No. 2100) and Ethical Review Committee (ERC) Appendix IV. A written informed consent was sought from the parents/guardians of all study participants before interview. The patients targeted were determined by a clinician and laboratory tests prescribed to confirm diagnosis. A stool specimen was used in laboratory diagnosis. There were no risks or inconveniences. The samples were identified using the outpatient number and a study reference number (the informed consent form no.). The participants received no tangible benefit. Voluntary participation was employed for the study. Confidentiality of information collected was assured and maintained. All the diagnosed cases were referred for management to the hospital staff.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of the Study Population

Three hundred and thirty one (331) participants who met all inclusion criteria were recruited. Of these, 298 stool specimen were examined in the final analysis and were the ones considered in study. A total of 164 boys and 134 girls participated in the study. The study included an age range below 60 months. These are the children brought to the hospital with diarrhoea and vomiting. After observation out of 164 boys 90.8% were hospitalized while out of 134 girls 92.5% were hospitalized for specialized management. As indicated in Table 4-1 majority (45.3%) of the participants were aged between 0 - 12 months followed by 32.6% those aged between 13-24 months while those between 25-36months, 37-48months and 49-60 months were 8.7%, 8.4% and 5.0% respectively. Among the study participants brought to the hospital 59% were breastfeeding, 71.5 % had been exclusively breastfeed for between 4 to 6 months, 20.8% for less than 3months, and only 6.7% had been exclusively breastfed for more than 7 months.

Table 4.1: Distribution of the study participants by age

Age range months	frequency	Percentage
0-12	135	45.3%
13-24	97	32.6%
25-36	26	8.7%
37-48	25	8.4%
49-60	15	5.0%
Total	**298**	**100%**

Z-score values are used to quantify growth status of children since they represent standardized measures comparable across age and sex. The measure indicates deviation from the population mean .About 77.5% of children presented for treatment had z-scores between -1 and +1 with majority 41% having a median score. (Figure 4-1). In this study this was being used to determine association between nutritional status and vulnerability to rotavirus. This indicated that majority of the children were within the expected population mean and therefore no significant association to infection with Rotavirus (p= 0.774)

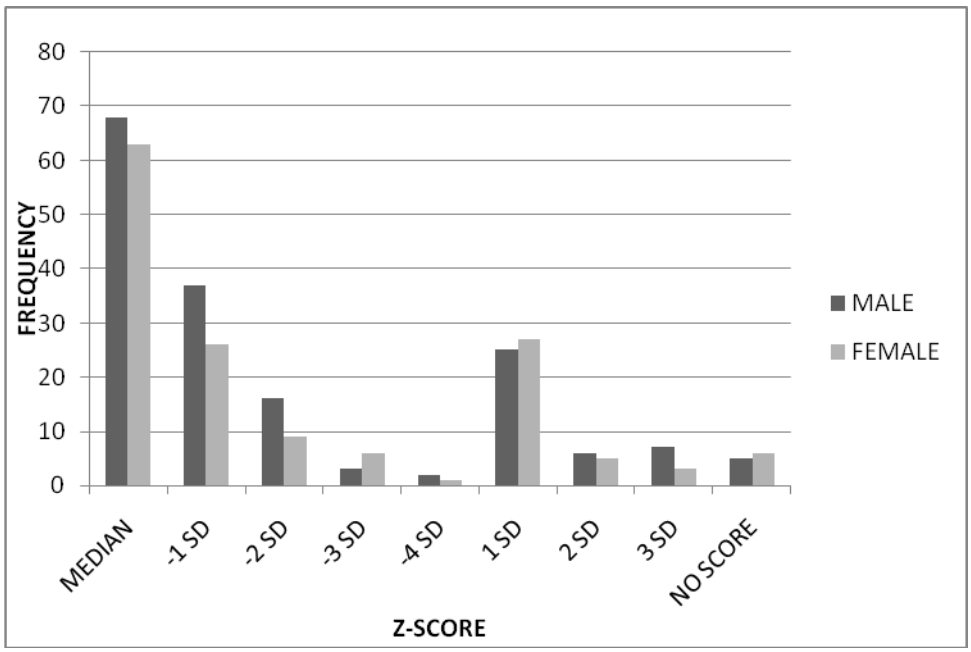

Figure 4.1: Z-Score ranges of the study population

A total of 120 (40.3%) children were vaccinated against Rota virus using different vaccines. Fifty nine (19.8%) were vaccinated with Rotarix®, twenty five (8.4%) were vaccinated with Rotateq® and thirty six (12.1 %) vaccine type unknown. One hundred and seventy six children (59.1%) were not vaccinated against Rotavirus while vaccination status of two children (0.6%) was unknown as indicated in Table 4-2.

Table 4.2: Vaccination status of study population

Children(patients)	Frequency	Percentage rate
Vaccinated		
Rotarix	59	19.8%
Rotateq	25	8.4%
Type of vaccine unknown	36	12.1%
Not vaccinated	176	59.1%
vaccination status unknown	2	0.6%
Total	**298**	**100%**

4.2 Clinical presentation of the study population

Fifty six percent (56%) of the study population was presented with fever (temperatures above 37.7degrees centigrade).

Majority of the children presented to the hospital seventy four percent (74.2%) had vomiting episodes of less than thrice and 76.8% had less than 3 bouts of diarrhea per day (Table 4-3) while 81.2% experienced vomiting for at least 3 days and 70.5% had diarrhea that lasted for 3 days (Table 4-4).

About eighty five percent (84.9%) of the children presented for treatment had some dehydration, 13.1% were not dehydrated and only 2% (six) patients were severely dehydrated. Twenty five (8.4%) were rehydrated orally and discharged while the remaining majority two hundred and seventy three (91.6 %) were admitted to the wards and rehydrated intravenously.

Table 4.3: Frequencies of vomiting and diarrhoea per day

Frequency of symptom per day	< or equal to 3	4< or equal to 7	>7
Vomiting	74.2%(221)	19.1%(57)	6.7%(20)
Diarrhea	76.8%(229)	21.5%(68)	1.7%(5)

Table 4.4: Duration of vomiting and diarrhoea

Duration of symptom	< or equal to 3days	>3 days <5days	>5 days
Vomiting	81.2% (242)	14.1% (42)	4.7%(14)
Diarrhea	70.5% (210)	14.1% (42)	15.4%(46)

During the period of the study no child died out of rotavirus infection. One hundred and seventy two (57.7%) were admitted for a short time (less than 3days) while one hundred (33.6%) were admitted for long (equal to or more than 4 days).

4.3 Prevalence of Rotavirus Infections

Among the 331 participants who were enrolled into the study only 298 stool specimens were examined in the final analysis. These were processed and screened for rotavirus antigen using ELISA. Overall, 94 of the 298 had detectable rotavirus antigen, representing a prevalence rate of 31.5%. About ninety four percent (93.6%) of the rotavirus infected patients were admitted into the wards while 6.4% of the infected children were rehydrated and discharged after observation. Of the rotavirus infected, 54.3% had a short stay (less than 3 days), 39.4% stayed for long (more than 4 days).

Rotavirus infection was most common in children below 2 years of age. Among those infected, males were 46 (48.9%) while the females were 48 (51.1%). Fifty seven (60.1%) were still breastfeeding while the majority approximately seventy two (72. 3%) had been exclusively breastfed for between 4- 6 months. (Table 4-5)

Table 4.5: Ratio of rotavirus infection in relation to patients' demography information

Characteristics	Rotavirus positive cases n =94 (%)	X^2	df	P value
Age in months				
0-12 months	35 (37.2)			
13-24 months	37 (39.4)			
25-36 months	06 (6.4)	13.585	8	0.093
37-48 months	08(8.5)			
49-60 months	08(8.5)			
Breastfeeding				
Yes	57 (60.6)			
Not	37 (39.4)	0.070	2	0.966
Exclusive breastfeeding				
0-3 months	21 (22.3)			
4-6 months	68 (72.3)			
7-9 months	4 (4.3)	2.698	8	0.952
Gender				
male	46 (48.9)	5.270	2	0.072
Female	48 (51.1)			

Among the rotavirus positive cases, 33 (35.1%) had been vaccinated using either of the rotavirus vaccines available as indicated in Table 4-6.

Table 4.6: The prevalence of rotavirus infection in vaccinated children

Vaccination records	Positive rotavirus antigen	Negative rota virus antigen
	n = 94 (%)	**n = 204 (%)**
Rotarix	21 (22.3)	38 (18.6)
Rotateq	4 (4.3)	21 (10.3)
Not vaccinated	61 (64.9)	115 (56.4)
vaccine type unknown	8 (8.5)	30 (14.7)

4.4 Clinical characteristics of rotavirus infection among vaccinated children

The major symptoms of rotavirus gastroenteritis in children include watery diarrhea, vomiting, respiratory symptoms, and fever. In this study among the vaccinated infected children 26 (78.8%) experienced diarrhoea for less than 3 days and only 2 had diarrhoea for more than 5 days while 25 (75.8%) had episodes of diarrhoea of equals to or less than 3 and 8 (24.2%) experienced more than 4 and less than 7 episodes of diarrhoea. Episodes of vomiting equal to or less than 3 was recorded in 23 (69.7%), 6 (18.2 %) experienced episodes more than 4 and equal to 7 while 4 (12.1%) had episodes of more than 7 among the vaccinated infected group. The duration of vomiting among the vaccinated was recorded in 25 (75.8%) for less than 3 days, 7 (21.2%) for more than 3 days but less than 5 days and only 1 (3%) had vomiting for more than 5 days. Of the vaccinated children 29 ((87.9%) had some dehydration, none were severely dehydrated and 4 (12.1%) were not dehydrated. Majority 29 (87.9) had to be rehydrated intravenously. Most of these 18 (54.5%) had a short stay of less than 3 days while 12 (36.4%) had a long stay of more than 4 days and 3 (9.1%) were not admitted. (Table 4-7)

Notably, from the results of this study although children vaccinated also had acute gastroenteritis the duration and severity of the infection was lower than that in children not vaccinated as shown in table 4-7.

Table 4.7: Clinical symptoms and treatment of vaccinated and non-vaccinated children infected by rotavirus

Clinical symptoms/treatment	**vaccinated**	**Not vaccinated**
	n = 33	**n = 61**
Diarrhoea		
Duration of diarrhoea		
Equal to or < 3days	26	41
>3<5 days	5	8
>5days	2	12
Frequency of diarrhea		
Equal to or < 3 days	25	43
4< or equal to 7	8	18
Vomiting		
Frequency of vomiting		
Equal to or < 3 days	25	49
>3<5 days	7	9
>5 days	1	3
Frequency of vomiting		
< or equal to 3	23	38
4 < or equal to 7	6	17
>7	4	6
Hospital stay		
Short " <3days"	21	36
Long " equal to or > 4 days"	12	25
Dehydration		
No dehydration	4	3

Some dehydration	29	54
Severe dehydration	0	4
Treatment		
Oral rehydration	2	3
Intravenous rehydration	31	58

4.5 Molecular characteristics of rotavirus

4.5.1 Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

In this study, bands characteristic of rotavirus double-stranded RNA were detected by PAGE in 57 (61.0%) of the 94 samples in which the presence of rotavirus had been demonstrated by ELISA. All of the visible electropherotypes exhibited the characteristic 4-2-3-2 distribution pattern of the 11 segments of dsRNA for group A rotaviruses. Strains isolated in this study displayed great genetic diversity, with 38 (40.4%) being long and 19 (20.2%) being short electropherotypes.

A basic variance in RNA profiles among the electropherotypes is the separate migration of all 11 RNA segments or co-migration of certain RNA segments (Figure 4-2). The variation was more common among the long electropherotypes whereas all short electropherotypes had the latter profile with co-migration of segments 2 and 3. Besides this basic difference, there were no other distinctive differences among different electropherotypes.

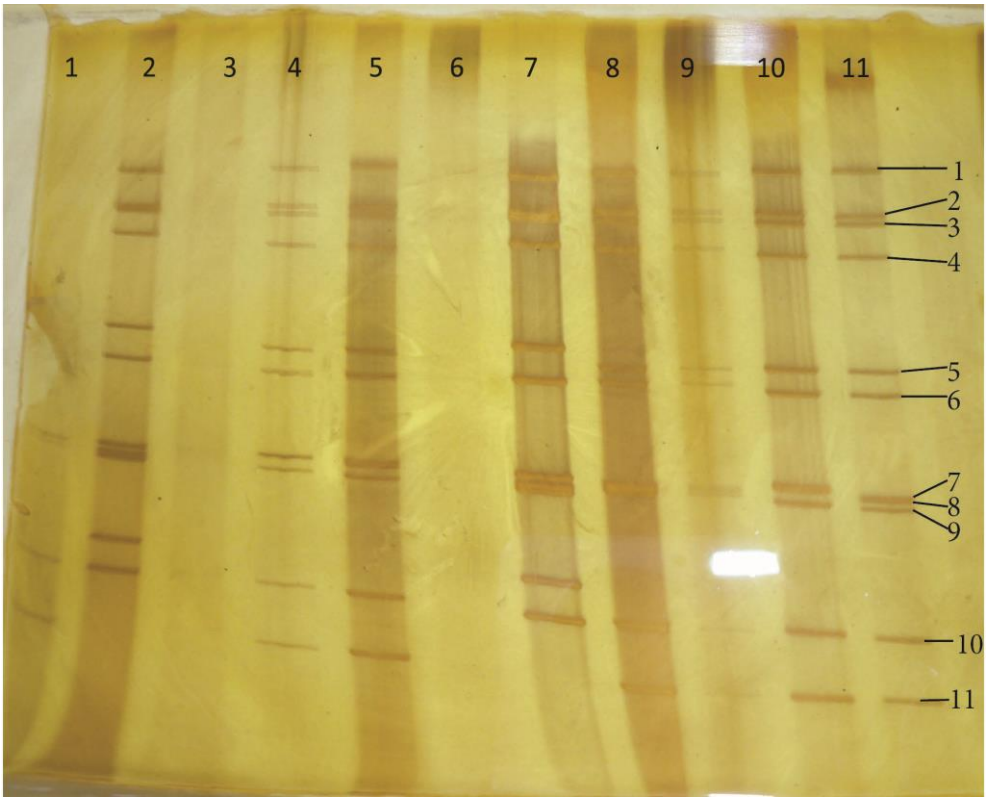

Figure 4.2: Pattern variability of rotavirus electropherotypes on 10% polyacrylamide gel

This was determined for some of 95 ELISA-positive samples from children who participated in the study. Lane 2 and 7 Short chain electropherotype; Lanes 1, 4, 5, 8, 10&11: Long chain electropherotypes, lane 3and 6 had no visible bands. The numbers on the left along the bands represent the segments.

In long electropherotypes, segments 2 and 3 of the large RNA segments 1-4 varied the most in relative migration positions that ranged from far apart to together. In contrast, segments l and 4 had basically the same mobility, or position in the gel. The mobility of segments 5 and 6 was essentially similar in all long electropherotypes. The closely running triplet of segments 7, 8 and 9 had the most number of variable patterns: the different positions of segment 8 relative to 7 produced migration patterns in which segment 8 was located equidistant to segments 7 and 9 or

progressively closer to segment 7. Segments 10 and 11 occupied rather similar positions in all electropherotypes.

Among short electropherotypes, the mobility of RNA segments 1 to 4 was similar in all the electropherotypes. Segments 5 and 6 had 2 different migration patterns. The migration patterns of the triplet segments 7, 8 and 9, and the small segments 10 and l1 were different in all the electropherotypes. In some stool samples more than 11 RNA segments were found, suggesting the possibility of simultaneous infection by more than one electropherotype and/or the occurrence of modification in the length of RNA segments during infection

Table 4.8: Association of some electropherotypes and genotypes

Genotype	Electropherotypes (Long/ Short)	Frequency
G1	L	**1**
P [6]	L	**1**
P [4]	S	**2**
G3P[4]	S	**5**
G9P[6]	L	**1**
G9	L	**4**
G9/3P[4]	L	**4**
G9/3P[6]	L	**1**
G9/3P[NT]	L	**7**
G12P[6]	L	**2**
G1/3P[4]	S	**1**

Notably no specific genotype is associated with an electropherotype and that most circulating rotavirus strains are long. Table 4.8 shows some of the genotypes isolated and the electropherotypes assigned.

4.5.2 Viral protein 7 Genotyping

Eighty three (83) which had adequate RNA extracted were subjected to genotyping using reverse transcription (RT-PCR). VP7 genotype was determined in 44 (53%), 33 (39.8%) non-typeable and 2 (2.4%) produced non-specific products from the existing primer set. The strains demonstrated were G1, G3, G9 and G12. The predominant G genotype observed was G3 (12 [27.3%] of the 44 samples), followed by G9 (11[25%]), G12 (4[4.3%]) and G1 (3 [6.8%]) respectively. Mixed infections

of G9/3, G3/9, and G1/3 genotype specificity were observed at (13 [29.5%]) as demonstrated in figure 4-3.

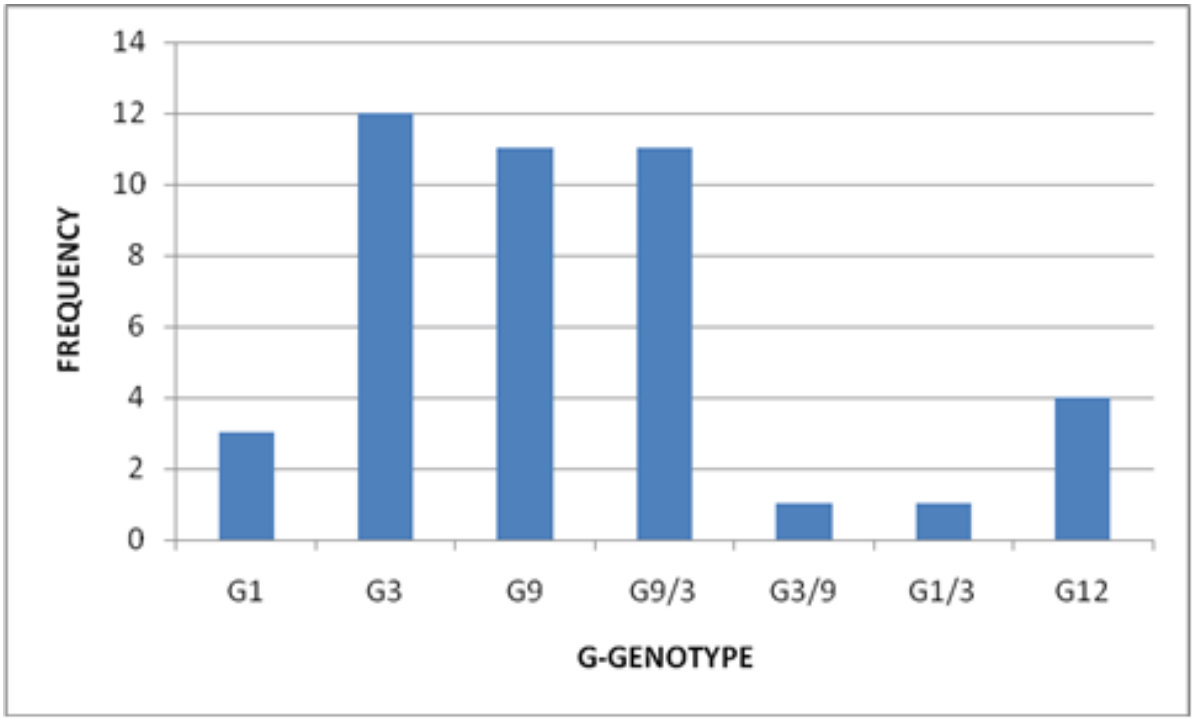

Figure 4.3: Frequency of rotavirus G genotypes isolated.

The distribution of rotavirus G genotypes determined by multiplexed semi-nested RT-PCR for the 83 ELISA-positive samples from children attending Gertrude's Children Hospital in Nairobi District, Kenya, 2012.

4.5.3 Viral protein 4 Genotyping

The frequency of rotaviruses P genotypes isolated during this study (Figure 4-4). Out of the 83 specimens analyzed from rotavirus positive fecal specimen genotyped by RT-PCR, 27 (32.5%) had VP4 genotypes assigned, 47 (56.6%) non-typeable (PNT) while 5 (6%) had non-specific products using the existing primer set. 2 P-types P [4]

and P [6] were observed. The predominant P-type was P [4] at 16 (59.3% of 27 samples with genotype specificity) while P [6] were 11 (40.7%).

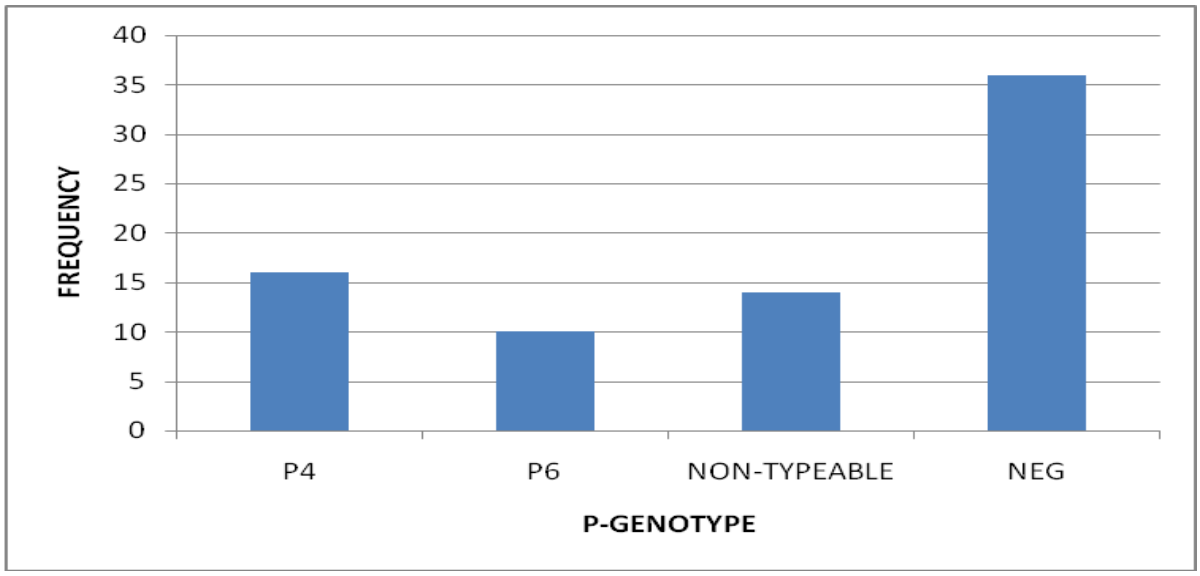

Figure 4.4: Frequency of rotavirus P genotypes isolated.

This is the frequency of rotavirus P genotypes determined by multiplexed semi-nested RT-PCR for the 83 ELISA-positive specimens that could be genotyped from children attending Gertrude's Children's Hospital Nairobi.

4.5.4 Distribution of rotavirus genotypes

The study indicates the most predominant rotavirus strains were G3P [4] (8.5%) followed by G12P [6] (4.3%), G9P [6] (2.1%) and G3P [6] (1.1%). The strains detected as mixed strains included G9/3P [4] (4.3%), G9/3P [6] (1.1%) and G1/3P [4] (1.1%). Combination G and P genotype specificities were possible for only 22 rotavirus positive strains. Others which were only partially G or P were as follows: G9P [NT] (9.6%), G9/3P [NT] (6.4%), G3P [NT] (3.2%), G1P [NT] (3.2 %), GNTP [4] (2.1%), and GNTP [6] (2.1%) (Table 4-9).

Table 4.9: Distribution of rotavirus genotypes circulating January 2012 to June 2012 among rotavirus positive and children

Strains	Rotavirus positive n =94 (%)	Unvaccinated n= 176	Vaccinated n = 120	Rotarix n =59	Rotateq n =25
G1P [NT]	3 (3.2)	3	0	0	0
G3P[4]	8 (8.5)	6	2	1	1
G3P [6]	1 (1.1)	0	1	1	0
G3P [NT]	3 (3.2)	2	1	1	0
G9P [6]	2 (2.1)	1	1	0	0
G9P [NT]	9 (9.6)	6	3	1	0
G9/3P [4]	5(5.3)	3	2	0	0
G9/3P [6]	1 (1.1)	0	1	0	1
G9/3P [NT]	6 (6.4)	3	3	2	1
G1/3P [4]	1 (1.1)	0	1	1	0
GNTP [4]	2 (2.1)	2	0	0	0
GNTP [6]	1(1.1)	0	1	0	0
GNTP [NT]	29 (30.9)	22	7	3	2
G12P [6]	4 (4.3)	2	2	2	0
Not genotyped	19 (20.2)	10	9	9	0

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

This study was undertaken to evaluate the diversity of rotavirus (RV) genotypes and prevalence of RV infection after the commencement vaccine use in Kenya around 2007.

Rotavirus was detected in overall 94 (31.5%) of the children presenting with gastroenteritis with prevalence of 10.7% (32) in children vaccinated against rotavirus using either of the vaccines in the market. The results from this study reveal an overall prevalence of rotavirus infection within the estimated ranges from previous studies. A study conducted from 1991 to 1994 demonstrated a prevalence of 22.5% (Nakata et al., 1999) while a review of rotavirus research in children with diarrhea conducted in Kenya between 1975 and 2005 revealed rotavirus prevalence ranging from 11% to 56.5% in children less than 5 years and 6% in neonates (Kiulia et al., 2008). Another study conducted at the Kenyatta National Teaching and Referral Hospital in children revealed a prevalence of 59% (Gatinu et al., 2007). A study conducted by Kiulia et al, between 2009 and 2011demonstrated a prevalence of 37.8% (Kiulia et al 2014) yet another study carried out in selected hospitals from Kiambu County in Kenya demonstrated a prevalence rate of 36.6 % (Apondi et, al., 2012). In Kenya rotavirus related diarrhea occurs throughout the year (Urasawa et al., 1987), but seasonal peaks are observed during the dry seasons (January–March and June–September) (Mutanda et al., 1984; Makino et al., 1983). This study was carried out between January to July 2012 during one of seasonal peak of rotavirus infection and therefore explains the high prevalence rate reported in this study. A study by Jain, with the introduction of rotavirus vaccine although morbidity and mortality reduce the prevalence remains the same (Jain et al., 2014). There may however, be variations in prevalence and viral strains exhibited from geographical

setting to another in distribution. While breast-feeding protects against all cause diarrhea in infants, no evidence shows that breastfeeding confers specific protection against viral gastrointestinal infection (Kramer et al., 2001). This study also found no association between breastfeeding and rotavirus infection (p=0.966). Among the children infected 68 (72.3%) were exclusively breastfed for between 4 and 6 months. Several studies have demonstrated that breastfeeding did not prevent acquisition of rotavirus (Heinig, 2001; Misra et al., 2007). However, a beneficial effect of breast-feeding against rotavirus infection has been reported in some studies (Al-Wardi et al., 1990) but the degrees of protection offered against rotavirus infection vary in different populations (Weinberg et al., 1984).

This study documents the genetic diversity of group A rotaviruses associated with severe acute gastroenteritis in children less than 5 years of age from a hospital where 120 (40.3%) of the study participants were vaccinated against rotavirus. Notably most children are currently vaccinated using Rotarix®. This could be because Rotarix® is currently cheaper than Rotateq® in the Kenyan market and parents pay for the rotavirus vaccine. Of all the children who presented with gastroenteritis those vaccinated using Rotarix® were 59 (19.8%) and out of which 21(35.6%) were infected with rotavirus genotypes G3P [4], G3P [6], G12P [6] and G1/3P [4] among the fully characterized strains while those vaccinated with Rotateq® were 25 and out of these 4(16%) were infected with rotavirus genotypes G3P [4] and G9/3P [6]. A study done in Bangladesh found some differences in the antigenic epitopes of the currently circulating homotypic G1, G2 and P [8] genotypes with the vaccine strains (Afrad et al., 2013). This variation could be due to the fact that the original vaccine strains were isolated quite a long time ago and the circulating strains in the meantime have changed through their natural evolution (Afrad et al., 2013). The current rotavirus vaccines are based on the strains that were isolated in the 1980s. A study done by Jin and colleagues showed that the tendency of continuing accumulation of point mutations in the antigenic sites of the outer capsid proteins greatly increased the possibility of escaping host immunity conferred by the vaccine virus (Jin et al 1996). However, clinical trials failed to confer adequate efficacy (<60%) in low

income countries such as India, Vietnam and Bangladesh (Narang et al., 2009; Zaman et al., 2010; Taneja and Malik, 2012). A key question that remains is why rotavirus vaccines did not provide sufficient protection in low income countries. One of the reasons could be the strain diversity and antigenic variations of rotavirus strains compared to the vaccine strains. The implication of such mixed infection is that reassortant strains are likely to emerge following the simultaneous infection of an individual with different isolates (Steele et al., 1988).The absence of the bands in some ELISA-positive specimens could be due to insufficient RNA concentration (herring et al., 1982) or its destruction during extraction by phenol/chloroform (Pennap et al., 2000). This study reported 38.9% (37/95) of ELISA positive specimens gave invisible profiles.This study found 3 single G-genotypes were G1, G3 and G9 and 2 single P-genotypes P [4] and P [6]. G3 and G1 genotypes are found in the Rotateq® vaccine combination while for Rotarix® has G1P [8] genotype which was not isolated in the current study. The prevalence of rotavirus G and P types have been monitored worldwide and the most common G types are G1, G2, G3, G4 and G9 (Arguelles et al, 2000; Estes, 2010; Van Man et al, 2005). A study done in India in a hospital setting in 2010 found five different G genotypes G1, G2, G3, G9 and G12 (Chakravarti et al.,2010). This relates with the current results which demonstrated three different G genotypes except for genotype G2 and G12.

This study reported mixed infection where 29.5% of the children were infected with multiple strains of the rotavirus at the same time. It is suspected that during mixed infections there is a possibility of reassortment of strains resulting into typical or novel strains.

This is a common occurrence as was reported in Brazil where 11%-30% of children had mixed rotavirus infections (Leite et al.,1996). G3/9 and G1/3 were the G genotypes that were mixed. Notably, all the mixed infections involved G3 with another G genotype in this study. Worldwide genetic diversity of circulating rotavirus strains is associated with the presentation of new emerging strains, causing variability in the geographical distribution of the virus (Najafi et al., 2012). The

proportion of mixed infections (12.8%) reported in this study is substantially lower than what was reported in Guinea-Bissau (59%), (Fischer et al.,2003) and Iran (60%)(Kargar et al .,2012) and slightly lower than other studies conducted in Ireland, Indonesia, Africa, and India, with the prevalence of 28.5%, 23%, 21.4% and 21%, respectively (Reidy et al., 2005; Radji et al.,2010; Esona et al.,2010; Jain et al.,2001). The current study documented rare genotype mix of G1/3 and G9/3 mixed infection in Kenya. This G type has been reported in young children with gastroenteritis in Mexico and Iran (Rodriguez et al., 2000; Najafi et al., 2012). The high prevalence of mixed infections may reflect the observed diversity of strains. This great diversity of genomic variants in the same locality, as indicated by the large number of electropherotypes described, may reflect the potential for active genetic re-assortment between different viral strains. Patients harbouring multiple rotaviruses may offer a unique environment for the re-assortment process, facilitating generation of novel rotavirus strains and the maintenance of the observed diversity (Jain et al., 2001). The high mixed infection with different rotavirus strains may be explained by greater environmental contamination with rotavirus, for example water resources, coupled with greater contact between children and the environment around them (Jain et al.,2001) Therefore, the frequency of mixed infection of rotaviruses and its effect on the development of rotavirus vaccine should be thoroughly investigated.

This study demonstrated six genotype combinations G3P [4], G3 [6], G9P [6], G12P [6], G9/3P [6] and G9/3P [4]. Interestingly, none of the specimens processed demonstrated genotype P [8]. According to these results genotype P [8] seems to be controlled well given that it is included in both vaccines available. G12P [6] was isolated in a recent study in India in two cases (Chakravarti et al., 2010). An emerging G genotype since its first identification in the Philippines in 1990 (Taniguchi et al, 1990), it has spread worldwide (Kolkata et al., 2003; Kang et al, 2005; Pietruchinski et al, 2006). Epidemiological data has shown the occurrence of diverse rotavirus strains in Africa, including the common G1 type to the less common G8, G9, G10 and G12 types (Steele et al., 2012). In Malawi, G12 was the

predominant circulating strain noted during the study by Steele and in 2009 it accounted for 5% (Nigel et al., 2009; Steele et al., 2012). The G12 was also reported in Nigeria accounting for 2% of the rotavirus strains identified by Ayolabi et al., 2013). G12 was in association with P [6] genotype and was found in young African children with symptomatic rotavirus infection. In the current study all the four G12 (4.3%) were in found to be in association with P [6] forming G12P [6]. This finding agrees with other early studies that have reported the occurrence of this combination G12P [6] and G12P [4], isolated for the first time in Bangladesh (Paul et al 2010). Hospital based studies conducted between 2005 and 2009 in India, showed an increase in the incidence of G12P [6] infections from 8% to 39% in Northern India and from 8% to 34% in Western India.

Genotype G4P [6] is considered to be rare globally, P[6] strains combined with G1, G2, G3, or G4 have been reported as a cause of outbreaks of nosocomial rotavirus infection in hospitals and newborn nurseries [Steele et al., 1995; Kilgore et al., 1996; Lee et al., 2001;Linhares et al.,2002]. The implication is that the prevalence of formerly common G/P combinations is falling in Kenya while that of uncommon G/P combinations is on the increase. It is notable the demonstration of rare combinations in this study that include G3P[4] (n=8) ,G3P[6](n=1) ,G9P[6](n=2), and G12P[6] (n=4). In addition, co-infections with two different G types and a single P type G9/3P [4], G9/3P [6] (n=1) and G1/3P [4] (n=1) were detected. In addition, 22 samples (46 %) were negative upon full-length VP4 gene amplification but positive with VP7 gene-specific primers. Further investigation of these findings is needed to confirm whether the rotavirus is undergoing genetic diversification. The data from this study demonstrate that in Nairobi , Kenya there is a high level of diversity among G-type and P type rotavirus, with prevalence of well-established or newly, emerging genotypes such as G3P [4], G3P [6], G9P [6] and G12P [6]. More surveillance studies are vital to increase the data available on the molecular epidemiology of unusual genotypes. However combination G3P [6] and G3P [4] have been reported in an earlier study and in a more recent study done in Ghana and were found to be predominant in both the studies (Binka et al.,2003; Enweronu-

Laryea et al.,2013). Similar reports have indicated rotaviruses undergo constant genetic variation via point mutations by antigenic drift, genetic reassortment or antigenic shift, genomic rearrangement or intragenic recombination, resulting in the emergence of new strains (Santos and Hoshino, 2005). However, unusual rotavirus P-G combinations may result from mixed rotavirus infections. Cyclic strain variations have been known to occur every 3-5 years, wherein the rotavirus strain predominance patterns undergo variations (Than et al., 2013). Together with the re-assortments that occur from animal strains, there could be a wide variability in the rotavirus types that cause infections in children (Nayak, 2014).

The great diversity and re-emerging rotavirus strains observed in this study especially in vaccinated children raises concerns about whether the vaccine strains G1P [8], G1, G2, G3 and G4 would evoke sufficient heterotypic protection against other strains not present in the vaccine. Cross protection with natural infection and vaccine-induced immunity has been reported (Vesikari *et al*., 2006; Madhi *et al.,* 2010; Richardson *et al*., 2010). However, as rotavirus vaccination is introduced into the routine immunization program it is important to closely monitor vaccine performance against heterotypic strains in Kenya.

5.2 Conclusions

From this study children aged less than 2 years recorded highest prevalence of Rotavirus gastroenteritis despite vaccination before the children are 6months of age. The clinical characteristics ranged from only a few days of diarrhea and vomiting, some dehydration and short stay in the hospital for intravenous rehydration. Six single genotypes were demonstrated G1, G3, G9, G12, P [4], and P [6] while genotypes G3P [4], G3P [6], G9P [6], G12P [6], were found to be combined. Mixed infections observed included G9/3P [4], G1/3P [4] and G3/9P [6]. Rotavirus strains isolated from children vaccinated included G3P [4], G3P [6], G1/3P [4], G12P [6], G9, G9/3 for those who had been vaccinated using Rotarix® while G3P [4], G9/3P [6] and G9/3 for those vaccinated using Rotateq®. Notably emerging strains of G12

and G9 were found among these children infected with Rotavirus. Vaccine strain of P [8] was not isolated.

In this study, prevalence of rotavirus infection remains high in spite of vaccination. Notably, vaccine uptake is still very low. Severity of the rotavirus associated gastroenteritis is reduced with a reduction in hospital stay. The great diversity with presence of re-emerging rotavirus strains observed in this study especially in vaccinated children raises concerns as to whether the vaccine strains G1P [8], G1, G2, G3 and G4 would evoke sufficient heterotypic protection against other strains not present in the vaccines. This emphasizes the need for long-term rotavirus surveillance in order to establish the effectiveness of vaccines. The findings of high prevalence of G9 and emergence of G12 strains in Kenya may have implications in the assessment of the efficacy of new rotavirus vaccines.

The limitation of this study was 33 G genotypes and 41 P genotypes were non-typeable from specimens that were positive for rotavirus antigens. A possible reason behind the failure of the semi nested multiplex RT-PCR to genotype could be the presence of a mutation at primer binding sites leading to mismatch of the primer-binding site. With the ever-changing and diversity of rotavirus a close monitoring and updating of oligonucleotide primers is recommended in order to minimize the percentage of non-typeable strains found during rotavirus surveillance studies .

5.3 Recommendations

The diversity of rotavirus strains and the high prevalence of mixed infections in Kenya are unique features of rotavirus epidemiology in Kenya. The ever-changing epidemiology of rotavirus may represent a substantial challenge to the development of effective vaccines. This emphasizes the need for long-term rotavirus surveillance to judge effectiveness of vaccines. Therefore we recommend that vaccines should be formulated with a broad range of strains for better protection including G9 and G12 genotype strains.

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APPENDICES

Appendix I: Publication

PanAfrican Medical Journal

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Research

Prevalence and genetic diversity of rotavirus infection in children with acute gastroenteritis in a hospital setting, Nairobi Kenya in post vaccination era: a cross-sectional study

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Abstract

Introduction: Rotavirus is the leading cause of severe diarrhoea among infants and young children. Each year more than 611 000 children die from rotavirus gastroenteritis, and two million are hospitalized, worldwide. In Kenya, the impact of recent rotavirus vaccinations on morbidities has not been estimated. The study aimed at determining the prevalence and identity of rotavirus strains isolated from rotavirus-associated diarrhoea in vaccinated children presenting with acute gastroenteritis. **Methods:** Two hundred and ninety eight specimen from children presented at Gertrude Childrens' Hospital from January to June 2012 were tested by EIA (Enzyme-linked Immunosorbent Assay) for rotavirus antigens. Molecular characterization was conducted on rotavirus-positive specimens. Extracted viral RNA was separated by polyacrylamide gel electrophoresis (PAGE) and the specific rotavirus VP4 (P-types) and VP7 (G-types) determined. **Results:** The prevalence rate of rotavirus was 31.5% (94/298). Of the rotavirus dsRNA, 57 (60.1%) gave visible RNA profiles, 38 (40.4%) assigned long electropherotypes while 19 (20.2%) were short electropherotypes. The strains among the vaccinated were G3P [4], G12P [6], G3P [6], G9P [4], G mixed G9/3P [4] and G1/3P [4]. Specifically, the G genotypes were G9/3 (5.3%), G9 (4.3%), G3 (4.3%), G12 (2.1%) and mixed G1/3 (1.1%). The P genotypes detected were P [4] (5.3%) and P [6] (5.3%). **Conclusion:** The present study demonstrates diversity in circulating genotypes with emergence of genotypes G3, G9, G12 and mixed genotypes G9/3 and recommends that vaccines should be formulated with a broad range of strains to include G9 and G12.

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Introduction

Diarrhea is a leading killer of children in Kenya, causing approximately 9 percent of deaths in children less than five years of age [1]. It is estimated that 27% of all under five diarrheal disease hospitalization in Kenya is caused by rotavirus infection [2]. Current global estimates of mortality attributed to rotavirus-associated disease is 611,000 in children less than 5 years old [3] and mortality figures for sub-Saharan Africa total 145,000 annually [4]. Studies in Nigeria [5], Tunisia [6] and Kenya [7] have demonstrated that close to 90% of all children are infected with rotavirus by 2 years of age. In Kenya, the peak age of contracting gastroenteritis in children is 6- 24 months [8-10]. It is estimated that, in Kenya, 68 deaths, 132 hospitalizations, and 21,800 clinic visits per 100,000 children aged less than 5 years annually are attributable to rotavirus diarrhoea [11]. Rotavirus belongs to the family Reoviridae, non-enveloped with a triple-layered icosahedral protein capsid and a genome of 11 double stranded RNA segments [12]. Rotavirus is the most common etiological agent associated with severe gastroenteritis leading to dehydration and death in young infants' worldwide [3]. Rotaviruses are classified into groups, subgroups, serotypes and on the basis of electrophoretic migration of gene segments. The group and subgroup specificity are present on the inner capsid VP6 . Thus, currently only rotavirus groups A, B and C have been identified as human and animal pathogens, while groups D, E, F, and G have only been identified in animals and birds [13-15]. Majority of rotavirus diarhoea infections are caused by group A rotaviruses [16]. Group B rotavirus infection are uncommon but have recently been associated with outbreaks in China and India [17]. Group C rotavirus has been isolated in Kenyan children [18]. The genes encoding the outer capsid VP7 and VP4 form the basis of classification of group A rotaviruses into G and P genotypes, respectively [19]. There are twenty P genotypes with P [4], P [6], and P [8] most frequently associated with human infections. Further, there are 14 G genotypes with G-types 1, 2, 3 and 4, accounting for 80% of human infections. G8 and G9 are emerging as common genotypes with G9 being the most common in Kenya [20]. There are currently two orally administered rotavirus vaccines; Rotarix® (GlaxoSmithKline, Rixensart, Belgium) and Rotateq® (Merck &Co., Whitehouse Station, NJ), which have been available in Kenyan hospitals since 2007. Rotateq® vaccine contains five human-bovine reassortant viruses [W179-9 (G1P [5], SC-2 (G2P [5]), W178-8 (G3P [5]), BrB-9 (G4P [5]) and W179-4 (G6P [8])]. These viruses were generated by crossing the naturally attenuated bovine rotavirus strain WC3 with five unique human rotaviruses each contributing a G1, G2, G3, or G4 VP7 or P[8] VP4 gene to one of the vaccine viruses [21]. Rotarix® vaccine is a human G1P [8] virus RIX4414 which was derived by serial passage in cell culture of a virus recovered from the stool of an infected child [22]. Human rotavirus vaccine has been shown to reduce hospitalizations as a result of gastroenteritis from any cause by up to 42% [22]. The present study was aimed at estimating prevalence of rotavirus gastroenteritis and the genetic diversity of rotavirus among the vaccinated children at the Gertrude's Children Hospital, Nairobi, Kenya.

Methods

Study setting and population

This study was conducted at the Gertrude's Children's Hospital (GCH) between January and July 2012. The GCH is a leading Pediatric Hospital in Kenya which offers a variety of healthcare services to infants, school-age children, and teenagers. The laboratory work was undertaken at the rotavirus laboratory in the Centre for Virus Research (CVR) of the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. The study population comprised children presenting with gastroenteritis and attending the Gertrude's Children's Hospital. All children aged less than 5 years presenting with gastroenteritis at the hospital's outpatient department, or during their first 48 hours of hospitalization and whose parents/guardians consented to the study, were included in this study. Those children who had developed gastroenteritis 48 hours after admission, or those with bloody stool with gastroenteritis and those whose parents/guardians did not give consent, were excluded from the study.

Ethical clearance

Ethical clearance for this study was obtained from the Scientific and Ethical Review Committees of the Kenya Medical Research Institute (KEMRI). Authorization to conduct the study was obtained from the Gertrude's Children Hospital (GCH).

esearch instruments

A structured questionnaire was used to collect the patients' clinical and demographic data, both from the hospital records and from the consenting parents/guardians.

Study design and sample size determination

This was a hospital based cross-sectional study. The required sample size was calculated using Fischer et al (1998) formula. The sample size was calculated based on a previous prevalence study of rotavirus gastroenteritis in an urban hospital in Nairobi, which had been established to be 28.4% [**23**]. To detect this with a precision of 5% and confidence level at 95%, at least 313 patients was required. Only 298 had adequate sample for analysis.

Specimen collection and processing

Stool specimen was collected in a sterile polypot from a patient presenting with symptoms of gastroenteritis. They were stored in a -20°C freezer then transported in cool boxes to the processing laboratory at the Kenya Medical Research Institute (KEMRI), Nairobi. All procedures for working with biosafety level 2 (BSL2) agents such as rotavirus were observed as indicated in the WHO biosafety manual. All biohazardous materials were disposed off in the KEMRI incinerator. One milliliter of 10% (w/v) feacal suspension was prepared in phosphate-buffered saline (PBS), vortexed and clarified by centrifugation. The clarified supernatant was tested for the presence of group A rotavirus antigen using the commercial rotavirus antigen-detection Enzyme Linked Immunosorbent Assay (ELISA) (ProSpectTM Rotavirus Microplate Assay, Oxoid Ltd, United Kingdom). The procedures for detection were undertaken according to the manufacturer's instructions.

Molecular analysis of ELISA-positive samples

The ELISA-positive samples were analyzed by polyacrylamide gel electrophoresis (PAGE) to identify the presence of rotavirus double stranded RNA. The procedure was as stipulated in the WHO Manual of Rotavirus detection and characterization methods [24]. The rotavirus dsRNA was run on 10% polyacrylamide resolving gels using a large format gel electrophoresis system (Hoefer SE600) and 3% spacer gel was used to enhance resolution of the ds RNA segments. Thirty microlitres (30µl) of each sample was loaded onto gels and electrophoresis was conducted at 100 V for 16-20h at room temperature. The gels were stained using silver staining to group the rotavirus in electropherotypes as described in in the Laboratory Manual developed by the African Rotavirus Workshop in South Africa, 2002 [25]. Reverse transcriptase/polymerase chain reaction (RT-PCR) amplification was performed on the rotavirus ds RNA as described by the South and West African Regional Rotavirus Laboratories. The extracted ds RNA was re-suspended in 20µl of sterile iodized water and stored for use in PCR reactions. Briefly, the dsRNA was denatured by boiling at 94° C for 5 min followed by chilling in ice. The ds RNA was then reverse transcribed by incubating with reverse transcriptase and deoxynucleotides for 30 min at 42° C. The resultant cDNA was amplified in magnesium-dependent PCR. Briefly, for the G typing, a full-length 1062 gene segment 9, encoding for the VP7 glycoprotein was reverse transcribed and amplified by using primers sBeg9 [nucleotide (nt) 1-21, 5'-GGCTTTAAAAGAGAGAATTTC-3'] and End 9 (nt 1062-1036, 5' GGTCACATCATACAATTCTAATCTTAAG-3') followed by genotyping with cocktail of primers specific to six human serotypes G1- G4, G8 and G9 (aBT1, aCT2, aET3, aDT4, aAT8 and AFT9) and the consensus primer RVG 9 as described by [26, 27] shown in Table 1. For VP4 genotyping a full length 876 gene segment 4, encoding for the VP4 was reverse transcribed and amplified by using outer primers Con3 and Con2 as previously described [28] shown in Table 2 followed by genotyping with cocktail of primers specific to the five human P genotype P[4], P[6], P[8], P[9], and P[10] (2T-1, 3T-1, 1T-1, 4T-1, 5T-1) and consensus primer as Gentsch describes. PCR fragments were analyzed on 2% TAE agarose gels at 80-90 volts with appropriate molecular weight marker to determine the genotype of rotavirus strain. PCR bands were compared with molecular weight markers.

Data management and analysis

Data coding and analysis was performed done using the SPSS Version 20.0 software. Pearson's chi-square was used to determine associations. Level of significance was fixed at 0.05 (p=0.05).

Results

Three hundred and thirty one (331) participants who met all the inclusion criteria were recruited. Of these, 298 stool specimens were examined in the final analysis. Ninety four (94) (31.5%) specimens were positive for rotavirus (Table 3). Rotavirus infection was most

common in children below 2 years of age. Among those infected, males were 46 (48.9%) while the females were 48 (51.1%) and 57 (60.1%) were still breastfeeding. Over 60% of the rotavirus cases had z- scores above median showing that they were of good nutritional status with the majority (41.5%) recording a median score (Table 3). Among the rotavirus positive cases, 33 (35.1%) had been vaccinated using either of the rotavirus vaccines available. Of these, 26 (78.8%) experienced diarrhoea for less than 3 days and only 2 had diarrhea for more than 5 days (Table 4). Only 12 among those who were vaccinated stayed long in the hospital (more than 4 days). All 29 who had some dehydration were treated with intravenous fluids. A total of 94 EIA rotavirus-positive stool samples were subjected to PAGE and visible dsRNA migration patterns were obtained in 57 (60.1%) samples. Long electropherotypes were 38 (0.4%) while 19 (20.2%) displayed short patterns. Reverse transcription was done on 83 samples which had adequate RNA extracted. Overall, the results indicated the most predominant rotavirus strains were G3P [4] (8.5%) followed by G12P [6] (4.3%), G9P [6] (2.1%) and G3P [6] (1.1%). Many strains detected as mixed strains included G9/3P [4] (4.3%), G9/3P [6] (1.1%) and G1/3P [4] (1.1%). Others which were only partially G or P were as follows: G9P [NT] (9.6%), G9/3P [NT] (6.4%), G3P [NT] (3.2%), G1P [NT] (3.2), GNTP [4] (2.1%), and GNTP [6] (2.1%) (Table 5).

Discussion

This study was undertaken to evaluate the diversity of rotavirus (RV) genotypes and prevalence of RV infection in vaccinated children. Rotavirus was detected in 31.5% of the children presenting with gastroenteritis attending GCH in Nairobi, Kenya. The results from this study reveal an overall prevalence of rotavirus infection within the estimated ranges from previous studies. A study conducted from 1991 to 1994 demonstrated a prevalence of 22.5% [23] while a review of rotavirus research in children with diarrhea conducted in Kenya between 1975 and 2005 revealed rotavirus prevalence ranging from 11% to 56.2% in children less than 5 years and 6% in neonates [29]. Another study conducted at the Kenyatta National Teaching Hospital in children revealed a prevalence 59% [30]. A study conducted by Kiulia et al, between 2009 and 2011 demonstrated a prevalence of 37.8% and yet another study carried out in selected hospitals from Kiambu County in Kenya demonstrated a prevalence rate of 36.6 % [31]. After the introduction of rotavirus vaccines in the Kenyan market around 2007 after which this study was conducted 39.4% of the RV infected children were between 13 to 24 months in age followed by those less than 12 months at 37.2%. Consistently, most studies indicate that children less than 2 years are most affected by RV infection. This onset of infection correlates well with the decline of maternally acquired antibodies that disappear around 5 months [32]. In the present study gender difference was not significant. This is in agreement with another study looking at risk factors in pediatric diarrhea [33]. Among the rotavirus positive children, fifty four (57.4%) who were not vaccinated had some dehydration as compared to twenty eight (30%) who were vaccinated. A similar percentage among vaccinated and unvaccinated children were treated by intravenous rehydration. In this study among vaccinated children, G9 genotype was most common genotype at 23.4%. This is in agreement with a recent studies in Kenya where prevalence of 13- 15% have been reported [9, 34, 35]. G9 has been recognized as the most widespread of the emerging genotypes. It was first reported in the United States in the early 1980s [36]. Soon after its detection, it disappeared for more than a decade; then re-emerged in the mid-1990s and has been affecting patients to date. Currently, the genotype comprises 4.1% of global rotavirus infections, and accounts for as high as 70% of rotavirus infections as reported by some studies [37]. Genotype G3 was the second most common genotype demonstrated in this study among the vaccinated children at 4.2%. In Kenya, genotype G3 strains were predominant circulating genotypes in the years 1999 and 2000 [29] but further reports on occurrence of this genotype declined. Studies done elsewhere around the world have reported a high occurrence of G3 genotype. A study in Korea and Ghana found G3 to be the second predominant G genotype at 26.4% and 12.7%, respectively [38, 39]. In Tunisia, a study in 2011 found that, G3 was the second most predominant at 25% [40]. Apart from being the predominant single G genotype, the Tunisian study established that it was also found in mixed infection with G9 and G1 at 17.5%. A study in India earlier mentioned detected 3% and 1.9% of diarrheal cases caused by G3 [41, 42]. The genotype G12 was also demonstrated in this study in both the vaccinated and unvaccinated children in equal percentage (4.3%). A study by Kiulia in 2009 to 2011 in Eastern Kenya [34] showed that G12 was found in 3.1% of the samples. Notably, G12 genotypes have been consistently detected in various countries, including in South Africa. [43, 44]. Since its first identification in the Philippines in 1990 [45], it has been reported worldwide [41]. In Malawi, G12 was the predominant circulating strain [46, 47]. G12 was in association with P [6] genotype and was found in young African children with symptomatic rotavirus

ifection. In the current study, all the four G12 were in found to be in association with P [6] forming G12P [6] combination. This finding concurs with other early studies that have reported the occurrence of this combination of G12P [6] and G12P [4], isolated for the first time in Bangladesh [48] Genotype G1 as single and combined with G3 was also detected in this study. Genotype G2 was not identified and has not been documented for over 8 years in Kenya as documented by Kiulia et al [34]. The current study did not document any case of G1 genotype among vaccinated children although numerous molecular epidemiological studies have indicated that G1 is the most common circulating G type around the world [37, 49, 50]. Interestingly none of the specimens processed demonstrated genotype P [8]. According to these results genotype G1 and P [8] seems to be controlled well given that both are included in both vaccines available.

Conclusion

In the current study, prevalence of rotavirus infection remains high in spite of vaccination. Notably, vaccine uptake is still very low. Severity of the rotavirus associated gastroenteritis is reduced with reduced hospital stay. The great diversity and re-emerging rotavirus strains observed in this study especially in vaccinated children raises concerns as to whether the vaccine strains G1P[8] ,G1,G2,G3 and G4 would evoke sufficient heterotypic protection against other strains not present in the vaccine. This emphasizes the need for long-term rotavirus surveillance in order to establish the effectiveness of vaccines. The findings of high prevalence of G9 and the emergence of G12 strains in Kenya may have implications in the assessment of the efficacy of new rotavirus vaccines. Therefore, we recommend that vaccines should be formulated with a broad range of strains for better protection to include G9 and G12. The limitation of this study was that some of the guardians or parents presenting the children to the hospital did not exactly know the vaccine that was used. Further surveillance on use of vaccines and diversity of strains against vaccine used need to be done.

What is known about this topic

- Rotavirus is the most common etiological agent associated with severe gastroenteritis leading to dehydration and death in young infants worldwide (Parashar et al., 2006);
- Close to 90% of all children are infected with rotavirus by 2 years in these settings (Nyangao et al., 2010);
- Safe and effective vaccines are needed, especially in poorer countries where most deaths from the disease occur (Parashar et al., 2009).

What this study adds

- Overall, 94 of the 298 had detectable rotavirus antigen, representing a prevalence rate of 31.5%.
- The study documents the genetic diversity of group A rotaviruses associated with severe acute gastroenteritis in children less than 5 years of age from a hospital where some of the children were already are vaccinated against rotavirus;
- Emerging strains of G12 and G9 were found among these children infected with Rotavirus.

Competing interests

The authors declare no competing interest.

Authors' contributions

MTA conceived the study, drafted the proposal, carried out data collection, laboratory examination, data analysis, interpretation of the results and ultimately finalized write up of the manuscript. JRO, JK and RWK gave technical advice in proposal development, in-process consultation and review of the manuscript. JN gave technical advice on sample testing and analysis, data analysis and review of the manuscript. JK, AAO and AB assisted in review of this paper. All authors have read and agreed to the final version of this manuscript and have equally contributed to its content and to the management of the case.

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Tables

Table 1: Oligonucleotide primers for G serotyping as designed by Gouvea et al., 1990 and Gault et al., 1999

Table 2: Oligonucleotide primers for P genotype PCR typing as designed by Gentsch et al., 1992

Table 3: Characteristics, clinical symptoms and treatment of children <5 years of age hospitalized with rotavirus and non-rotavirus gastroenteritis

Table 4: Clinical symptoms and treatment of vaccinated and non-vaccinated children infected by rotavirus

Table 5: Distribution of rotavirus genotypes circulating January 2012 to June 2012 among rotavirus positive and vaccinated children

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Table 1: Oligonucleotide primers for G serotyping as designed by Gouvea *et al.*, 1990 and Gault *et al.*; 199

Primer	Sequence (5'-3')	Position(nt)	Strain (genotype)
SBeg	GGCTTTAAAAGAGAGAATTTC	1-21	Group A
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Group A
End9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	Group A
EndA	ATAGTATAAAATACTTGCCACCA	922-944	Group A
aAT8	GTCACACCATTTGTAAATTCG	178-198	69M (G8)
aBT1	CAAGTACTCAAATCAATGATGG	314-335	Wa (G1)
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	DS-1 (G2)
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	ST-3 (G4)
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	P (G3)
aFT9	CTAGATGTAACTACAACTAC	757-776	WI61 (G9)
RVG9	GGTCACATCATACAATTCT	1062-1044	Group A

Table 2: Oligonucleotide primers for P genotype PCR typing as designed by Gentsch et al., 1992

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
1T-1	ACTTGGATAACGTGC	339-356	KU [P8]
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5 [P4]
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076 [P6]
4T-1	TGAGACATGCAATTGGAC	385-402	K8 [P9]
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M [P10]
Con3	TGGCTTCGCCATTTTATAGACA	11-32	Group A
Con2	ATTTCGGACCATTTATAACC	868-887	Group A

Table 3: Characteristics, clinical symptoms and treatment of children <5 years of age hospitalized with rotavirus and non-rotavirus gastroenteritis

characteristics	Rotavirus-positive cases	Rotavirus-negative
	n=94(%)	n=204(%)
Sex		
Male	46(48.9)	118(57.8)
Female	48(51.1)	89(43.6)
Age in months		
0-12	35 (37.2)	101(49.5)
13-24	37(39.4)	59(29)
25-36	6(6.4)	23(11.3)
37-48	8(8.5)	16(7.8)
49-60	8(8.5)	8(3.9)
Vaccination status		
Rotarix®	21(22.3)	38(18.6)
Rotateq®	4 (4.3)	21(10.3)
Vaccine used unascertained	8 (8.5)	30(14.7)
Not vaccinated	61(64.9)	115(56.4)
Breast feeding	57(60.1)	119(58.3)
Z-scores		
Median	39 (41.5)	83(40.7)
-1 sd	14(14.9)	46(22.5)
-2 sd	9(9.6)	14(6.9)
-3 sd	1(1.1)	8(3.9)
1 sd	16(17)	33(16.2)
2 sd	4(4.3)	6(2.9)
3 sd	5(5.3	5(2.5)

Table 4: Clinical symptoms and treatment of vaccinated and non-vaccinated children infected by rotavirus

Clinical symptom/ treatment	**vaccinated**	**Not vaccinated**
	n=33	**n=61**
Diarrhoea		
Duration of diarrhoea		
Equal to or < 3 days	26	41
>3<5	5	8
>5 days	2	12
Frequency of diarrhoea		
< or equal to 3	25	43
4< or equal to 7	8	18
Vomiting		
Duration of vomiting		
Equal to or < 3 days	25	49
>3<5	7	9
>5 days	1	3
Frequency		
< or equal to 3	23	38
4< or equal to 7	6	17
>7	4	6
Hospital stay		
Short < 3 days	18	33
Long "equal to or > 4 days	12	25
Dehydration		
No dehydration	4	3
Some dehydration	29	54
Severe dehydration	0	4
Treatment		
Oral rehydration	2	3
Intravenous rehydration	29	57

Table 5: Distribution of rotavirus genotypes circulating January 2012 to June 2012 among rotavirus positive and vaccinated children

Strains	Rotavirus positive n=94 No. (%)	Vaccinated n=132	Rotarix n=64	Rotateq n=27
G1P[NT]	3 (3.2)	0	0	0
G3P[4]	8 (8.5)	2	1	1
G3P[6]	1 (1.1)	1	1	0
G3P[NT]	3 (3.2)	1	1	0
G9P[6]	2 (2.1)	1	0	0
G9P[NT]	9 (9.6)	3	1	0
G9/3P[4]	4 (4.3)	2	0	0
G9/3P[6]	1 (1.1)	1	0	1
G9/3P[NT]	6 (6.4)	3	2	1
G1/3P[4]	1 (1.1)	1	1	0
GNTP[4]	2 (2.1)	0	0	0
GNTP[6]	2 (2.1)	1	0	0
GNTP[NT]	29 (30.9)	7	3	2
G12P[6]	4 (4.3)	2	2	0
Not genotyped	18 (19.1)	107	52	22

Appendix IIA: Informed Consent form

Title of project: Identification of Rotavirus strains among children with gastroenteritis

at the GGCH, Nairobi.

Investigators: Mary-Theresa Agutu, Principal Investigator (ITROMID, JKUAT)

Dr. Juliette R. Ongus, Co-investigator (JKUAT)

Dr. Janeth Kombich, Co-investigator (KEMRI)

Dr. Rose W. Kamenwa, Co-investigator (AKUH)

Introduction

My name is……and I am a student at the Jomo Kenyatta University of Agriculture and Technology. This study intends to determine the different types of rotavirus circulating and also prevalence of the disease. We need to know which

types of this virus cause infection in the children who have been vaccinated. The information we gather is useful to the government and other policy makers who may consider including the vaccines already in use in routine immunization schedule for all babies in future. We will summarize our findings from this study and disseminate it to various stakeholders including Ministry of Health, Ministry of Public Health and Sanitation, KEMRI, WHO-EPI and others. The KEMRI's ethical review committee, who are responsible for conducting such reviews at national level, has approved this study.

Research Procedures: Your child is here to have his or her stool collected because your child's clinician has recommended some tests**.** If you allow your child to participate in this study, we will ask you a few questions regarding the rotavirus vaccine that was used . Then stool will be collected by you. We will give you a sterile polypot which is clean and safe. The stool from your child will be transported to the KEMRI laboratories in Nairobi for analysis for the rotavirus types. In order to ensure complete confidentiality of the test results, no names will be attached to the stool samples, but a number assigned to your child will be used to label the sample.

Benefits: This study will not directly benefit your child. However the study will benefit the country as a whole since by helping us and the government the types of Rotavirus in circulation will be known. We will also be able to recommend the immunization of all babies with these vaccines which will minimize the impact of this infectious disease.

Risk: There will be no pain during stool collection.

Your child's Rights: Your child's participation in this study is voluntary and if you disallow participation, you will not be denied any services that are normally available to you.

Confidentiality: Only the research team and staff will have access to the study results. All collected data will be kept secure and confidentiality ensured by use of passwords. The research team and staff will receive appropriate training on securing and maintaining confidentiality and safeguarding data. Data will be coded in SPSS software for archiving and any other identifiable confidential data will be destroyed.

Contact of Principal Investigator: In case of any questions about the study contact Mary-Theresa Agutu at anytime on 0722-983405 or 0738-671816.

Contact of KEMRI/NERC: If you have questions now or in the future regarding your child's rights or this study you can contact KEMRI ERC, who gave approval for this study on Postal address, P.O Box 54840-00200, Nairobi and telephone number 0722-205901 or 0733-400003.

Storage, Exportation of Samples and Further Studies: Samples in this study will be stored in a freezer at the KEMRI laboratory for a period of up to 2 years. During this time there may be need to send the samples to Regional Laboratories outside the country for confirmation testing or more advanced characterization.

Consent for the individual for stool sample:

May I now ask if you will allow your child to participate in the study?

The above details about the study and the basis of participation have been explained to me and **I allow my child** to take part in the study. I understand that I am free to allow my child to be part of the study. I also understand that if I do not want him/her to go on with the study, I can withdraw at any time. **I give my consent** for my child's stool to be tested for Rotaviruses.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark----------

Date ----------

Consent for sample storage, export and further testing

Do you give consent for the sample collected to be stored, and exported for further testing?

I am aware that the sample I have provided will be stored and may be shipped to Regional Laboratories outside the country for further testing for Rotaviruses. I give my consent to have the sample stored and even be exported for further testing.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark----------
Date ----------

Appendix IIB: Karatasi cha kufahamisha idhini au ridhaa

Kichwa cha utafiti: Utafiti wa virusi vya kuharisha 'Rotavirus' kwa watoto wanaotibiwa katika hosipitali ya watoto ya Gertrude's, Nairobi.

Watafiti: Mary-Theresa Agutu, Mtafiti mkuu

Dkt. Juliette R. Ongus, Mtafiti msaidizi (JKUAT)

Dkt. Janeth Kombich, Mtafiti msaidizi (KEMRI)

Dkt. Rose W. Kamenwa, Mtafiti msaidizi (AKUH)

Jina langu ni ……Mimi ni mwanafunzi katika chuo kikuu cha Jomo Kenyatta cha ukulima na Teknologia. Tuna fanya utafiti juu ya virusi vinavyosababisha kuharisha na kutapika 'rotavirus'. Katika utafiti huu tunakusudia kugunduwa aina za rotavirus zilizoko katika jamii. Matokea ya utafiti huu yatasambazwa kwa serikali na wadau wengineo kama Wizara za Afya na ile ya Afya ya Umma, WHO-EPI, KEMRI na wengineo ambao watatengeneza miradi mbalimbali zinazolenga kupunguza maambukizi na kuboresha huduma kwa watoto wanaoathirika katika jamii. Utafiti huu umepitishwa na kuruhusiwa na kamati inayo husika na utoaji wa vibali vya utafiti ya KEMRI, yaani ERC.

Utaratibu:

Daktari wa mtoto amesha kueleza ya kwamba anahitaji mtoto apimwe choo ili ajue kama mtoto huyu anamagonjwa fulani. Ikiwa utakubali mtoto wako ahusike na utafiti huu, tutakuuliza maswali machache juu ya chanjo ya rotavirus. Baadaye, tutakupa chupa cha kuweka choo cha mtoto. Choo kita safirishwa hadi maabara huko KEMRI kwa upimaji wa virusi 'rotavirus'. Ili kuhakikisha usiri wa jina la mtoto wako katika utafiti huu, jina na maelezo yake hayata andikwa kwenye sampuli ya choo. Sampuli hii ita tambuliwa na nambari.

Faida/ Mapungufu: Mtoto wako hata sikia maumivu unapochukuwa choo. Aidha mtoto wako hatapata faida yoyote kibinafsi kutoka na kushiriki kwake katika utafiti huu. Utafiti huu utasaidia jamii yote kwa jumla kwa sababu tukitambua virusi vinavyo ambukiza watoto tutaweza kushauri wadau wanaoshugulikia miradi mbalimbali ya kupunguza athari za kuambukizwa na virusi vya rotavirus.

Haki za mtoto wako: Kushiriki kwa mtoto wako katika utafiti huu ni kwa hiari yako. Ukikataa asishiriki mtoto wako hata nyimwa huduma zinazotolewa kwa kawaida.

Usiri/Utunzaji wa taarifa: Watafiti na wafanyikazi wengine katika maabara pekee yao ndio watapata kujua matokeo ya utafiti. Matokeo yatawekwa kwa usiri na nambari za siri kutumika kwenye tarakilishi zisipate kujulikana waziwazi. Pia watakaohusika wote watapata mafunzo maalum ili kuzuia usambazaji wa matokeo na

utunzaji wa zile taarifa. Matokeo yatawekwa kwenye tarakilishi kuhifadhiwa na nyingineo kuharibiwa.

Mawasiliano na mtafiti mkuu: Iwapo utakuwa na swala kuhusu utafiti huu wasiliana na mtafiti mkuu Mary-Theresa Agutu wakati wowote kwa nambari za simu 0722-983405 au 0738617816.

Mawasiliano na afisi za KEMRI/NERC: Iwapo utakuwa na maswala kuhusiana na haki za mtoto wako au utafiti huu, unaruhusiwa kuwasiliana na KEMRI ERC, walioruhusu utafiti huu kwa Sanduku la posta 54840-00200, Nairobi au nambari za simu 0722-205901 au 0733-400003.

Kuhifadhiwa, na Kusafirishwa ng'ambo kwa utafiti wa ziada: Sampuli zinazo tumika katika utafiti huu zitahifadhiwa kwenye barafu katika maabara ya KEMRI kwa mda wa miaka miwili. Wakati huu kuna uwezekano wa sampuli hizi kusafirishwa hadi maabara zinginezo hapa nchi na hata ng'ambo kwa sababu ya utafiti wa ziada.

<u>Ridhaa ya kutoa sampuli cha choo:</u>

Napenda kuuliza ridhaa yako kushirikisha mtoto wako katika utafiti huu kwa kutoa choo

chake?

Nimeelewa maelezo ya hapo juu yanayo husu utafiti huu. Nina kubali mtoto wangu kushiriki katika zoezi hili. Naelewa kuwa ushiriki wa mtoto wangu ni wa hiari na iwapo sitakubaliana wakati wowote ninaruhusa wa kumtoa katika zoezi hili. Natoa ridhaa kutoa choo cha mtoto wangu kwa upimaji. Sahihi/dole gumba…… Sahihi ya msimamizi…… Tarehe…… Tarehe…….

<u>Ridhaa ya Kuhifadhiwa kwa sampuli na kuzisafirisha sampuli kwa ajili ya utafiti wa ziada</u>

Napenda kuuliza ridhaa yako kuhifadhi sampuli au kuzisafirisha ng'ambo kwa ajili ya utafiti nyingineo?

Natoa ridhaa yangu na kuruhusu kuhifadhiwa kwa sampuli nilizotoa kutumika kwenye utafiti huu na pia kusafirishwa kwa sampuli hizo kwa utafiti wa ziada humu inchi na ng'ambo.

Sahihi/dole gumba…… Sahihi ya msimamizi……

Tarehe…… Tarehe…….

Appendix III: Questionnaire

CASE ASSESSMENT ON ROTAVIRUS GASTROENTERITIS

Serial number……..Date……

Patient reference…….

1. **Biodata**

Age of the child/infant in months ( )

Gender Male= 1 Female= 2 ( )

Weight in kgs ( )

Height in cms ( )

Is the child breast feeding Yes = 1 No= 2 ( )

Period of exclusive breast feeding ( )

2. **Z-score** ( )

3. **Assessment of symptoms**

Axiliary temperature ( )

Frequency of diarrhoea/day ( )

Duration of diarrhoea ( ) days

Frequency of vomiting /day ( )

Duration of vomiting ( ) days

Clinical assessment of severity of dehydration

No dehydration = 1; some dehydration = 2; severe dehydration =3; shock = 4

4. Management of gastroenteritis

Rehydrated at triage and discharged = 1 Admitted to wards = 2 ( )

Method of rehydration; Oral = 1 Intravenous = 2 ( )

Duration of hospital stay; Short = 1(less or equal to 3 days)

Long= 2(equal to or more than 4 days)

Dead= 3

5. Vaccination history

Vaccinated Yes = 1 No= 2 ( )

Vaccine used: Rotarix only = 1; Rotateq only = 2; Rotarix and Rotateq=3; other= 4

If Rotarix:

No. of doses administered: 1dose= 1; 2doses= 2; more=3 ( )

Age at first dose: before 6wks=1; between 6 and 14 wks=2; after 14wks=3 ( )

Duration after first dose: less than 4 wks=1; 4wks=2; more than 4wks=3 ( )

Age at final dose: before 24 weeks=1 ; after 24 weeks=2 ( )

Duration between last dose of vaccination and onset of diarrhoea ( ) wks

If Rotateq;

No. of doses administered: 1dose= 1; 2doses= 2; 3 doses=3; more=4 ( )

Age at first dose: before 6wks=1; between 6 and 14 wks=2; after 14wks=3 ( )

Duration after first dose: less than 4 wks=1; 4wks=2; more than 4wks=3 ( )

Duration after second dose: less than 4 wks=1; 4wks=2; more than 4wks=3 ( )

Age at final dose: before 32 weeks=1 ; after 32 weeks=2 ( )

Duration between last dose of vaccination and onset of diarrhoea ( ) wks

6. **Stool antigen Elisa for rotavirus:** Positive = 1 Negative = 2 ( )
7. **Electropherotypes:** Group A = 1; Non-group A = 2; Non typeable= 3 ( )
8. **G genotype:** G1=1; G2=2; G3=3; G4=4; G8=5; G9=6 ; Others=7specify ( )
9. **P genotype:** P4=1; P6=2; P8=3; P9= 4; P10= 5; others=6specify ( )

Appendix IV: Ethical Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **May 14, 2013**

TO: **Ms. MARY-THERESA AGUTU (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. FREDRICK OKOTH,**
THE DIRECTOR, CVR,
NAIROBI

Forwarded
May 21st 2013
FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
Box 54628
NAIROBI

Dear Madam,

RE: **SSC PROTOCOL No. 2100 (*RESUBMISSION APPLICATION FOR CONTINUING REVIEW*): IDENTIFICATION OF ROTAVIRUS STRAINS AMONG CHILDREN WITH GASTROENTERITS AT THE GERTRUDE GARDEN CHILDREN'S HOSPITAL, NAIROBI**

Reference is made to your letter dated May 10, 2013. The ERC Secretariat acknowledges receipt of the application on 14th May 2013.

This is to inform you that in response of the ERC secretariat letter dated 25th September 2012, the Ethics Review Committee (ERC) determines that the issues raised have been adequately addressed.

The Committee was of the opinion that the progress made in the reporting period is satisfactory. Consequently, the study was granted approval for continuation effective the **14th day of May 2013**. Please note that authorization to conduct this study will automatically expire on **May 13, 2014**.

If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **April 01, 2014**.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received. Please note that any unanticipated problems resulting from the conduct of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinue.

You may continue with the study.

Yours faithfully,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
24 MAY 2013
P.O. Box 54628 - NAIROBI

In Search of Better Health

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **November 29, 2011**

TO: **MARY T AGUTU,**
PRINCIPAL INVESTIGATOR

THRO': **DR. FREDERICK OKOTH,**
THE DIRECTOR, CVR,
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

RE: **SSC PROTOCOL NO. 2100 REVISED (*RE-SUBMISSION*): IDENTIFICATION OF ROTAVIRUS STRAINS AMONG CHILDREN WITH GASTROENTERITS AT THE GERTRUDE GARDEN CHILDREN'S HOSPITAL (GGCH) NAIROBI**

The Committee is satisfied that the issues raised at the initial review are adequately addressed. The study is granted approval for implementation effective this **29th day of November 2011**. Please note that authorization to conduct this study will automatically expire on **28th November 2012**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **20th September 2012**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC.

You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,
CKithinji
Caroline Kithinji,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
30 NOV 2011
P. O. Box 54628 - NAIROBI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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ESACIPAC/SSC/9730 **9th September, 2011**

Mary T. Agutu

Thro'
Director, CVR
NAIROBI

Forwarded 12.9.11

REF: SSC No. 2100 (Revised) – Identificaion of rotavirus strains among children with gastroenteritis at the Gertrude's Garden Children's hospital Nairobi

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **182nd** meeting held on **6th September, 2011** and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised protocol to SSC for onward transmission to ERC office.

We advise that work on this project can only start when ERC approval is received.

Christine Wasunna, PhD
FOR: SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
12 SEP 2011
P.O. Box 54628 - NAIROBI

Appendix V: Authorization to conduct research at the GCH

Gertrude's
CHILDREN'S HOSPITAL
Quality Healthcare for Children

May 17, 2011

To the Graduate Programme Coordinator
Institute of Tropical Medicine and Infectious Diseases (ITROMID)

Dear Sir/Madam

Identification of rotavirus strains among children with gastroenteritis at the Gertrude's Children's Hospital, Nairobi by Dr. Mary-Teresa Ogutu.

Dr Mary-Teresa Ogutu has expressed interest in conducting the above study at Gertrude's Children's Hospital.

This is to confirm that we will be willing to support her in this effort by allowing the study to be conducted in the hospital.

Yours faithfully

Dr Thomas Ngwiri
Head of Clinical Services

P.O. Box 42325-00100, Nairobi, Kenya I Tel: (+254 20) 3763474/5/6/7 I Fax: (+254 20) 3763281
E-mail: info@gerties.org I www.gerties.org

Trustees: JG Bell, Chairman, AR Davis, Mrs. EA Russell, GA Maina, NR Pavitt, Dr. SJ Nesbitt, TM Davidson, K Shah
Administrator & Chief Executive: GO Odundo