Prevalence and Epidemiology of Enteric Viruses in Children Attending Lwak Mission Hospital in Asembo, Western 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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JKUAT, Kenya

DEDICATION

I dedicate this thesis to my parents, Mr. and Mrs. Ahenda who have given me the opportunity of an education from the best institutions and support throughout my life. Without their patience, understanding and most of all, love, it would not have been possible to complete this work.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABI	Applied Biosystems
Adeno	Adenovirus
Astro	Astrovirus
BLAST	Basic Local Alignment Search Tool
CA	California
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic acid
C. I.	Confidence Interval
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside Triphosphates
DSS	Demographic Surveillance System
DTT	Dithiothreitol
EIA	Enzyme Immuno Assay
ELISA	Enzyme-linked Immunosorbent Assay
GA	Georgia
GIS	Geographic Information System
gm	Grams
ID	Identification
ICT	Information and Communication Technology
IEIP	International Emerging Infections Program

KEMRI	Kenya Medical Research Institute
µg	Microgram
µl	Microliters
mM	Milli Molar
MOH	Ministry of Health
NCBI	NationalCenter for Biotechnology Information
Nm	Nanometer
Noro	Norovirus
ORS	Oral rehydration solution
ORT	Oral rehydration therapy
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
Rota	Rotavirus
Rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Sapo	Sapovirus
SARI	Severe Acute Respiratory Illness
SSC	Scientific Steering Committee
U	Units
USA	United States of America
USD	United States Dollar

UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Enteric viruses have been recognized as the most important etiologic agents of gastroenteritis worldwide in young children. The major enteropathogenic viruses include: rotavirus, norovirus, adenovirus, astrovirus and sapovirus. Worldwide, rotavirus is considered to cause a greater proportion of diarrhoea in children. Studies have investigated the prevalence and epidemiology of these viruses in many countries, however, mostly in hospitalized children. There is limited data available of viruses causing diarrhea amongst outpatient cases including the circulating aetiologies, prevalence and seasonality. The aim of this study was therefore to determinethe prevalence of these five most important diarrhoeal viruses among children below14 years of age who visited the outpatient clinic atLwakMissionHospitalin Asembo with mild to severe symptoms of diarrhoea.This was a sub-study within a major Morbidity study SSC No. 932: Active Population-based Study of Infectious Disease Syndromes in Western Kenya and Nairobi. A total of 206 stool specimens collected from children below the age of fourteen years who visited the outpatient clinic in Asembo with diarrhoea, between January 2007 and June 2010 were screened for rotaviruses, noroviruses, adenoviruses, astroviruses and sapoviruses. Enzyme immunoassay technique was used to test for the presence of rotavirus and adenovirus, while reverse transcriptase multiplex polymerase chain reaction (RT-PCR) assay was used for norovirus, astrovirus and sapovirus detection. At least one viral agent was detected in 26.7% (55/206) of the children. Rotavirus was the most prevalent with 13.6% (28/206), whereas norovirus was detected in 6.3% (13/206), adenovirus in 4.9% (10/206),

astrovirus in 2.9% (6/206) and sapovirus in 1.5% (3/206). Mixed infection (co-infection of viruses) was found in 9.1% (5/55) of the positive samples, with the majority of co-infections attributable to rotavirus dual infections.In most cases the viruses were detected in children aged 13-24 months (≤ 2 years) as the average age of children infected with these agents was less than five years. Vomiting and fever were the most common clinical features detected in these children especially amongst those who had rotavirus and norovirus infections. These findings suggest that at least five enteric viruses are potentially important agents of diarrhoeain this rural site in western Kenya. Defining clinical and epidemiologic characteristics predictive of viral etiology may have implications for the management of diarrhea in children in Kenya and similar settings.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Diarrhoea affects millions of people around the world, and is well known to have a greater impact on children. Approximately 1.8 million deaths occur each year in young children thus making diarrhoeal diseases among the top five causes of childhood deaths worldwide (Victoria *et al.,* 2007). It is one of the principal causes of morbidity and mortality among children in developing countries where numerous cases occur without identification of the specific etiologic agents (Kapoor *et al.,* 2009).It is estimated that diarrhoea is responsible for 25 – 30% of deaths among children younger than five years of age in the developing world (Basu *et al.,* 2003). Studies show that every child will contract a diarrhoeal disease several times a year during their first five years of life (Malasao *et al.,* 2008). In Africa alone, the incidence of diarrhoea is approximately 3 episodes per child per year in children aged 6 – 11 months (Basu *et al.,* 2003). According to the 2008 Kenya Demographic and Health Survey, every Kenyan child under the age of five yearshas an average of three episodes of diarrhoea annually with 86 children dying every day.

Diarrhoea isa result of an infection of the intestinal tract due to many causes including viruses, bacteria, parasites, chemicals and poisons and other unknown causes (Basu *et al.,* 2003; Guandalini 2004). Enteric viruses have been recognized as the major etiologic agents of viral diarrhoea.The most important of these enteric viruses are rotavirus,

norovirus, adenovirus, astrovirus and sapovirus (Nakanishi *et al.,* 2009). In addition, over the past decade, there have also been more advances in the understanding of the causes of viral diarrhoea and other newly recognized etiologic agents such as torovirus, human parechovirus 1, picobirnavirus and bacovirus due to the development of new, rapid and molecular methods of viral diagnosis (Román *et al.,* 2003; Clark and McKendrick, 2004; Nakanishi *et al.,* 2009).

These viruses are found in the human gut, excreted in human faeces and transmitted by the faecal oral route (Greening, 2006).Upon contamination of food and water by these enteric viruses, they are ingested and shed in faeces of the infected humans and the cycle continues (Sair *et al.,* 2002). With the infectious dose presumed to be low, these enteric viruses are able to attach and penetrate the mature enterocytes at the tips of the small intestinal villi where they replicate (Sair *et al.,* 2002).Viral attachment and entry into the epithelial cell without cell death is presumed to be enough to initiate diarrhoea (Clark and McKendrick, 2004).

Based on the duration of illness and other associated factors, diarrhoea can be clinically classified into acute and chronic (persistent) (Moyo *et al.,* 2007). In both cases, there is an increased frequency of bowel movementsranging from 4 – 5 to more than 20 times per day due tothe water content in the stools (Guandalini, 2004). This water is as a result of an imbalance in the physiology of the small and large intestinal processes involved in the absorption of ions, organic substrates and the subsequent water (Guandalini, 2004).

Acute diarrhoea is more widespread and a contributing factor to most childhood morbidity and mortality in tropical countries (Mathan, 1998).Vomiting and diarrhoea are actually non-specific symptoms in children but dehydration is by far the most common complication of both acute and chronic diarrhoea (Webb and Starr, 2005).

Surveys on the prevalence ofthese viruses have reported rotavirus as the most common enteric pathogen causing severe diarrhoea in children worldwide (Basu *et al.*, 2003).These viruses are more prevalent in developing countries than in developed countries possibly due to the differences in hygienic conditions between these countries (Nakata *et al.,* 1998).However, in recent studies carried out in countries such as Spain, Japan, Netherlands, Tunisia, Brazil, China, South Korea and France, norovirus has actually been shown to be the dominant cause of viral diarrhoea in children(Barreira *et al.*, 2010; Harada *et al.,* 2009). In most cases, noroviruswas associated withepidemic outbreaksin schools, hospitals and day-care centres(Nguyen *et al.,* 2008; Bruggink and Marshall, 2009; Xu *et al.,* 2009). These studies also found a considerable proportion of co-infections between rotavirus and norovirus among children (Enriqueta *et al.,* 2003; Oh *et al.,* 2003; Victoria *et al.,* 2007; Harada *et al.,* 2009). This may or may not be the probable cause in the increase of severity of diarrhoeal diseases (Oh *et al.,* 2003).

All the following viruses have relevant predominant strains circulating among children particularly: Group A rotavirus (family *Reoviridae*), Norovirus (family *Caliciviridae*), Adenovirus 40/41 (subgenus F), Astrovirus (family *Astroviridae*) and Sapovirus (family

Caliciviridae) (Oh *et al.,* 2003; Greening 2006). However, some other strains do arise when more precise and adequate molecular methods are used (Oh *et al.,* 2003). The genetic diversity of these viruses in different countries worldwide is also dependent on the seasons throughout the year, climatic changes and the different geographical settings (Moyo *et al.*, 2007; Malasao *et al.*, 2008).

Diarrhoea is usually self-limited, however dehydration is what mainly causes morbidity and mortality in children below the age of five years (Webb and Starr, 2005). Management is therefore aimed at preventing or treating the child with mild to moderate dehydration that so often accompanies this disease (CDC, 2003).For many years treatment of diarrhoea has relied on the simple but overwhelmingly effective therapy of oral rehydration and saved the lives of millions in developing countries (Santosham, 2002).In addition, education, proper hygiene and sanitation should always be considered to prevent future infections and spread and this will eventually lower the rate of morbidity and mortality in infants and young children (Guandalini, 2004).

Effective control of diarrhoeal diseases in any community depends upon an accurate understanding of the relative importance of specific etiological agents, particularly in relation to the disease burden in various age groups. It is quite likely that the most cost-effective control measures are those aimed at diarrhoeal diseases requiring admission to hospital as they are at the severe end point. However, it is also as important to concentrate on outpatients with viral diarrhoea who consult medical officers in clinics

from their surrounding communities. This studysought to investigate theepidemiological and clinical characteristics of specific etiologic agentsof viral diarrhoea in young children from an outpatient clinic in a rural community in Western Kenya.

1.2 PROBLEM STATEMENT

In Kenya, data on the specific causes of viral diarrhoea in children below the age of five years is mostly on rotavirus and from hospitalized patients detected using sentinel surveillance systems. Very few studies have been carried out on outpatient populations in Kenya to assess the relative importance of the other four enteric viruses- norovirus, adenovirus, astrovirus and sapovirus. Hence the exact epidemiology of viral diarrhoea from outpatient children remains unknown. Moreover, information on Kenyan studies regarding the number and distribution of viruses responsible for diarrhoeain infants and young children is scarce. Viral and epidemiologic data is required to study the association of these viruses from the environment to the children.

1.3 JUSTIFICATION

Most epidemiological data on viral diarrhoea is from hospitalized children. Few reports have been published on the viral status of children with mild to severe diarrhoea who are usually managed in outpatient clinics.

This study was designed to determine the enteric epidemiology of viral diarrhoea in children who visit the LwakMissionHospital clinic in Asembo, Western Kenya. The introduction of commercial assays based on monoclonal antibodies and highly sensitive molecular techniques as the diagnostic tools of these enteric viruses in stools, has improved the rate of epidemiological studies. It is important to determine the characteristics of circulating enteric viruses as a prerequisite to vaccine development and to the understanding of genetic diversity in Kenya.

1.4 RESEARCH QUESTIONS

1. Which enteric viruses are responsible forcausing diarrhoeal diseases in children below the age of fourteen years attendingLwakHospital clinic in Asembo, Western Kenya?
2. What are the epidemiologic characteristics of viral aetiologies of diarrhoea in children below the age of fourteen years?
3. What are the clinical characteristics of viral diarrhoea in children below the age of fourteen years?

1.5 OBJECTIVES

1.5.1 General Objective

To determine the prevalence of enteric viruses: rotavirus, norovirus, adenovirus, astrovirus and sapovirus as agents of diarrhoea in children who attended

LwakMissionHospital clinic in Asembo, Western Kenya between January 2007 and June 2010.

1.5.2 Specific Objectives

1. To determine the prevalence ofspecific viral aetiologies ofdiarrhoea in children below the age of fourteen years.
2. To determine the epidemiologic characteristics of viral aetiologies of diarrhoea in children below fourteen years of age.
3. To determine the clinical characteristics of viral diarrhoea in children below fourteen years of age.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background

By the year 1980, there was an estimated 15 million childhood deaths occurring each year worldwide due to eminently preventable and treatable conditions such as

Family	Genus	Type of Virus	Nucleic Acid Type	Size of Virion (nm)	Genome Size (kb)	Culturable[c]

pneumonia, diarrhoea, malaria, measles and malnutrition (Parashar*et al.,* 2003). In 1982, a review was done using existing data from reports published from 1950 through 1970 to get crude estimates of specific disease burdens. Despite the uncertainty of these findings based on the relatively sparse data during this period and variations in study methods, diarrhoea was estimated to cause 4.6 million childhood deaths each year (Kosek *et al.*, 2003). By the year 2000, diarrhoea related deaths had declined by half to 2.5 million deaths per year (Guerrant *et al.,* 2002; Parashar*et al.,* 2003). This reduction however, was only in mortality as morbidity still remains the same from the past decades to present (Kosek *et al.,* 2003). In studies carried out in Africa, this reduction has been mainly due to safe drinking water, better hygiene, reduced malnutrition and a positive change in cultural attitudes such as increased clinical visits (Feikin *et al.,* 2011).

Enteric viruses as important causative agents of diarrhoea first came to light from the detection of Norwalk virus by Kapikian *et al.* (1972) in the stool of a patient with diarrhoea (Wilhelmi *et al.,* 2003). This was subsequently followed by the discovery of rotavirusand astrovirus by Bishop *et al.*(1973) and (1975) respectively (Wilhelmi *et al.*, 2003). Other viruses have also since been discovered including coronaviruses and picobirnaviruses (Clark and McKendrick, 2004).

Adenoviridae	*Mastadenovirus*	Human adenoviruses (HAdV)[a]	dsDNA	70-90	28-45	Yes[c]
Astroviridae	*Mamastrovirus*	Human astroviruses (HAstV)[a]	ssRNA	28-30	7-8	Yes[c]
Birnaviridae	*Picobirnavirus*	Human picobirnavirus[b]	ssRNA	35	-	-
Caliciviridae	*Norovirus*	Norovirus (Norwalk-like virus)[a]	ssRNA	28-35	7.4-7.7	No
Caliciviridae	*Sapovirus*	Human sapovirus[a]	ssRNA	28-35	7.4-7.7	No
Coronaviridae	*Coronavirus*	Human coronavirus[b]	ssRNA	80-220	20-30	Yes[c]
Hepeviridae	*Hepevirus*	Hepatitis E	ssRNA	32-34	7.2	No
Parvoviridae	*Parvovirus*	Human parvovirus[b]	ssDNA	20-30	5	No
Picornaviridae	*Enteroviruses*	Polio virus, Coxsackievirus, Echovirus	ssRNA	28-30	7.2-8.4	Yes[c]
Picornaviridae	*Hepatovirus*	Hepatitis A	ssRNA	27-32	7.5	Yes[c]
Rotaviridae	*Rotavirus*	Human rotavirus[a]	dsRNA	60-80	16-27	Yes
Toroviridae	*Torovirus*	Human torovirus[b]	ssRNA	100-150	20-25	Yes

Table 2.1 Characteristics of clinically significant human enteric viruses causing diarrhoea

Source: Greening, 2006

[a] Viruses responsible for a greater percentage of viral diarrhoea in infants and young children resulting to morbidity and mortality worldwide.

[b]Novel viruses that have been recently identified due to the development of new and rapid molecular methods of viral diagnosis.

[c]Not all strains within the genus are culturable. The wild-type strains are often difficult to culture.

2.2 Description of Viral Agents of Diarrhoea

2.2.1 Rotavirus

Rotavirus was first discovered by Ruth Bishop and her colleagues in 1973 through electron microscopy of fecal extracts from children with acute diarrhoea (Widdowson *et al.*, 2009). Over the next 10 – 15 years, it became noticeably evident that rotavirus was the leading cause of severe diarrhoeal illness in infants and young children worldwide. Approximately 95% of children experience rotavirus diarrhoea by the age of five years (Bernstein, 2009). In developed countries like the United States, rotavirus is responsible for 5 – 10 % of cases of diarrhoea among children below five years of age (Bernstein, 2009). Deaths due to rotavirus in developed countries are uncommon, but in developing countries it is a very important contributor to childhood morbidity and mortality (Munford *et al.*, 2009). Of the estimated 527,000 child deaths occurring globally from rotavirus diarrhoea each year, >85% occurred in Africa and Asia (Kosek*et al.*, 2003). About 110,000 – 150,000 of these deaths occurred in sub-Saharan Africa alone (Arvay *et al.*, 2009). In Kenya, it is estimated that rotavirus diarrhoea is associated with 4,500 deaths, 8,800 hospitalizations and 1,444,000 clinic visits each year in children below the age of five years (Tate *et al.*, 2009). The difference in the incidence of rotavirus infection between the developed and developing countries is not much, meaning improved sanitation does not decrease the virus transmission (Bernstein, 2009).

Rotaviruses belong to the genus *Rotavirus* within the family *Reoviridae* (Table 2.1) (Munford *et al.*, 2009). They exhibit a characteristic wheel-like appearance and hence

the rotavirus name which means “wheel” in Latin (Greening, 2006). The particles are 70nm, nonenveloped icosahedral structures composed of three concentric layers; the outer capsid, inner capsid and a core containing the viral genome (Estes and Kapikian, 2007). They have 11 segments of double-stranded RNA as a genome encoding six viral capsid proteins (VP1, 2, 3, 4, 6 and 7) and six non-structural proteins (NSP1 – 6) (Clark and McKendrick, 2004). This is as described in Figure 2.1 (Pesavento *et al.*, 2003).

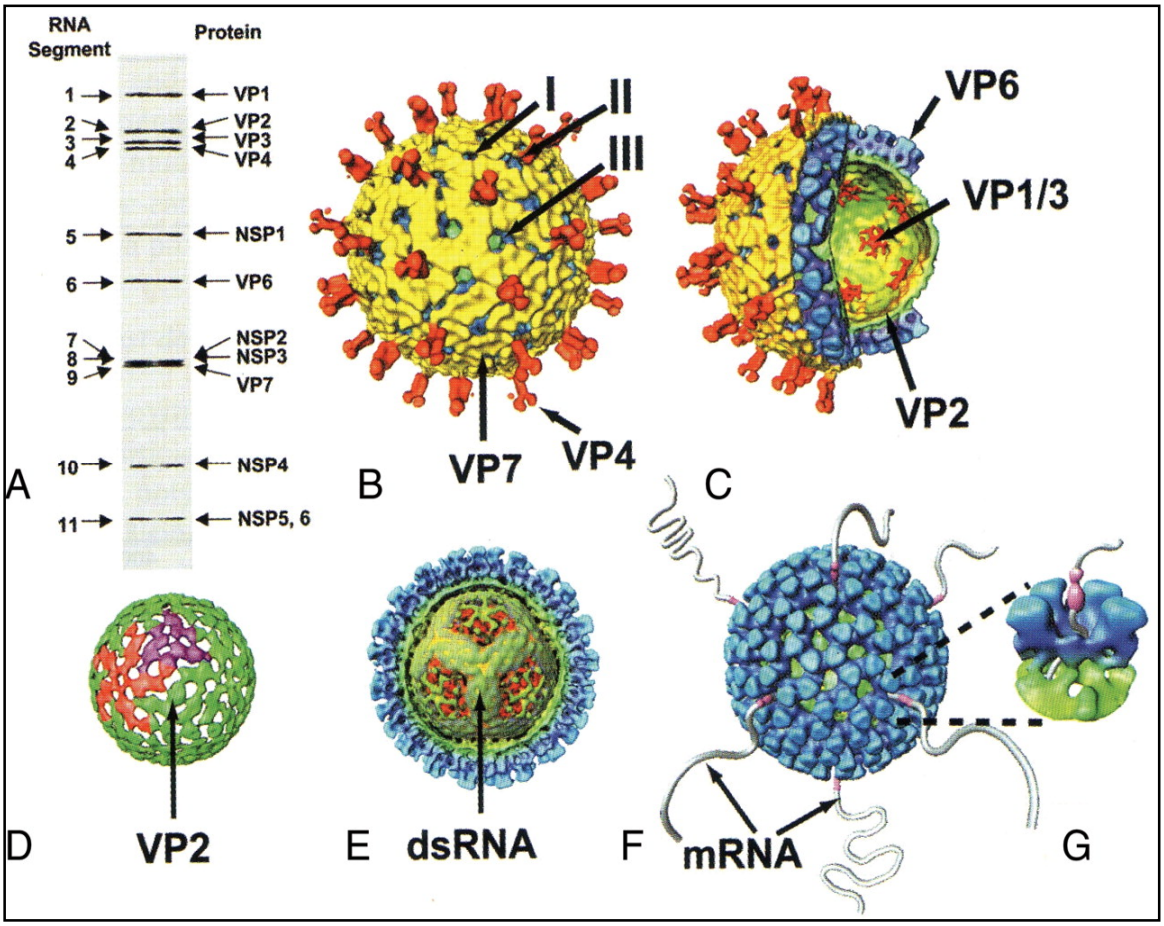

Figure 2.1: Structural Organization of a Rotavirus Virion (Pesavento *et al.*, 2003)

(A) Polyacrylamide gel showing rotavirus RNAs 1-11 with gene–protein assignment on the right.

(B) Surface representation of rotavirus structure. Channels of classes I–III are indicated, the VP4 spikes are in red, and the VP7 capsid layer is in yellow.

(C) Cut-away of the rotavirus structure showing the intermediate layer (VP6, blue), the core (VP2, green) and the flower-shaped VP1/VP3 complexes at the inside of VP2 opposite of class I channels (red).

(D) Structural organization of the VP2 layer (some of the 60 dimers are shown in red and purple).

(E) Genomic RNA in the rotavirus structure. The VP6 and VP2 layers are partially cut-away to expose the RNAs, which at the outside have a dodecahedral appearance.

(F) Structure of the actively transcribing double-layered particles with nascent mRNAs (grey) exiting through the class I channels.

(G) A close-up cut-away view of the exit pathway in one of the channels. The bowling pin-shaped density of the exiting transcript (pink) is seen in actively transcribing double-layered particles.

Panels **(B)–(G)** are delineated from image reconstructions of cryo-electron micrographs.

The outer capsid has two structural viral proteins (VP): VP4, the protease-cleaved protein (P protein) and VP7, the glycoprotein (G protein) while the inner capsid has the VP6 protein (Aung *et al.,* 2009). VP4 gives the virus its characteristic spoke-like ‘wheel’ feature and is responsible for its attachment to cells, whereas VP7 gives the virus its

smooth surface (Clark and McKendrick, 2004). VP4, VP7 and VP6 are antigenically dominant and used to classify rotavirus strains into G (VP7) and P (VP4) types and their subgroup specificities (VP6) (Estes and Kapikian, 2007). To date, there are at least seven serogroups (A – G) based upon the antigenic properties of the inner capsid protein VP6 and genomic characteristics, of which groups A – C are human pathogens (Clark and McKendrick, 2004; Aung *et al.,* 2009; Munford *et al.,* 2009). There are also 22 G and 31 P genotypes and 4 serogroups established so far in human and animal group A rotaviruses (De Grazia *et al.,* 2009). Group A rotavirus is the single most common cause of acute severe diarrhoea in young children worldwide (Aung *et al.,* 2009).

2.2.1.1 Epidemiology of Rotavirus

The severity of rotavirus infection is age dependent as itcommonly infects almost all children below the age of five years(Parashar *et al.,* 2003; CDC, 2006). Severe, dehydrating rotavirus diarrhoea primarily occurs among children aged 3 – 35 months (Bernstein, 2009). Children below the age of 3 months hardly get these diarrhoeal diseases, probably due to the transplacental antibodies they receive from their mothers which begin to decline after the first few months (Dennehy, 2008).

In temperate climate countries, rotavirus outbreaks exhibit a seasonal pattern with infection peaks during the winter season (Bernstein, 2009). Countries lying closer to the equator however have no distinct seasonal pattern but the disease is more pronounced

during the drier and cooler months. The reason for this seasonality remains unknown (Dennehy, 2008).

Rotavirus is genetically diverse and according to the WHO rotavirus surveillance carried out in different countries worldwide between 2001 and 2008, G1, G2, G3, G4 and G9 and P[8] were the most common rotaviral strains whose geographical distributions also varied(Kosek*et al.,* 2003; Widdowson *et al.,* 2009) with95% of childhood rotavirus diarrhoea being attributed to these strains (Bernstein, 2009). G1 is predominant in North America, Australia and Europe witha 70% rate of infection but lower in South America, Asia and Africa with only 20% - 30% rate of infection (Santos and Hosino, 2005).

2.2.1.2 Pathogenesis and Clinical Manifestations of Rotavirus Infection

Transmission of rotavirus is through the faecal-oral route and is usually spread by children or their caregivers failing to wash their hands and also contact with contaminated environmental surfaces (Nokes *et al*., 2008).They may also possibly be spread via faecally contaminated food and water and respiratory droplets (Parashar *et al.*, 2003; CDC, 2006). Transmission through air droplets however is still a theory (CDC, 2009). Very few infectious virions are needed to cause disease in susceptible human hosts (Bishop, 1996). This follows an incubation period of 1 – 3 days before the rotavirus illness begins abruptly (Bernstein, 2009).

Rotavirus illness exhibits non-specific clinical features whichare also similar to those caused by other diarrhoeal pathogens but are more severe (Greening, 2006). The clinical spectrum ranges from mild, watery diarrhoea of limited duration to severe diarrhoea with vomiting and fever. Vomiting remits after two days from onset but the other symptoms take about 3 – 7 days before resolving (CDC, 2009). These clinical presentations vary with age as the most severe infections in children occur at 3 months of age (Bernstein, 2009). Rotavirus diarrhoea is generally more severe than other causes of diarrhoea as it more often results to dehydration, hospitalization and if not treated leads to shock, electrolyte imbalance and death (CDC, 2006). According to other studies that have been carried in particular situations, patients with immunodeficiency are bound to experience more severe or prolonged diarrhoea upon infection (Clark and McKendrick, 2004).

2.2.1.3 Diagnosis of Rotavirus

It is not quite possible to diagnose rotavirus diarrhoea solely by clinical examinations however, suggestive features such as fever, dehydration and the regions' seasonal patterns may be conclusive enough (Bernstein, 2009). The presence of rotavirus in stools is best done by polymerase chain reaction (PCR) (Clark and McKendrick, 2004) but the most widely used methods are enzyme immunoassay and latex agglutination (Bernstein, 2009). This is far much better than electron microscopy and serological methods as it is highly sensitive and less time-consuming (Clark and McKendrick, 2004).

2.2.1.4 Treatment and Control of Rotavirus Diarrhoea

Diarrhoea is usually a self-limited infection, however dehydration is what mainly causes morbidity and mortality in children below the age of five years (Webb and Starr, 2005). Children usually confer immunity progressively after the first diarrhoeal infection as there is greater protection after every subsequent attack (CDC, 2006). Management is therefore aimed at preventing or treating the child with mild to moderate dehydration and restoring the normal physiological functions (CDC, 2003).

For many years, treatment of acute diarrhoea has relied on the simple but overwhelmingly effective therapy of oral rehydration and saved the lives of millions in developing countries. Oral rehydration therapy (ORT) consists of rehydration, continued feeding of normal diet and the replacement of ongoing fluid loss (Santosham, 2002). Nutrition in combination with oral rehydration is important as it safely and effectively helps a patient with a bout of diarrhoea (Guandalini, 2004). Oral rehydration solutions (ORS) are the safest, physiologic and effective way with fewer adverse effects than intravenous therapy of managing diarrhoea (Webb and Starr, 2005). This ideal ORT solution is generally composed of sodium, chloride, bicarbonate, potassium and glucose (Santosham, 2002). In addition zinc supplements have proven effective in children with diarrhoea in developing countries (Guandalini, 2004). It promotes optimal absorption of electrolytes, water and nutrients to maintain and replace what is already lost as this is the primary goal, treatment of dehydration (Sentongo, 2004).

The other form of treatment that is best used is the live vaccines which elicit an active immunization to increase resistance to the viral infection (CDC, 2009). Currently, there are two licensed types of vaccines;RotaTeq (Merck) and Rotarix (GlaxoSmithKline Biologicals) (Parashar *et al.,* 2009; Widdowson *et al.,* 2009). RotaTeq, approved in 2006 by the US Food and Drug Administration, is a pentavalent vaccine comprising five reassortant bovine-human viruses, which together express the human rotaviruses G1, G2, G3, G4 and P[8] antigens, and is administered as a 3-dose course at 2, 4, and 6 months of age.In contrast, Rotarix is a monovalent vaccine prepared from a single human attenuated strain of G1 P[8] that replicates well in the gut, and orally administered in two doses at 2 and 4 months of age (Dennehy, 2008; Bernstein, 2009; Widdowson *et al.,* 2009). Large trials of both of these vaccines have been carried out in the Americas and Europe to study the clinical efficacy and this is also currently ongoing in Africa and Asia. In Africa, RotTeq vaccine clinical trials are being carried out in Kenya, Ghana and Mali while Rotarix is underway in South Africa and Malawi(Widdowson *et al.,* 2009).

2.2.2 Norovirus

Noroviruses are considered the most common cause ofdiarrhoeal outbreaks worldwide **(**Barreira *et al.*, 2010).They are a genetically diverse group of viruses that possess single-stranded, positive-sense RNA genomes of 7400 – 7700 nucleotides classified as the genus *Norovirus* within the family *Caliciviridae* (Table 2.1)(Bruggink and Marshall, 2009; Xu *et al.*, 2009). The name *Caliciviridae* was derived from the Latin word *calix*, meaning cup or chalice like the 32 cup-shaped depressions found on the surface of the virion arranged in icosahedral symmetry (Green *et al.*, 2000) Figure 2.2.

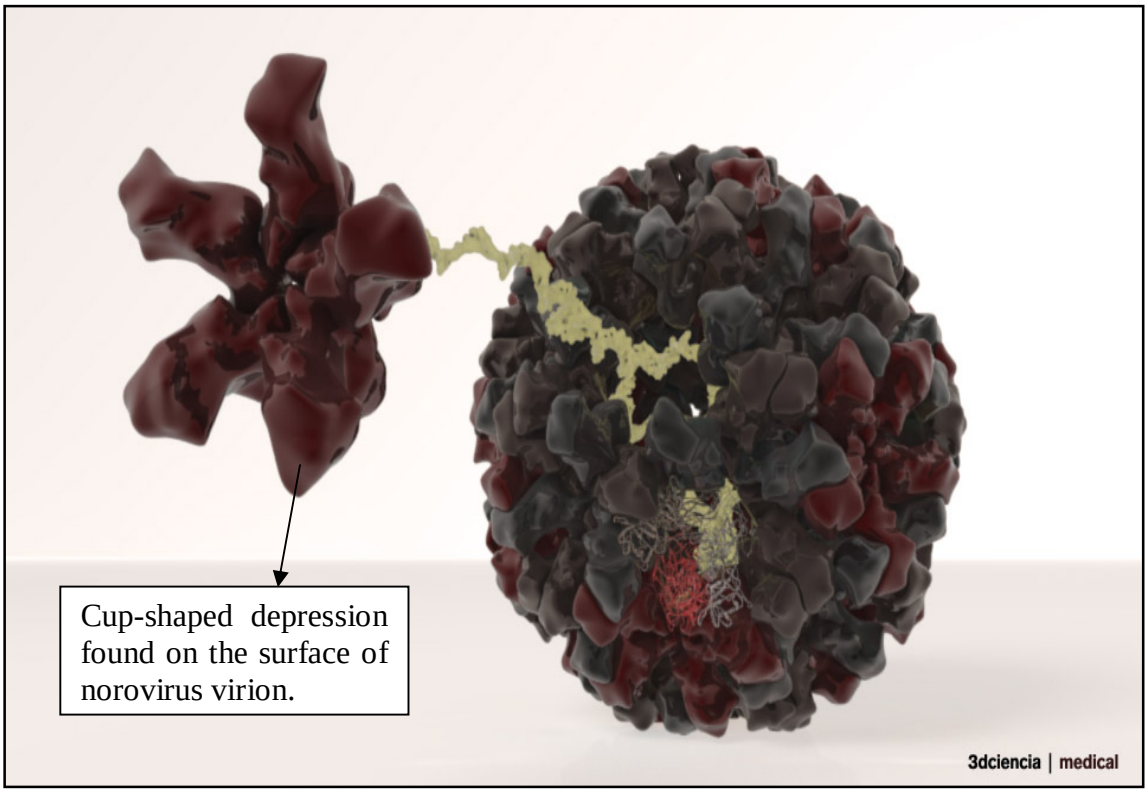

Figure 2.2: Diagrammatic structure of Norovirus showing the 32 cup-shaped depressions on the surface of the virion. Source: 3Dciencia, Accessed on 16th April, 2012.

Noroviruses however have a more ragged surface as seen under a direct electron microscope thus distinguishing them from other caliciviruses like sapovirus (Greening, *et al.,* 2006). On the basis of genome analysis and antigenic variability, human caliciviruses have been divided into at least three genogroups: genogroup I represented by Norwalk virus, genogroup II represented by SnowMountain virus and genogroup III represented by Sapporo virus (Nakata *et al.,* 1998).

Noroviruses were previously known as small round structured viruses and Norwalk-like viruses (Greening, 2006). They are genetically classified into five distinct genogroups, GI to GV of which GI, GII and GIV have been identified as the main causes of diarrhoeal infection in children (Barreira*et al.,* 2010; Bruggink and Marshall, 2009). Further classification into genotypes within the same genogroup has also been well documented and the genotype GIIb is regarded as the cause of both sporadic and outbreak cases of norovirus induced diarrhoea (Victoria *et al.,* 2007; Barreira *et al.,* 2010; Bruggink and Marshall, 2009).They are often known to cause major outbreaks of diarrhoea in different areas such as hospitals, schools and restaurants (Nguyen *et al.,* 2008).

2.2.2.1 Epidemiology of Norovirus

During the last three decades, there has been a dramatic increase in the number of newly recognized etiologic agents of diarrhoea. Norwalk virus was originally discovered in 1968 during a gastroenteritis outbreak in schoolchildren in Norwalk, Ohio by Adler and

Zickl but the norovirus prototype strain was first identified later in 1972 by Kapikian*et al.* (Glass *et al.,* 2000). This was the first viral agent to be discovered as an important cause of diarrhoea especially in children before rotavirus and the others (Wilhelmi *et al.,* 2003). The total burden of disease caused by norovirus has yet to be clearly documented as most cases of norovirus diarrhoea are as a result of an outbreak (Greening, 2006). A few epidemiologic studies, particularly in children have provided some clues to the potential prevalence of infections with these viruses and of their importance as a cause of sporadic disease and outbreaks (Victoria *et al.,* 2007; Nguyen *et al.,* 2008; Barreira *et al.,* 2010; Bruggink and Marshall, 2009; Harada *et al.,* 2009; Xu *et al.,* 2009). Outbreaks commonly occur in closed community situations such as homes, schools, camps, hospitals, resorts and cruise ships where food and water sources are shared (Bruggink and Marshall, 2009).

Various studies have been carried out on norovirus associated diarrhoea and they indicate norovirus as either the first or second most common cause of viral diarrhoeain infants and young children with a prevalence ranging from 5% to 40% (Barreira*et al.,* 2010; Xu *et al.,* 2009). This variation is probably due to differences in geographical areas and detection assays (Xu *et al.,* 2009). Seasonally, norovirus infections exhibit different seasonal patterns in various regions (Nguyen *et al.,* 2007; Xu *et al.,* 2009). Some studies such as those carried out in temperate climate countries, have reported a higher frequency in winter, spring or rainy seasons, whereas in tropical countries no obvious peak season has been reported (Nguyen *et al.,* 2008).

2.2.2.2 Pathogenesis and Clinical Manifestations of Norovirus Infection

Norovirus infection results from ingestion of viral particles from fecally contaminated food or water or direct contact from person to person. Airborne transmission of viral particles has also been documented as a secondary contaminant (Clark and McKendrick, 2004; Greening, 2006). A study was carried out in the United Kingdomon airborne transmission of noroviral particles after guests in a restaurant were infected with norovirus from an individual guest who vomited at the table during a meal. This airborne transmission gives norovirus an attack rate of about 50% - 70% or even higher depending on the source (Marks *et al.*, 2000).

Human noroviruses are uncultivable hence its pathogenicity has yet to be well understood. However, some investigation has been done using a mouse norovirus (Wobus *et al.*, 2004). A low infective dose of about 10 – 100 viral particles is all that is required to cause an epidemic as this is extremely infectious (Greening, 2006). It is assumed that they infect the mature enterocyte cells in the small intestines where they multiply and are excreted in large numbers in faeces from the onset of symptoms for about two weeks. The symptoms include an acute-onset projectile vomiting, watery non-bloody diarrhoea with abdominal cramps, nausea and at times low-grade fever (Greening, 2006).Norovirus epidemic is inevitable due to the high attack rate combined with a low infectious dose, prolonged viral shedding, short-term immunity and the environmental stability it exhibits (Greening, 2006).

2.2.2.3 Diagnosis of Norovirus

A more rapid and sensitive method of detection is what is required for a pathogen known to cause most diarrhoeal outbreaks. Therefore the best method for norovirus diagnosis is PCR(Gunson *et al.,* 2003). Other methods that have been employed in different studies include ELISA, electron microscopy and immune transmission electron microscopy (Clark and McKendrick, 2004).

2.2.2.4 Treatment and Control of Norovirus Diarrhoea

There is no specific treatment for norovirus infections but an adequate management system is required if an outbreak occurs,rapid diagnosis of the virus and infection control measures such as cleaning surfaces using disinfectants (Clark and McKendrick, 2004).

2.2.3 Adenovirus

Enteric adenoviruses belong to the *Adenoviridae* family and are classified into the genus *Mastadenovirus* (Greening, 2006) Figure 2.3. There are six species of human adenoviruses in this genus, HAdV-A to HAdV-F (van Regenmortel *et al.,* 2000). Currently, 51 human adenovirus serotypes (Ad1 – 5) and six subgenera (A-F) have been discovered in humans (Clark and McKendrick, 2004). Among these, serotypes 40 and 41 comprising the subgenus HAdV-F species are the most important etiological agents of acute infantile gastroenteritis transmitted mainly through the faecal-oral route. However, the other serotypes can also be shed in human faeces (Greening, 2006). In some cases, death has been recorded though quite rare (Bresee and Glass, 1999).

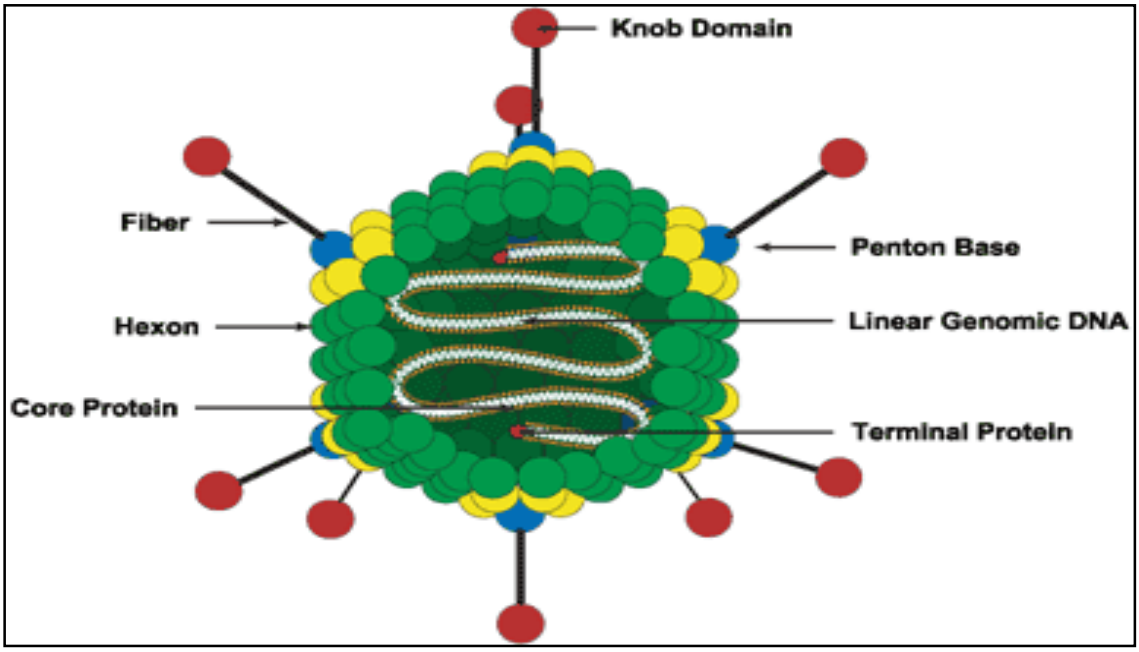

Figure 2.3: Diagram of an adenovirus virion.Source: The encyclopedia of Science website. Accessed on 16th April, 2012. Adenoviruses are medium-sized (90-100nm), nonenveloped icosahedral viruses containing double-stranded DNA.

2.2.3.1 Epidemiology of Adenovirus

Enteric adenoviruses are strongly associated with acute gastroenteritis in children and are said to be the second most important cause after rotavirus according to some studies (Bresee and Glass, 1999). Other studies show a prevalence rate of 2% - 13% in most infantile cases with diarrhoea (Basu *et al.*, 2003). Seroprevalence studies have also shown that 50% of children less than four years have antibodies against adenovirus indicating that children get exposed to enteric adenoviruses at an early age (Basu *et al.*, 2003). Adenovirus causes diarrhoeal infections throughout the year (Greening *et al.*, 2006).

2.2.3.2 Pathogenesis and Clinical Manifestations of Adenovirus Infection

Adenovirus diarrhoea causes persistent asymptomatic infections in severe cases, however, they generally result to a mild and self-limiting diarrhoea (Clark and McKendrick, 2004). The virus is shed largely in faeces for months or years (Clark and McKendrick, 2004) after an incubation period of 8 – 10 days which is much longer than all other enteric viruses (Greening *et al.*, 2006).

2.2.3.3 Diagnosis of Adenovirus

Diagnosis is usually by ELISA in most studies carried out for detection of adenovirus (types 40 and 41) however, PCR as always has been known to be more sensitive and faster (Clark and McKendrick, 2004).

2.2.3.4 Treatment and Control of Adenovirus Diarrhoea

Adenovirus diarrhoea is usually mild in most cases and self limiting (Clark and McKendrick, 2004).

2.2.4 Astrovirus

Astroviruses were first discovered in 1975 by Madeley and Cosgrove and were named according to their star-like appearance through electron microscopy (Greening, 2006). The name *astron* is a Latin word meaning star (Clark and McKendrick, 2004). They belong to the family *Astroviridae*which is divided into two genera:*Mamastrovirus* the human astrovirus (Table 2.1) and *Avastrovirus* the avian astrovirus(Clark and McKendrick, 2004). Astroviruses are described as 28 – 35 nm diameter non-lipid enveloped, single-stranded positive sense RNA viruses with a genome ranging from 6.4 to 7.3 kb (Kapoor *et al.*, 2009) Figure 3.4.

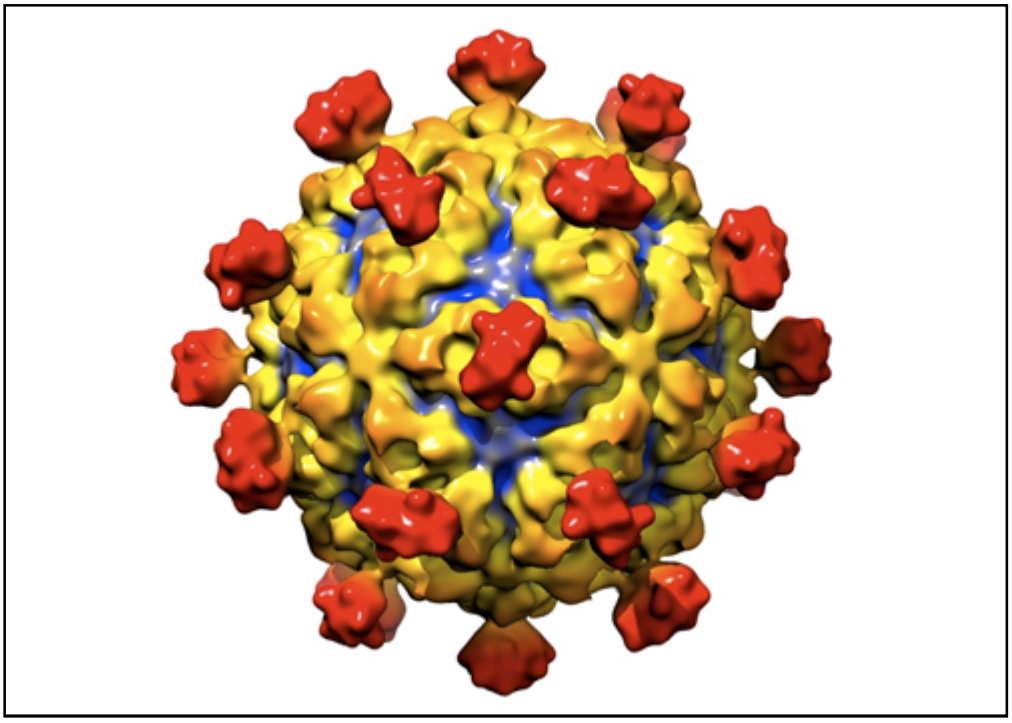

Figure 2.4: The spiky capsid shell of the astrovirus. Source: (Dong *etal.*, 2011)

There are eight known human astrovirus serotypes (HAstV-1 to HAstV-8) known to date and all associated with childhood diarrhoea (Clark and McKendrick, 2004; Kapoor *et al.*, 2009).

2.2.4.1 Epidemiology of Astrovirus

Astrovirus infections have not been well studied to confirm their pathogenesis in humans as it is generally considered to be a minor cause of viral diarrhoea(Nakanishi *et al.*, 2009).Studies carried out in both developed and developing countries have some similarities in prevalence rates of about 2% - 16%in hospitalized diarrhoeal cases and about 5% - 17% among children with diarrhoea in community based studies (Kiulia*et al.*, 2007). Most cases of infection have been detected in children below one year of age, outbreaks in schools and hospitals, the elderly and immunosuppressed (Greening, 2006; Kiulia *et al.*, 2007). Some of these studies have been carried out in African countries including Nigeria, South Africa, Malawi and Ghana and also in Europe, central and southern America, Australia and Asia. Most of these studies have reported difficulties in astrovirus detection and its association with gastroenteritis due to high levels of mixed infections (Pennap *et al.*, 2002). However, no studies on the prevalence of this pathogen have been documented in East Africa and the Horn of Africa (Kiulia *et al.*, 2007).

2.2.4.2 Pathogenesis and Clinical Manifestations of Astrovirus Infection

Astrovirus is transmitted through the faecal-oral route and has been recently identified as a cause of human gastroenteritis and diarrhoea (Kapoor *et al.,* 2009). Clinically, astroviruses cause symptoms similar to those of caliciviruses after an incubation period of 3 – 4 days consisting of watery diarrhoea and less commonly vomiting, headache, fever and abdominal pains (Kapoor *et al.,* 2009). More severe symptoms however, are seen in young children and those who are immunocompromised due to a particular serotype 3 (Clark and McKendrick, 2004).

2.2.4.3 Diagnosis of Astrovirus

Initially, studies were carried out using electron microscopy for the detection of astroviruses in stools. This reported significantly low cases of astrovirus as a cause of diarrhoea in children compared to recent studies which have employed new serological assays based on monoclonal antibodies (Pennap *et al.,* 2002). However, according to another study carried out to assess the sensitivity of ELISA and multiplex PCR as methods of detection of astrovirus, PCR is the most sensitive method of detection as the true prevalence of astrovirus could be underestimated if other methods are used solely (Dalton *et al.,* 2002).

2.4.4.4 Treatment and Control of Astrovirus Diarrhoea

There are no vaccines available for the treatment of astrovirus diarrhoea but in most case patients, the disease usually resolves without any specific treatment (Clark and McKendrick, 2004).

2.2.5 Sapovirus

Sapovirus is a diarrhoeal pathogen that was initially known as Sapporo-like virus (Greening, 2006) causing relatively small epidemics with mild gastroenteritis in infants and young children (Nakanishi *et al.,* 2009). Sapovirus, just like norovirus, belongs to the family *Caliciviridae* (Johnsen *et al.,* 2009). They are divided into five genogroups, GI to GV where GI, GII, GIV and GV have been specifically identified in infected humans, while GIII strains have been identified in porcine species (Hansman *et al.,* 2007; Nguyen *et al.,* 2008). Sapovirus has the typical calicivirus appearance which is the distinct cup-shaped indentations on the surface of the virions unlike norovirus (Greening, 2006).

2.2.5.1 Epidemiology of Sapovirus

Diarrhoea caused by sapovirus is usually milder than that caused by norovirus (Nguyen *et al.,* 2008). Despite the limited information on the epidemiology of sapovirus, studies have shown that the infection in humans is relatively harmless and of a short duration (Johnsen *et al.,* 2009). There is no clear seasonal pattern for this virus however, a study by Pang *et al.* (2000) in Finland suggested seasonality for sapovirus infections with a peak in March to May though not as clear as for rotavirus or norovirus.

2.2.5.2 Pathogenesis and Clinical Manifestations of Sapovirus Infection

Human to human infection is one of the known possible routes of infection according to studies that have been carried out in children with diarrhoea attending the same school and all had similar sapovirus strains detected in their stools (Harada *et al.,* 2009). Sapovirus infections have similar symptoms like norovirus infection though they do not cause an epidemic (Greening *et al,* 2006).Diarrhoea caused by sapovirus is usually milder than that caused by norovirus (Nguyen *et al.,* 2008). Despite the limited information on the epidemiology of sapovirus, studies have shown that the infection in humans is relatively harmless and of a short duration (Johnsen *et al.*, 2009).

2.2.5.3 Diagnosis of Sapovirus

PCR has been developed using specific primers for the detection of sapovirus in clinical specimens in most molecular epidemiologic studies (Harada *et al.,* 2009). Other methods also include electron microscopy, immune transmission electron microscopy and ELISA (Clark and McKendrick, 2004).

2.2.5.4 Treatment and Control of Sapovirus Diarrhoea

Since sapovirus expresses similar diarrhoeal symptoms to norovirus, then its treatment and control is not any different. In addition, prompt implementation of infection control should also be considered during an outbreak likewise to norovirus (Clark and McKendrick, 2004).

2.3 Other Viruses with potential to cause diarrhoea

Recently, the number of viral agents associated with diarrhoea in humans has steadily increased from the availability of diagnostic tests mainly immunoassays or molecular techniques (Wilhelmi *et al.,* 2003). These include: parvoviruses, coronaviruses, toroviruses and picobirnaviruses (Table 2.1). Some of these viruses have recently been discovered while others that were discovered earlier are yet to be well studied such as torovirus detected first in 1984, coronavirus in 1975 and picobirnavirus in 1988 (Wilhelmi *et al.,* 2003).They are known to cause diarrhoea in animals however, they are now emerging as causes of viral diarrhoea in humans according to several studies (Nakanishi *et al.,* 2009; Harada *et al.,* 2009; Clark and McKendrick, 2004). Just as other viruses they are also transmitted via the faecal-oral route by both humans and animals. However, there is little evidence on their mode of transmission to cause acute diarrhoea in infants and young children as they are less common (Greening, 2006). Most of these viruses cause mild infections which hardly result to death. Those affected are either young children or immunodeficient patients (Clark and McKendrick, 2004).

2.4 Viral Diarrhoea in Adults

The cause of diarrhoea has not been well studied in adults as most studies concentrate on infants and young children. However, there are viruses that have been known to cause epidemic diarrhoeal outbreaks in adults and the elderly (Wilhelmi *et al.,* 2003).

Caliciviruses are the most common aetiologic agents of viral diarrhoea in adults(Kittigul *et al.,* 2009).

2.5 Socioeconomic Impact of diarrhoea

Active surveillance programs of potentially infectious diseases such as diarrhoea are often difficult to maintain in developing countries mainly due to the limitations experienced in healthcare sectors (Tornheim *et al.,* 2010).Population-based surveillance at field clinics and households can provide data to decision makers about the need for targeted interventions (Feikin *et al.,* 2011). According to various reports from African studies, more children are hospitalized with diarrhoea than adults (Tornheim *et al.,* 2010). This in most cases is attributed to the poor health and environmental conditions and lack of clinical consultations by those especially in rural communities (Kosek *et al.,* 2003). However, a higher number of these children who are hospitalized or seek medical attention are from urban areas than those from rural areas most probably due to better health facilities, health education awareness and close proximities and access to health centres (Feikin *et al.,* 2011).Children residing in a low socioeconomic status such as rural areas have also been reported to have higher incidences of diarrhoea than children from urban areas (Seigel *et al.,* 1996).The cost of treating a child with diarrhoea is a major burden in rural areas hence most children seek care from traditional healers or thecommunity (Tate *et al.,* 2009).Distances from health facilities and poor road conditions especially during rainy seasons are major contributors to these differences in incidence rates in rural communities from developing countries (Feikin *et al.,* 2011).

2.6 Risk Factors

Malnutrition has been documented as a contributing factor to diarrhoeal diseases to some extent (Guerrant *et al.,* 2002). In poverty stricken areas where many children are born malnourished, neonates and young infants particularly, areat risk (Guandalini, 2004). Children usually have multiple episodes of diarrhoea in one season resulting to a drastic reduction of the body's essential nutrients and thereby leading to subsequently more severe diarrhoeal attacks. For this reason, children are recommended to have their nutritional intake increased and maintained after their first diarrhoeal infection (CDC, 2003). Some specific cases, such as those who are immunosuppressed, experience malabsorption due to impaired intestinal functions from prolonged diarrhoea and infections (Guerrant *et al.,* 2002). Generally, the magnitude and impact of early childhood diarrhoea can result to a lower general growth and physical fitness, cognitive impairment and a decrease in school performance.

Apart from children, others who are also prone to infectious diarrhoeal diseases include adults, those who are immunocompromised from organ transplants or HIV-infection and the elderly (Anderson and Weber, 2004; Anderson, 2008). Adults with diarrhoea are usually from closed communities with a history of routine exposures to virally-infected children (Anderson and Weber, 2004).Transmission of viruses such as rotavirus is usually within families from handling infected infants or young children while changing their diapers or cleaning them up after a visit to the toilet (Anderson and Weber, 2004). Washing hands thoroughly after such incidences has shown to greatly reduce the impact

of diarrhoeal diseases among children and adults as well. Hands are usually the best vectors which transmit these viruses to foods and surfaces and to mouths of susceptible hosts (Curtis and Cairncross, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This wasa laboratorybased study assessing the prevalence, epidemiologic and clinical characteristics of viral diarrhoea in children below the age of fourteen years in Western Kenyabetween January 2007 and June 2010. Stool samples that were archived for downstream testing for the Morbidity study SSC Nos. 932, were extracted from freezers for this study.

3.2 Study Site

The study was conducted at the LwakMissionHospital in Asembo, Western Kenya. This is a rural study site near Lake Victoria in Siaya District, NyanzaProvince in Western Kenya (Figure 3.1 and Figure 3.2). Since September 2001, KEMRI and CDC have collaborated to operate a health and demographic surveillance system (HDSS) in Bondo and Siaya Districts of Western Kenya (SSC Nos. 647).The DSS covers three rural study sites near Lake Victoria; Asembo, Gem and Karemo.The DSS area in Western Kenya has a population of approximately 200,000 people living in about 34,000 households. The area is large (almost 400 km^2), culturally homogeneous (95% Luo), with subsistence farming and fishing constituting the principal economy.

Around Lwak hospital, a central referral clinic in Asembo within a subset of the DSS population of 24,000 people living within 5 km radius of the hospital, a population-based

morbidity surveillance project of the International Emerging Infections Program (IEIP) is taking place (SSC # 932) (Figure 3.2). The 5 km radius defines the geographical area that clinical services are accessed physically by most people. The area is one of the most impoverished in Kenya with 60-70% of people living below the poverty line (Kenya Bureau of Statistics, 1997). The area has a high level of malaria transmission and a high rate of HIV infection among other infectious diseases. Consequently, the area has mortality figures that reflect this burden of diseases with infant mortality rate at 120 per 1,000 live births and life expectancy at birth of 38 years. The study site for this project was therefore located at Lwak Mission hospital, since the active population based study of major infectious disease syndromes provided an opportunity to capture children with diarrhoeal diseases.

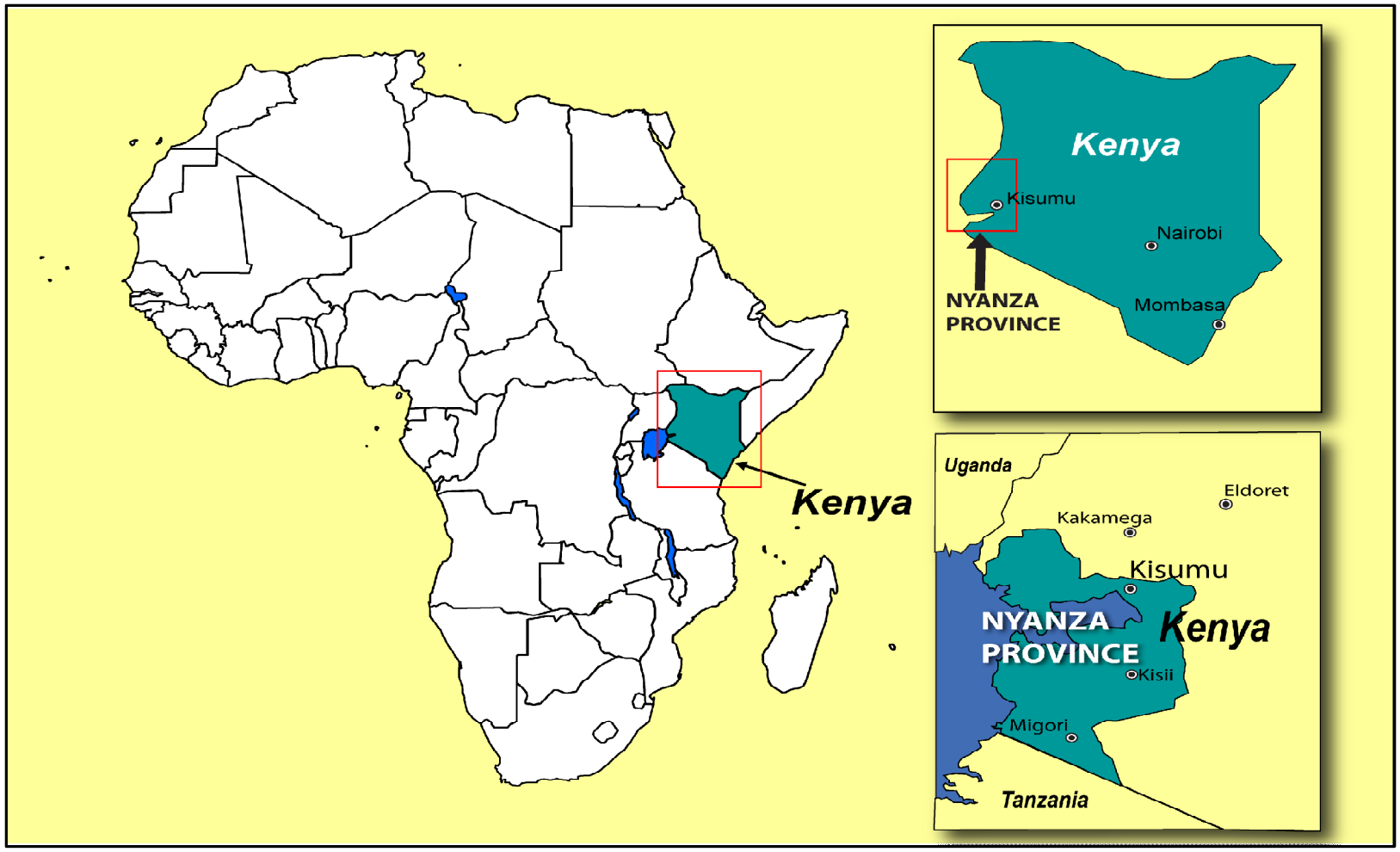

Figure 3.1: Map of Nyanza Province in relation to Kenya and Africa. (Source: KEMRI/CDC, Unpublished Data)

Figure 3.2: Map of the Demographic Surveillance System (DSS) study area in Asembo, Western Kenya. (Source: KEMRI/CDC, Unpublished Data)

3.3 Study Population

The study population consisted of children below the age of fourteen years with mild to severe symptoms of diarrhoea participating in the IEIP surveillance for infectious disease syndromes (SSC # 932) and attendedLwakMissionHospital clinic in Asembo.

3.3.1 Inclusion Criteria

Stool samples from children aged fourteen years and below who presented with mild to severe symptoms of diarrhoea at LwakMissionHospital clinic during the IEIP population-based morbidity surveillance in Asembo between January 2007 and June 2010, were incorporated in this study.

3.3.2 Exclusion Criteria

Stool samples from children, who were above the age of fourteen years with similar diarrhoeal symptoms, from the same study site during the IEIP population-based morbidity surveillance in Asembo between January 2007 and June 2010, were excluded in this study.

3.4 Sample Size Determination

According to a study carried out in Dar es Salaam, Tanzania on “The prevalence of enteropathogenic viruses among children with diarrhoea”, the proportion of children with diarrhoea caused by at least one viral agent was detected in 32.2% of the

children(Moyo *et al.*, 2007). This indicates the population proportion of children with viral diarrhoea in Tanzania an East African country where Kenya is also geographically situated. Hence, the sample size that was required to estimate the true proportion of children with viral diarrhoea attending LwakHospital clinic in Asembo,Western Kenya, with a desired 95% confidence interval and aprecision of 0.05, was computed in the following formula.

$$\mathbf{n = \frac{Z^2\,PQ}{d^2}}$$ (Fisher *et al.*, 1983)

Where:-

n = Desired sample size

P = 32.2%; Proportion of children with viral diarrhoea (Moyo *et al.*, 2007)

Q = 1 – P

Z = 1.96; Standard error (95% confidence interval)

d = 5%; Desired precision

Therefore, using these figures and substituting them in the formula, the minimum sample size was**335**.

3.5 Sampling Design

A computerized stratified random sampling method was used to select the archived stool samples based on age(0-14 years old)of child and month the stool sample was collected between January 2007 and June 2010.Selection was done by first obtaining the total number of patients with diarrhea who were seen at LwakMissionHospital in Asembo between January 2007 and June 2010 from the IEIP database at KEMRI-CDC in Kisumu. From this, the total number of children ≤ 14 years old who visited the outpatient clinic at LwakMissionHospital during this study period with diarrhea, was extrapolated. Out of these children, information was further obtained of the number of children with diarrhoea who were sampled after meeting all the Morbidity study's inclusion criteria. Next, this batch number was stratified further into age and month as indicated in Figure 3.3. This was to ensure an equal distribution of all cases in each age set per month to prevent any selection biasness. However, since the total number of children below the age of two years was highest as has also been seen in other diarrhoeal case studies (Basu *et al.*, 2003), this age group was further stratified into months.Approximately 1-2 samples were chosen for each age set per monthat randomdepending on their availability.

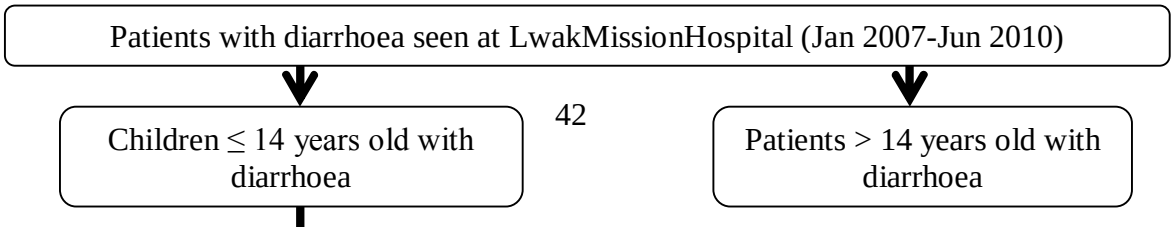

Figure 3.3: Sampling DesignFormat

3.6 Faecal Specimen Collection and Handling

Faecal samples of children below the age of fourteen years collected in sterile specimen collection bottles had already been stored in -80°C freezers, at CDC/KEMRI enterics laboratory in Kisian, Kisumu.These faecal samples had been collected for the isolation, identification and antibiotic susceptibility testing of enteric bacterial pathogens for the IEIP surveillance study (SSC # 932).Each sample had been aliquoted into four vials and archived for future testing such as the current study. All the samples had a barcode number attached hence patient information was retrieved from the database to identify the required samples for the current study.

3.7 Ethical Consideration

This project was approved by the Scientific Steering Committee and the Ethical Review Committee at the Kenya Medical Research Institute (KEMRI). Consent from parents/guardians for their children had already been done by the personnel involved in the IEIP population-based morbidity surveillance study (SSC # 932) at the Lwak hospital in Asembo. The study participants had assented where appropriate and the respective caregivers had consented for the storage and future testing of their faecal samples. All the personnel involved in this study were trained and certified in the ethical conduct of human studies by CDC/KEMRI requiring them to adhere to an unwavering code of conduct regarding the confidentiality of patients and patient's information.

3.8 Laboratory Procedures

There were two types of techniques that were used for detection of enteric viruses from the stool samples, ELISA and RT-PCR. ELISA was used to detect rotavirus and adenovirus, whereas RT-PCR was used to detect norovirus, astrovirus and sapovirus. The use of two different techniques was adopted from the Global Enterics Multicenter Study (GEMS) that was ongoing in KEMRI-CDC in Kisumu during the study period. GEMS study was conducted in Gem, one of the three DSS rural study sites near Lake Victoria (SSC # 1155).

3.8.1 Detection of Rotavirus and Adenovirus

3.8.1.1 ELISA

Group A rotaviruses and human adenoviruses were detected using commercial ELISA kits, ProSpecT™ Rotavirus and ProSpecT™ Adenovirus (Oxoid Ltd., Basingstoke, UK) according to the manufacturer's instructions and as previously described (Nitiema *et al*., 2011).The test kits for both Rotavirus and Adenovirus had break-apart microwells pre-coated with the viruses' specific antibodies to detect specific antigens present in both rotaviruses and adenoviruses. Both methods utilized the antibody capture in a solid-phase, sandwich ELISA techniques according to the manufacture's protocol. First, 2 drops (100µl) of each faecal diluted suspension or control was added to the microwells and incubated simultaneously with 2 drops (100µl) of the viruses' specific antibody conjugated to horseradish peroxidase. After 60 minutes incubation at room temperature, the microwells were washed with adiluted (x10) Wash Buffer (phosphate buffered solution containing antimicrobial agent and detergent). 2 drops (100µl) of chromagen (Substrate) was then added to the microwells and incubated for 10 minutes at room temperature. The presence of specifically bound enzyme labeled antibodies in the microwells resulted in a colour change, which was stopped by the addition of an acidic Stop Solution (100 µl). This coloured product was read spectrophotometrically at 450nm using an absorbance microplate reader (BioTek, Elx800, US).These results werethen interpreted against specific negative and positive controls included in each plate. The positive controls for both rotavirus and adenovirus assays from the test kits, were inactivated viruses containing antimicrobial agents, whereas the negative controls

constituted tris buffered saline containing antimicrobial agent and red dye. The cut-off value between the negative and the positive was calculated by adding 0.100 absorbance units to the negative control value. This interpretation was done as follows: Positive: Sample absorbance value > the cut-off value; Negative: Sample absorbance value < the cut-off value; Equivocal: Sample absorbance value within 0.010 absorbance units of the cut-off value (These samples are retested).

3.8.2 Detection of Norovirus, Astrovirus and Sapovirus

3.8.2.1 Viral Isolation (RNA)

A pea size amount of 0.1 gm solid stool or an equivalent 0.1 ml liquid/mucoid stool specimen was first transferred using a sterile spatula or a micropipette respectively into specific ID labeled microcentrifuge tubes containing 500 µl distilled deionized water and 500 µl Vertrel XF (1,1,1,2,3,4,5,5,5- Decafluoropentane) (Miller-Stephenson, Danbury, USA). Vertrel XF is a solvent and dispersion media used to separate the viral particles from the stool specimen after vortexing and centrifuging(8,000 rpm) using a microcentrifuge (Eppendorf Centrifuge, 5417R) for 1 minute each resulting into formation of a supernatant. The supernatant was then carefully transferred into newly labeled microcentrifuge tubes and stored at 4°C before later extraction of viral RNA. Viral RNAwas extracted using a QIAamp Viral RNA Mini kit (Qiagen, HildenGermany) according to the manufacturer's protocoland as previously described (Oh *et al*., 2003). One hundred and forty µlof faecal supernatant was mixed with 560µl ofAVL viral lysis

buffer containing carrier RNA by pulse-vortexing for 15 seconds. The mixture was incubated at room temperature for 10 minutes and 560µl of ethanol added. This mixture was vortexed for about 15 seconds and centrifuged again to bring down the contents of the tube to the base and avoid any spillage when opening. The mixture was then transferred carefully into a QIAamp Mini spin column with a membrane specific to viral RNA binding, in a 2 ml collection tube without wetting the rim and centrifuged at 8000 rpm for 1 minute at room temperature. The column was placed into a new 2 ml collection tube and 500µl of AW1 buffer was added. The column was centrifuged at 8,000 rpm for 1 minute to remove unbound materials, and washed by addition of 500µl of AW2 buffer and then centrifuged again. Then, the column was centrifuged at full speed (about 14,000 rpm) for 3 minutes and placed into a new 1.5 ml microcentrifuge tube. Finally, 60µl of AVE buffer was added directly onto the column to elute RNA. After incubating at room temperature for 1 minute, the column was centrifuged at 8,000 rpm for 1 minute. The viral RNA was spun down into the collection tube and used as a template for the reverse transcription polymerase chain reaction (RT-PCR).

3.8.2.2 Synthesis of cDNA (Reverse Transcription)

First, 3 µl of the extracted viral RNAwas mixed with2.05 µl of5X First strand Buffer (Invitrogen), 0.75 µl of 10 mM dNTPs (Roche), 0.75 µl of 10 mM DTT (Invitrogen), 0.5 µl of40 U/µl RNase Inhibitor (Roche), 0.375 µl of 1µg/µl Random Primer (TaKaRa), 0.75µl of 200 U/µl Superscript II Reverse Transcriptase (Invitrogen)and 8.825µl of nuclease-free water was added to give a total volume of 14 µl for 1 sample

reaction.thereverse transcription step was carried out at 42°C for 1 hour for 99°C for 5 minutes to inactivate the enzyme and cooling at 4°C immediately. Synthesized cDNA was then used in multiplex PCR detection of the respective viruses accordingly (Li *et al*., 2008).

3.8.2.3 Viral target genes and specific-primer pairs binding regions

A diagram of capsid genes with nucleotide position of primer binding regions of norovirus group I (NVGI), norovirus group II (NVGII), sapovirus (SV) and human astrovirus (HAstV) is shown in Figure 3.4(Yan*et al*., 2003). Two sets of primers G1SKF/G1SKR and COG2F/G2SKR were used for amplifying the partial capsid gene of NVGI and NVGII, which generated a 330 bp and a 387 bp PCR product, respectively. The SLV5317 and SLV5749 primers, which generated a 434 bp PCR product, were used for amplifying the partial capsid gene for all genogroups of SV. For HAstV, the primers PreCAP1 and 82b were used to generate a 719 bp PCR product of its partial capsid gene. The primer sequences and relative locations of the primers binding are shown in Table 3.1 (Yan*et al*., 2003).

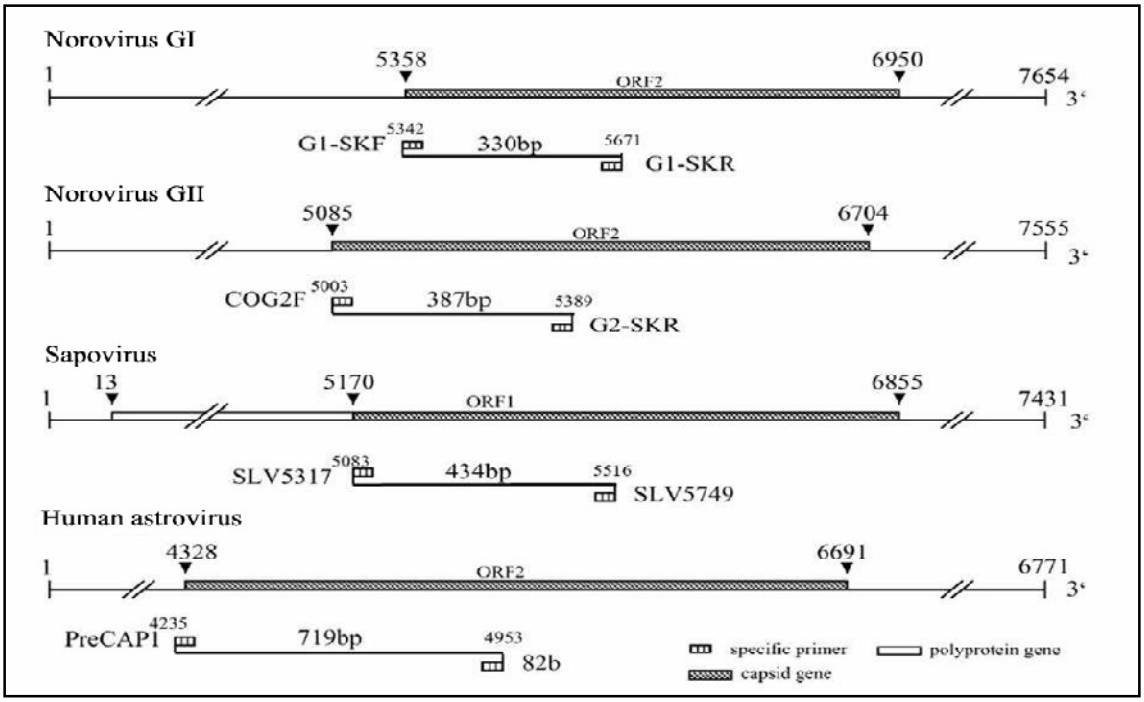

Figure 3.4: Diagram illustrating the amplification of partial capsid genes of NVGI, NVGII, SV and HAstV by PCR (Yan*et al*., 2003).

The above diagram indicated the positions of primer pairs relative to the plus sense strand of RNA genome as shown for representative strains Norwalk/68 (GenBank accession no. M87661), Lordsdale/93 (GenBank accession no. X86557), Manchester/93 (GenBank accession no. X86560) and human astrovirus serotype 1 Oxford (GenBank accession no. L23513) in NVGI, NVGII, SV and HAstV respectively.The arrows denote the location of the first AUG in the predicted ORF encoding the viral capsid protein. Capsid gene sites in ORF2 of NVGI and NVGII and HAstV genome are also indicated. In SV genome, the capsid gene is fused with a polyprotein gene in a single ORF (ORF1) as described in the text.

Table 3.1: Specific primers used for the detection of NVGI, NVGII, SV and HAstV

Virus	Primer	Polarity	Sequence (5' to 3')	Position (nt number)	Amplicon size (bp)
Norovirus Group I (NVGI)	GISKF*	+	CTGCCCGAATTYGTAAATGA	5342-5361	330
	GISKR*	-	CCAACCCARCCATTRTACA	5653-5671	
Norovirus Group II (NVGII)	COG2F*	+	CARGARBCNATGTTYAGRTGGATGAG	5003-5028	387
	G2SKR*	-	CCRCCNGCATRHCCRTTRTACAT	5367-5389	
Sapovirus (SV)	SLV5317*	+	CTCGCCACCTACRAWGCBTGGTT	5083-5105	434
	SLV5749*	-	CGGRCYTCAAAVSTACCBCCCCA	5494-5516	
Human Astrovirus (HAstV)	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	4235-4255	719
	82b	-	GTGAGCCACCAGCCATCCCT	4934-4953	

Source: Yan*et al*., 2003

*IUB codes: B = C, G or T; H = A, C or T; N = any base; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

+ Forward Primer

- Reverse Primer

3.8.2.4 Multiplex PCR Assay

Multiplex PCR was conducted using the cDNA from RT step as the template with specific mixed primer-sets as previously described(Malasao *et al.*, 2008). The primers (0.4µl each) were mixed together with 2.5µl of 10X Taq Polymerase Buffer (Applied Biosystems), 2 µl of 2.5 mM dNTPs,0.25 µl of 5U/µlAmplitaq DNA Polymerase (Applied Biosystems) and 12.05 µl of nuclease-free water to give a total volume of 20 µl for 1 sample reaction.then the amplification was performed for 35 cycles under the following thermal conditions: 94°C for 3 minutes to initiate denaturation, 94°C for 30 seconds, 55°C for 30 seconds and a final extension at 72°C for 1 minute and then the mixture was cooled at 4°C immediately. The respective PCR products were thendetected and analyzed using agarose gel electrophoresis.

3.8.2.5 Gel Electrophoresis

PCR products were subjected to electrophoresis on a 2% agarose gel, stained with 5µl Ethidium Bromide. 10µl of each PCR product was mixed with 1µl of gel loading buffer and loaded into a sample well on the gel made prior to its preparation. The voltage was set at 120 Volts and ran for 55 minutes. The size of the amplification products generated by NVGI, NVGII, SV and HAstV specific primers were identified by comparing with EZ Load Molecular 100bp DNA Molecular Ruler. These were also compared against reference strains from pooled positive samples (kindly provided by GEMS study, KEMRI-CDC, Kisumu). Nuclease-free water was used as the negative control. The

resultant gel was then viewed under UV light and images captured using a gel documentation system (UVP BioImaging Systems, Upland CA.,USA).

3.8 Data Management

3.8.1 Data Collection Tools

Identifier information was abstracted from the questionnaires which had been administered to the respondents seeking medical attention at LwakHospitalclinic in Asembo. This wasdone at the ICT department at CDC/KEMRI in Kisian, Kisumu. The data was delinked to remove the patient personal identifiers before analysis.

3.8.2 Data Storage

All the data collected including patient information and laboratory results were entered into a Microsoft excel database.

3.8.3 Data Processing and Analysis

Data processing involved abstracting patient information from questionnaires and entering them into a Microsoft excel database. Once all the information required was transferred, the data was checked to resolve any discrepancies of double entry. The final cleaned patient information database was merged with the laboratory results database using their unique identification numbers to ensure accurate matches. After successful merging, data analysis was conducted using Epi Info (version 3.5.2, CDC, Atlanta, GA,

USA) which has procedures to calculate frequencies, summarize data into tables and graphs and produce reliable confidence intervals.Confidence intervals were calculated using the Fisher's Exact method. A p-value of less than 0.05 was considered significant. The detailed results with their corresponding 95% confidence intervals are presented in appendix II.

CHAPTER FOUR

4.0 RESULTS

4.1 Study Population and Demographic Features

From January 2007 to June 2010, a total of 4,077 children aged below 14 years had with diarrhoea attended Lwak Mission Hospital (Figure 4.1). Only 342 of these children were sampled for the IEIP population-based morbidity surveillance study.

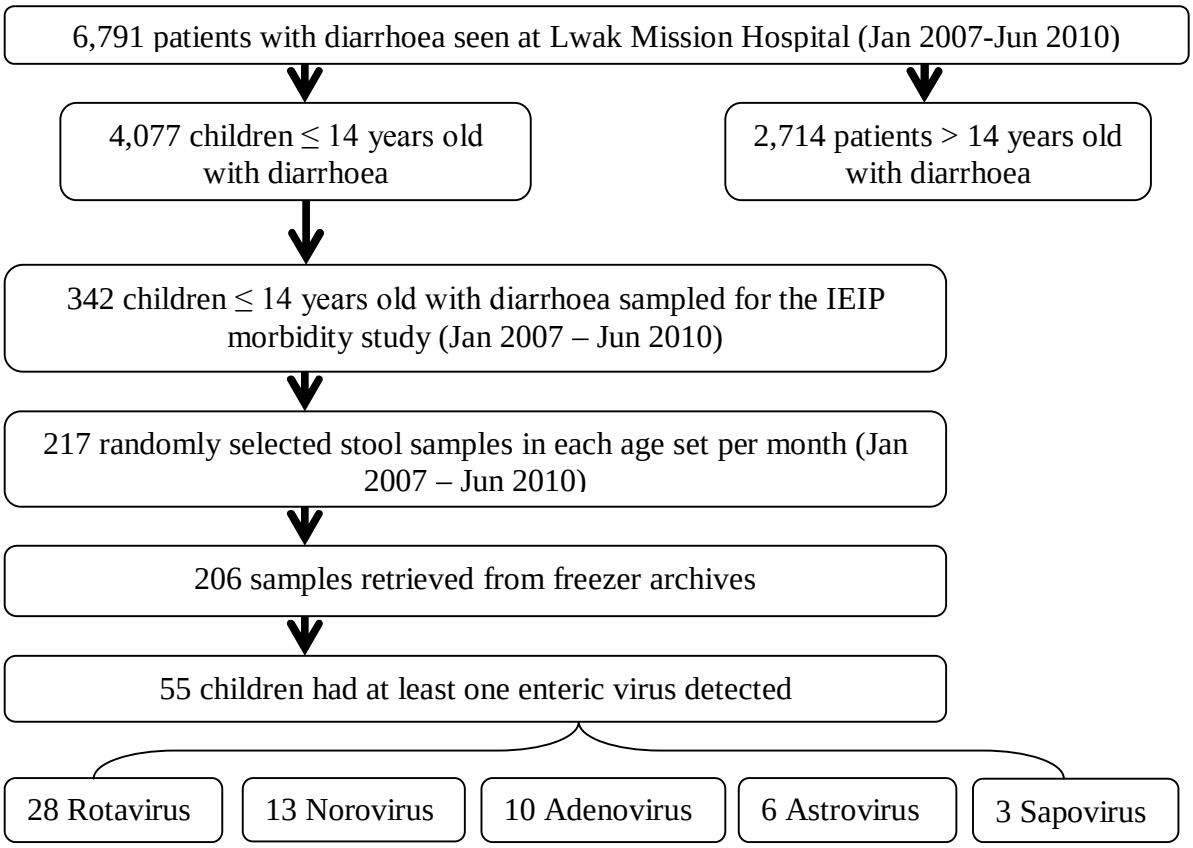

Figure 4.1: Overall coverage of study subjects

There were 217 samples randomly selected for the study, however, only 206 were able to be extracted from the freezers for testing as some stool samples were not enough.

The demographic information of all children, who presented to the Lwak clinic with diarrhoea during the study period, was collected according to their ages, gender and dates (Table 4.1). Children aged below two years of age constituted half (50.9%) of the total number of children who visited the hospital with diarrhoea during this period.

Table 4.1: Demographic data of study participants with diarrhoea from Western Kenya

	Number of children with diarrhoea		Number of specimen tested	
	N = 342	**n/N %**	**N = 206**	**n/N %**
Age group (years)				
0 - 2	174	50.88	92	44.66
3 - 5	67	19.59	37	17.96
6 - 8	35	10.23	26	12.62
9 - 11	26	7.60	23	11.17
12 - 14	40	11.70	28	13.59
Sex				
Males	174	50.88	111	53.88
Females	168	49.12	95	46.12
Time Period				
Jan 07 - Jun 07	20	5.85	13	6.31
Jul 07 - Dec 07	44	12.87	23	11.17
Jan 08 - Jun 08	36	10.53	18	8.74
Jul 08 - Dec 08	62	18.13	42	20.39
Jan 09 - Jun 09	46	13.45	27	13.11
Jul 09 - Dec 09	59	17.25	36	17.48
Jan 10 - Jun 10	75	21.93	47	22.82

The yearly number of children who attended the hospital with diarrhoea varied, with peaks between July and December of each year. However, there was a steady increase in attendance of children with diarrhoea during the 3 ½ year period.

The total number of children randomly selected for the study was evenly distributed within each age set and monthly period.The ages of the children varied from 3 months to 14 years while the mean was 4.6 years and standard deviation, 0.67. The ratio of males to females was 1.2:1 as males accounted for 53.9%. During the morbidity surveillance follow-up studies, there were 15 deaths recorded from unknown causes. Among the 206 samples selected, six were already dead prior to this study.

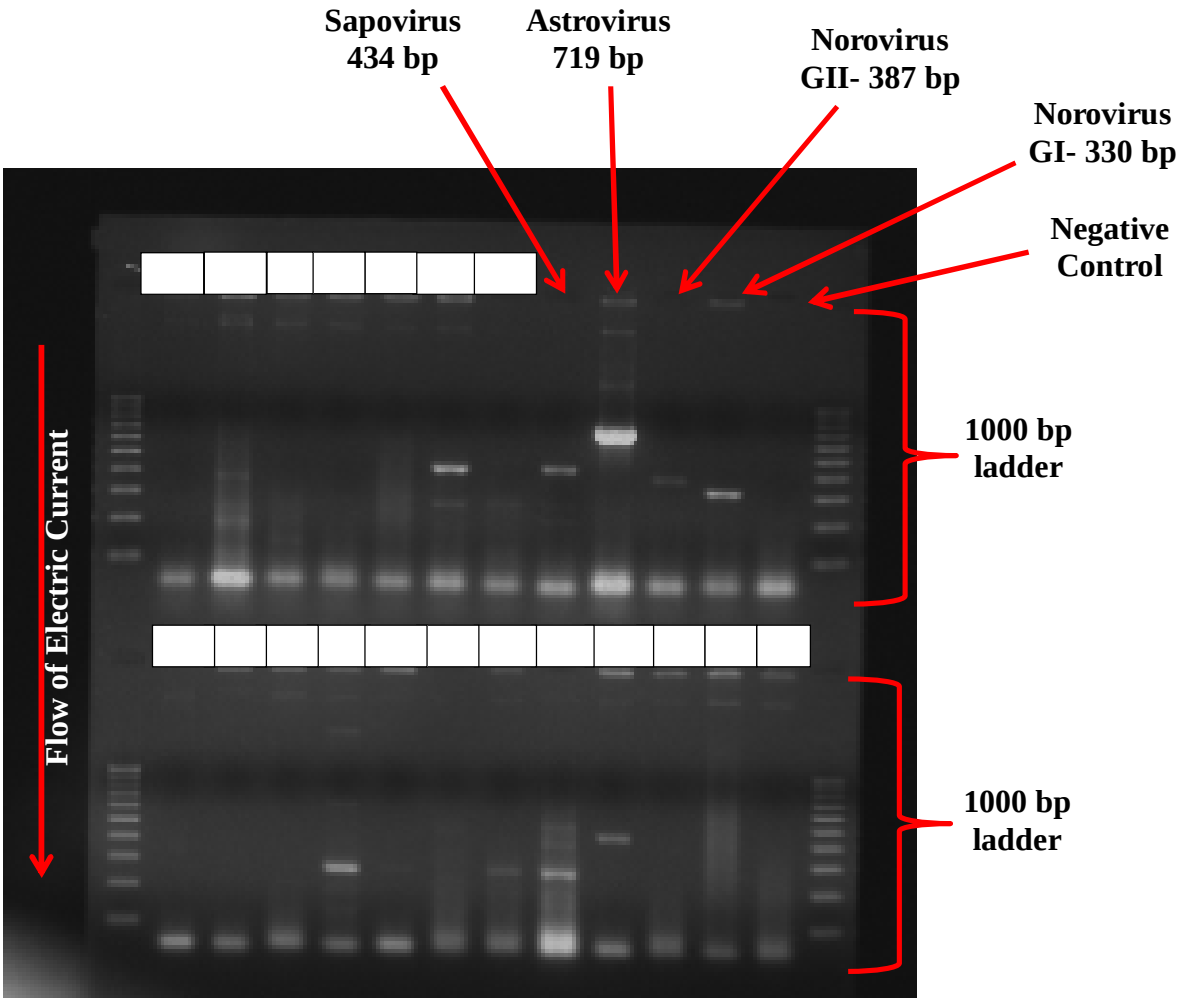

Figure 4.2: A two dimensional view of a gel electrophoresis run captured using a UVP BioImaging gel documentation system on the 18th of August 2010. Each gel run contained 19 samples (1- 19) loaded in two rows. The upper row contained both the negative and positive (NVGI, NVGII, AstV and SV) controls for each gel run. A 1000 bp ladder was loaded on the flanking end wells of each gel for both the upper and bottom rows as seen in the picture above. A clear band without smears was considered an amplified DNA .For those that had smears, a repeat gel electrophoresis run was done to confirm the result.

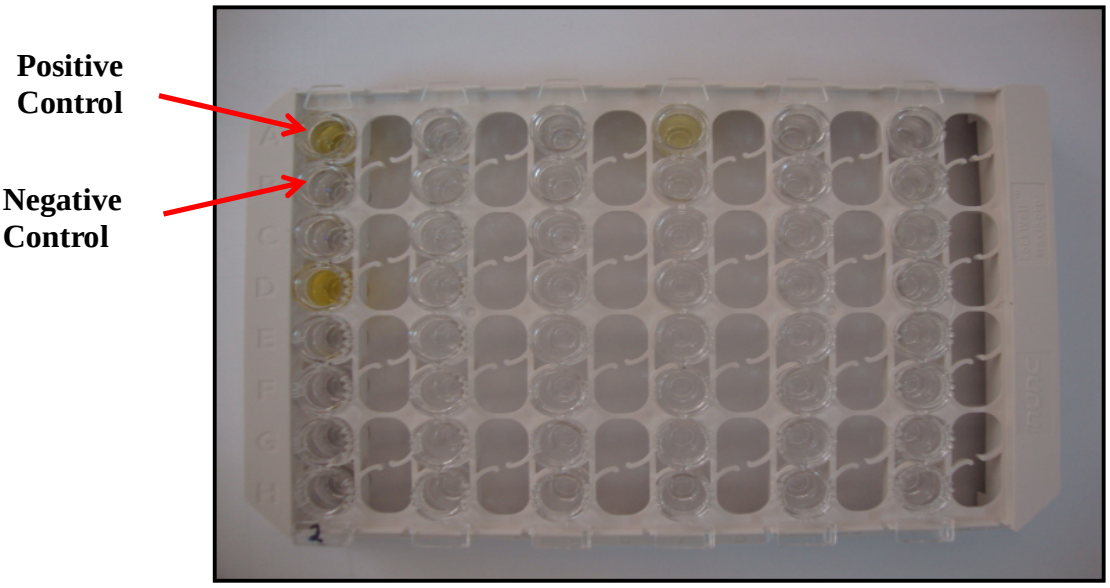

Figure 4.3: An aerial view picture of an ELISA plate assay taken on the 20th of August, 2010. Each plate had 45 samples for every assay run including a positive and negative control. After a complete assay, positive samples for the specific target virus turned yellow as seen in the diagram after a addition of stop solution.

4.2 Prevalence of Enteric Viruses in children ≤ 14 years with diarrhoea from Lwak hospital, Western Kenya, 2007-2010

Diarrhoea viruses were detected in 26.7% (95% CI: 20.79, 33.29) of the samples tested. Among the diarrhoeal viruses detected, rotavirus was the most prevalent (13.59%), followed by norovirus (6.31%), adenovirus (4.85%), astrovirus (2.91%) and sapovirus (1.46%) respectively (Figure 4.4).

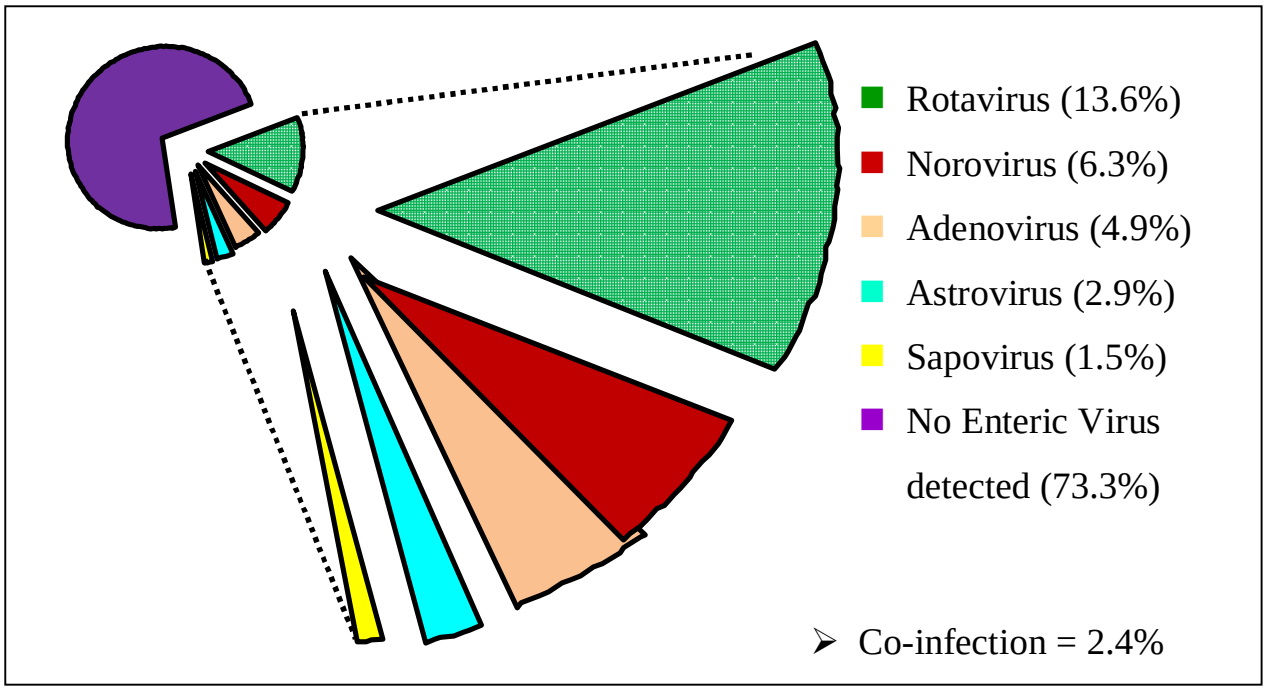

Figure 4.4 Percentage prevalence of enteric viruses detected in children ≤ 14 years with diarrhoea, Western Kenya, 2007- 2010

Among the rotaviruses detected, all were Group A rotaviruswhereas for the norovirus positive samples, 9/13 were norovirus group I and 4/13 were norovirus group II.There were 5/206 samples that tested positive for two different viruses each. The combinations

included rotavirus and norovirus (1/5), rotavirus and astrovirus (2/5), rotavirus and adenovirus (1/5) and norovirus and adenovirus (1/5). These children, who had dual infections, were aged below five years, only one of them was 11 years.

Children below two years had more enteric viruses detected (32.6%) than those aged between 12 and 14 years old (25%)(Figure 4.5). Interestingly, children aged 12 – 14 years had more viral infections than those aged 9 – 11 years, 25% and 13% respectively. Rotavirus was evidently highest among children aged 0 and 2 years (19.6%) and those aged 12 and 14 years (17.9%). Norovirus was highest in children aged 3-5 years (10.8%). There was no sapovirus detected in children aged 3-5 and 9-11 years. Likewise, children aged 9-11 years had no astrovirus detected while those aged 12-14 years were the only children without adenovirus detected in their stools.

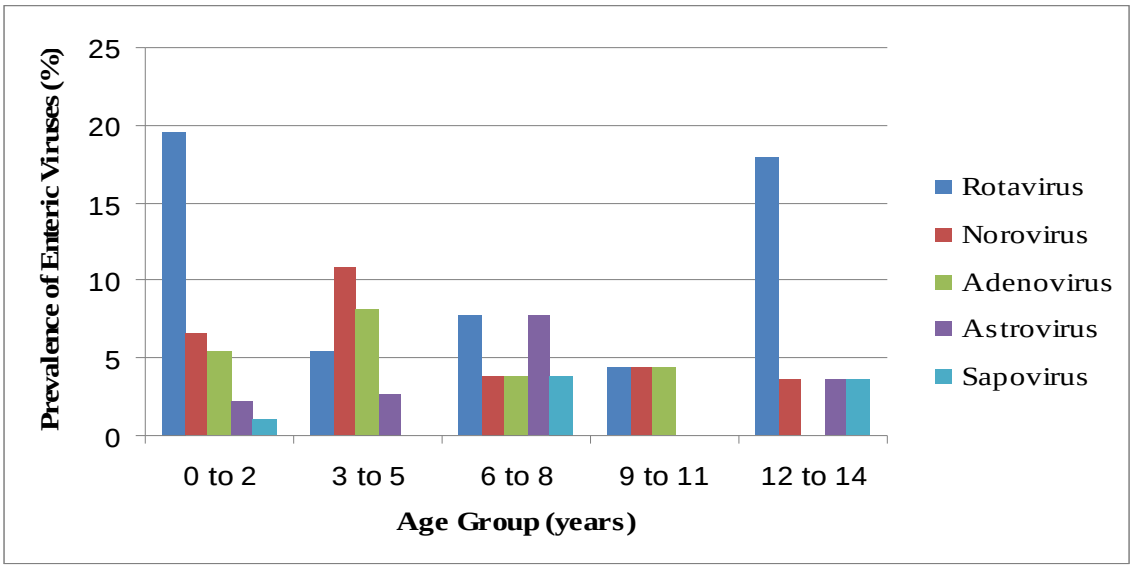

Figure 4.5 Distribution of enteric viruses in children with diarrhoea in different age groups

Table 4.2 shows the distribution of all the enteric viruses detected in both males and females. Out of the total number of specimen tested from children with diarrhoea, male children had a higher proportion of enteric viruses detected in them (28.83%) than females.

Table 4.2 Prevalence of Enteric Viruses detected in children by gender

			Overall viruses detected in stool samples = 55 (26.70%)				
	Number of specimen tested (N)	**Total number of viruses detected n/N %**	**Rota n (%)**	**Noro n (%)**	**Adeno n (%)**	**Astro n (%)**	**Sapo n (%)**
Sex							
Males	111/206	32 (28.83)	15 (13.51)	9 (8.11)	7 (6.31)	3 (2.70)	1 (0.90)
Females	95/206	23 (24.21)	13 (13.68)	4 (4.21)	3 (3.16)	3 (3.16)	2 (2.11)

4.3 Epidemiology of Diarrhoea viruses in Western Kenya, 2007-2010

There were no consistent distinct peaks throughout the 3 ½ years to show seasonal infections for any of the five viruses detected (Figure 4.4). Rotavirus was highest in most half year periods, however, in 2008 between July and December, norovirus recorded a higher prevalence than rotavirus 11.9% and 14.3% respectively. Sapovirus was not detected in 2007 as well as astrovirus in 2010. Children with norovirus I had this infection at different times of the year while those with norovirus II were during the months of June to September.

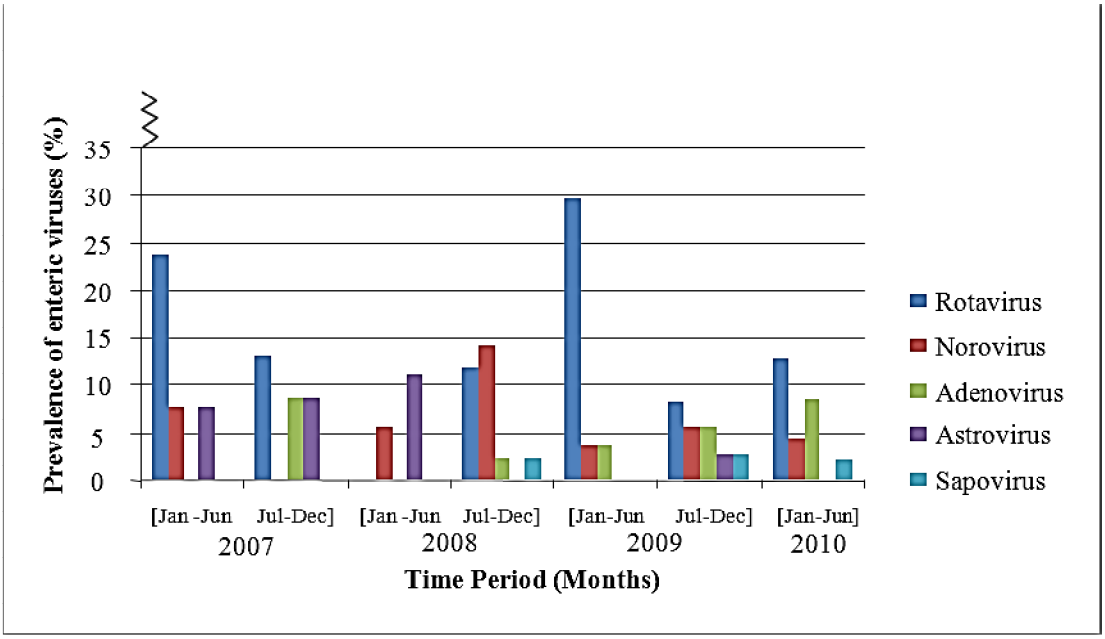

Figure 4.6: Distribution of enteric viruses in children with diarrhoea over time

4.4 Clinical Characteristics of children who presented with diarrhoea at Lwak hospital in Western Kenya, 2007- 2010

Almost four-fifths (79.1%) of the study children had diarrhoea with a duration of 1 to 3 days (Table 4.3). Some children had longer duration but only 3.9% had diarrhoea which lasted more than a week.

Table 4.3 Clinical features presented by children ≤ 14 years with diarrhoea

	Number of Case Patients	
	N = 206	**n/N %**
Clinical Features		
Vomit	78	37.86
Fever	162	78.64
Cough	106	51.46
Number of Diarrhoea Days		
1 to 3	63	79.13
4 to 6	19	9.22
≥ 7	8	3.88
Unknown	16	7.77

Most children had at least one clinical feature associated with diarrhoea. The most common clinical symptoms were fever (78.6%) and cough (51.5%). About a third(28.6%) of these children were admitted due to the severity of the diarrhoeal disease during this study period, with the rest being treated and discharged.

In all age groups, fever was the most common clinical feature, followed by cough then vomiting (Table 4.4). This was predominant in children aged 3 – 5 years (83.8%). Vomiting on the other hand was experienced mostly by children aged 6 – 8 years (46.2%) compared to those aged below two years of age (45.7%) (p value = 1). Male children generally had higher frequency of presentation with these clinical features than females, though females had a higher percentage of cough (54.7%). This was not significant to the number of male children with a cough (p value = 0.4645).

Table 4.4 Distribution of clinical features in children with diarrhoea by age and sex

		Clinical Features n/N (%)		
	Case Patients N (%)	**Vomit**	**Fever**	**Cough**
Age group (years)				
0 – 2	92 (44.66)	42 (45.65)	74 (80.43)	54 (58.70)
3 – 5	37 (17.96)	7 (18.92)	31 (83.78)	20 (54.05)
6 – 8	26 (12.62)	12 (46.15)	20 (76.92)	10 (38.46)
9 – 11	23 (11.17)	9 (39.13)	18 (78.26)	12 (52.17)
12 – 14	28 (13.59)	8 (28.57)	19 (67.86)	10 (35.71)
Sex				
Males	111 (53.88)	43 (38.74)	89 (80.18)	54 (48.65)
Females	95 (46.12)	35 (36.84)	73 (76.84)	52 (54.74)

4.5 Clinical characteristics associated with enteric viruses detected in children with diarrhoea from Western Kenya, 2007-2010

Children exhibited other symptoms of vomit (37.9%), fever (78.6%) and cough (51.5%) in addition to diarrhoea. Most children presented with fever and of the total number with this symptom, 30.3% had a viral infection. Rotavirus was the most prevalent virus detected among those with fever (14.2%).Those who had rotavirus detected in their stools presented more clinical features in addition to diarrhoea compared to other viruses (Figure 4.7). Sapovirus was associated with the least number of clinical features in these children.

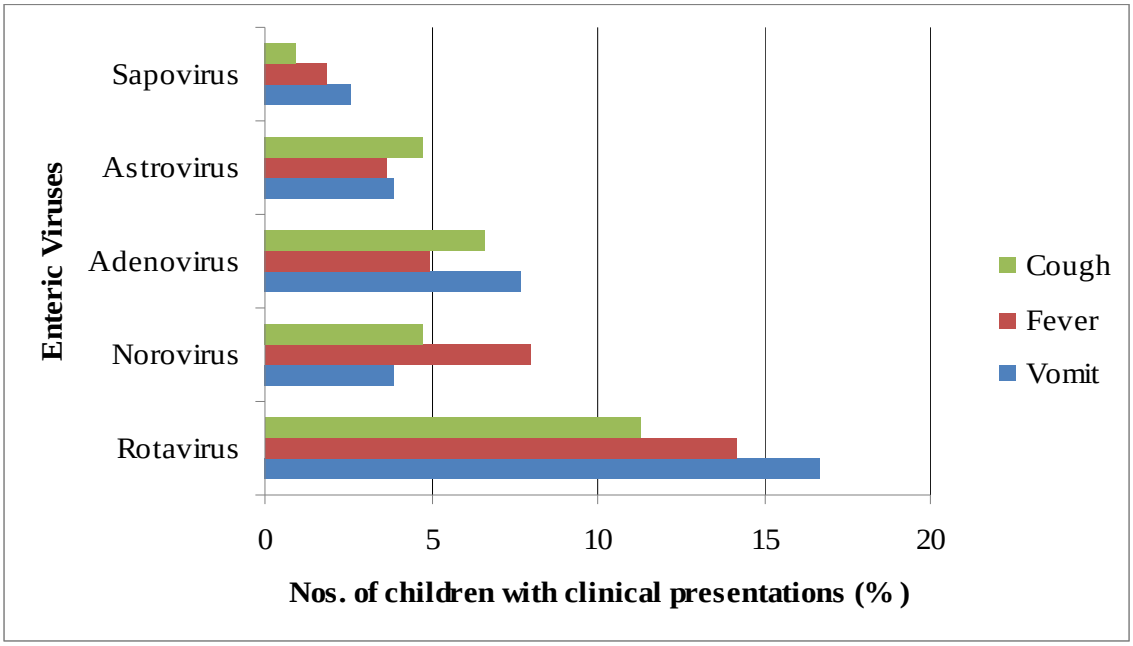

Figure 4.7 Proportions of children presenting with a clinical presentation accompanying an enteric virus isolation

Enteric viruses were detected more in children with shorter diarrhoea days between 1 and 3 days (Table 4.5). No virus was detected in children with diarrhoea lasting between seven and more days.

Table 4.5 Estimation of enteric viruses detected with prolonged diarrhoeal days in children, Western Kenya

		Viral Etiologies n (%)				
Number of Diarrhoea Days	**Number of case patients N = 206**	**Rota**	**Noro**	**Adeno**	**Astro**	**Sapo**
1 to 3	163 (79.13)	25 (15.34)	10 (6.13)	8 (4.91)	5 (3.07)	3 (1.84)
4 to 6	19 (9.22)	3 (15.79)	2 (10.53)	2 (10.53)	1 (5.26)	0 (0.00)
≥ 7	8 (3.88)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Unknown	16 (7.77)	0 (0.00)	1 (6.25)	0 (0.00)	0 (0.00)	0 (0.00)

Among those who had all the three clinical features (vomit, fever and cough), the prevalence of viral infection was 32.4% (Table 4.6). However, there were a higher number of children who had fever and cough as combined symptoms (43.7%). Among them, 26.7% had a virus detected of which rotavirus (11.1%) was the highest, followed by adenovirus (6.7%).

Table 4.6 Children with multiple clinical features associated with enteric viruses detected in children with diarrhoea

			Viral Etiologies n (%)				
Clinical Features	**Number of case patients N = 206**	**Total number of viruses detected n/N %**	**Rota**	**Noro**	**Adeno**	**Astro**	**Sapo**
Vomit + Fever	62 (30.10)	20 (32.26)	10 (16.13)	3 (4.84)	4 (6.45)	3 (4.84)	2 (3.23)
Vomit + Cough	40 (19.42)	13 (32.5)	5 (12.50)	2 (5.00)	4 (10.00)	2 (5.00)	1 (2.50)
Fever + Cough	90 (43.69)	24 (26.67)	10 (11.11)	5 (5.56)	6 (6.67)	5 (5.56)	1 (1.11)
Vomit + Fever + Cough	34 (16.50)	11 (32.35)	4 (11.76)	2 (5.88)	3 (8.82)	2 (5.88)	1 (2.94)

4.6 Distribution of enteric viruses in children with diarrhoea from Western Kenya, Jan 2007- June 2010

Amongst those who tested positive for any of the five enteric viruses, the GIS map shows their distribution alongside the rivers flowing into the mainland from Lake Victoria (Figure 4.8). It is also evident that most study participants who visited Lwak hospital during this study period with diarrhoea came from areas close to the rivers. All children who had an enteric virus detected in their stool, came from different areas within the study area. There was no distinct cluster of a specific virus.

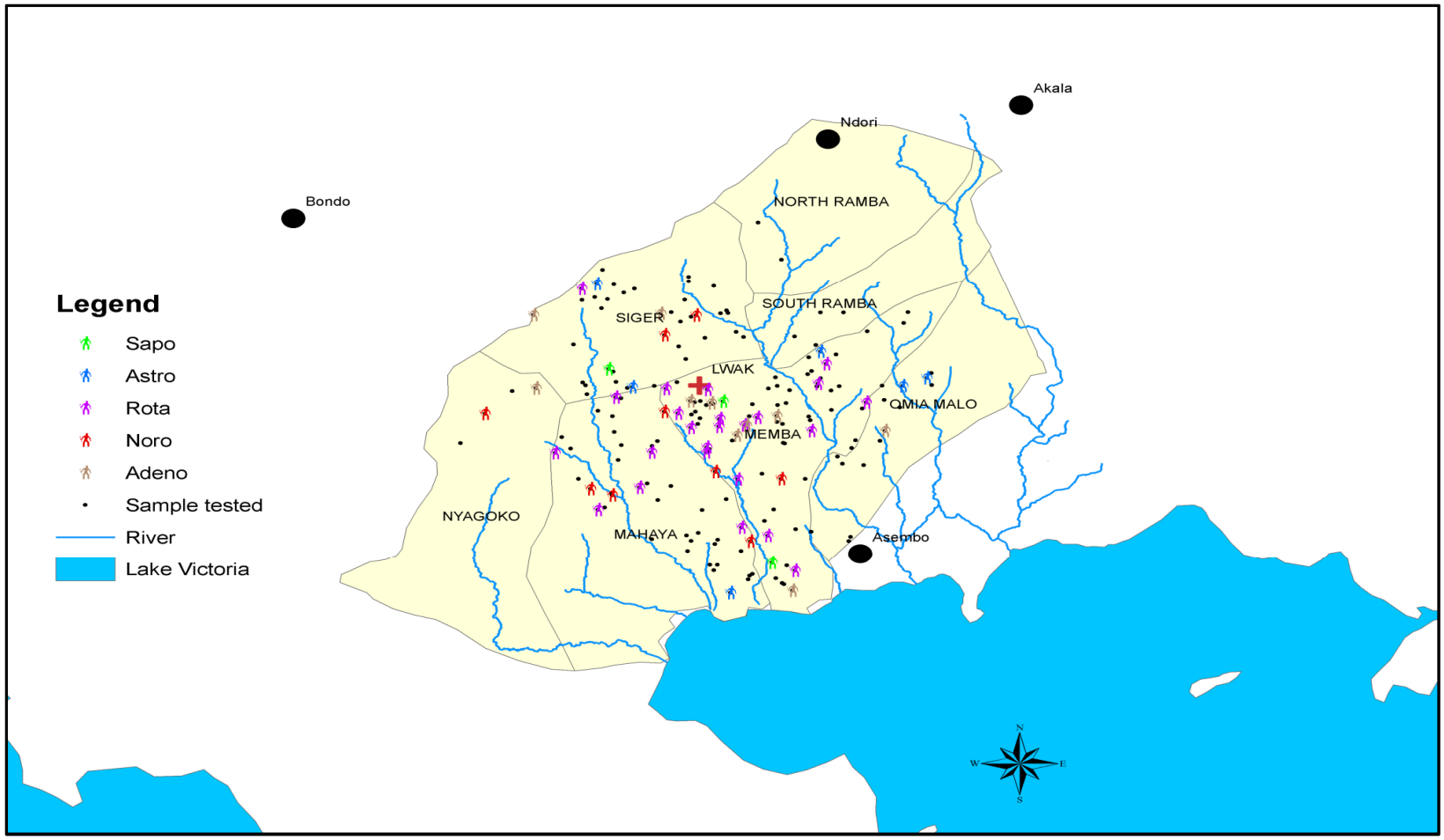

Figure 4.8: GIS Map of study participants showing their distribution within the study area of Asembo in Western Kenya. (Source: KEMRI/CDC: Unpublished Data)

CHAPTER FIVE

5.0 DISCUSSION

Diarrhea is one of the major health problems confronting children in low-income societies. Enteric viruses, mainly rotavirus, norovirus, adenovirus, astrovirus and sapovirus, have been noted to cause a significant proportion of viral diarrhea in infants and young children worldwide. Studies on viral etiologies of diarrhoea in Kenyan children, has mostly focused on rotavirus. This focus has mainly been driven by the fact that it is still responsible for a greater proportion of diarrhoeal deaths in Africa (Armah *et al.,* 2010).

In this study, enteric viruses were detected in 26.7% of stool samples in children less than 14 years of age. Thisis slightly lower than what was reported from a study in Tanzaniawhere the prevalence was 32.2% (Moyo *et al.,* 2007). The Tanzania study included only hospitalized children under five years old from Dar es Salaam.This could, therefore explain the higher rates in the Tanzania study due to possible selection of more ill children as opposed to this current study where both mild and severe diarrhea cases were enrolled into the study. This is the only study to date done in East Africa of at least four enteric viruses as causative agents of diarrhoea. All other studies that have been carried out in Kenya mainly include two viruses at most (Nakata *et al.,* 1998; Kiulia *et al.,* 2007; Nokes *et al.,* 2008; Tate *et al.,* 2009; Armah *et al.,* 2010; Magwalivha *et al.,* 2010). Other similar studies of enteric viruses that have been carried out worldwide in the recent past indicated a prevalence of 59% in Germany (Oh *et al.,* 2003), 37% in

France (Marie-Cardine *et al.,* 2002) and 37.2% in Japan (Nakanishi *et al.,* 2009). There are a number of possible reasons as to the differences in the number of enteric viruses detected in children. First, the total number of children recruitedand their ages are different in various studies. The age group in this study was 0 and 14 years where the number of children used had to be stratified to obtain a near equal distribution. These other studies concentrated on children under five years of age who were hospitalized. Secondly, tropical countries hardly experience seasonal weather patterns as compared to tropical countries. Thus, countries like Germany and France are bound to have seasonal peaks and outbreaks depending on the weather patterns. Other reasons could be the use of more sensitive methods such as molecular biology techniques, for viral detection in stool samples. PCR techniques are considered useful for confirmation of the results of other techniques and also genotyping (Wilhelmi *et al.,* 2003).

Rotavirus and adenovirus were detected in 13.6% and 4.9% of stool samples respectively using EIA technique. Likewise, norovirus, astrovirus and sapovirus were detected in 6.3%, 2.9% and 1.5% of stool samples respectively using RT-PCR assay. As in many other countries, Group A rotavirus was found to be the most common enteric viral pathogen associated with diarrhoea in children from Western Kenya between January 2007 and June 2010. Several studies in Kenya (Nokes *et al.,* 2008; Sanchez-Padilla *et al.,* 2009; Tate *et al.,* 2009; Widdowson *et al.,* 2009) have demonstrated the important role rotavirus plays in diarrhoeal disease in children. This highlights the socio-economic importance of effective prevention, including the current efforts in the

development and introduction of rotavirus vaccines e.g., Rotateq®. Most studies have always incorporated RT-PCR as a more sensitive diagnostic and laboratory test technique though there are others where EIA, electron microscopy or latex agglutination has been used (Sanchez-Padilla *et al.,* 2009). This is possibly, one of the main contributors to the difference in rotavirus prevalence in different countries worldwide due to the difference in sensitivities (Oh *et al.,* 2003).

The second-most frequent enteric viral pathogen was norovirus, which was detected at a higher rate (6.3% of all stool samples) than it was among children who visited outpatient clinics in three districts in Kenya from August 1991 to July 1994 (0.1% of all stool samples) (Nakata *et al.,* 1998). These test results confirm recent observations indicating that norovirus is the second most common enteric virus among young children (Oh *et al.,* 2003; Moyo *et al.,* 2007; Levidiotou *et al.,* 2009; Li *et al.,* 2009; Nakanishi *et al.,* 2009).However, this proportion is lower than what has been reported, that norovirus is responsible for 10-15% of all diarrhoeal cases in both developed and developing countries (Moyo *et al.,* 2007). Norovirus occurs predominantly in adults and causes epidemic outbreaks (Wilhelmi *et al.,* 2003). Therefore, the detection rates could indicate either a seasonal variation or the absence of an epidemic during this study period.

Among the norovirus-infected children, norovirus group I was predominant (4.4%) compared to norovirus group II with 1.9% of the total stool samples tested. This finding isinconsistent to the prevalence reported by other studies(Clark and McKendrick,

2004;Victoria *et al.,* 2007; Malasao *et al.,* 2008; Nguyen *et al.,* 2008; Bruggink and Marshall, 2009; Xu *et al.,* 2009; Barreira *et al.,* 2010) indicating norovirus group II as the most prevalent. The discrepancy of the prevalent rates between this study and others might be due to the difference in the duration and/or geographical area where those studies have been conducted.

During the present study period, adenovirus serotypes 40/41 were responsible for 4.9% of the diarrhoea in children from Western Kenya. These results corresponded to that found in another recent study in Tunisia (2.3%) also relying on EIA diagnosis (Sdiri-Loulizi *et al.,* 2009). There is however a difference in detection rates in developing countries of adenovirus infection which varies widely between 2.8% in Vietnam and 31.2% in Guatemala (Sdiri-Loulizi *et al.,* 2009). Another recent study that was also carried out in Kenya reported a much higher prevalence of 37.4% using nested PCR, a technique that reportedly detects all described human adenovirus species compared to the group or species specific EIAs (Magwalivha *et al.,* 2010). This reinforces the limitations of the immunological-based methods for adenovirus detection. Thus, published data on adenovirus should be interpreted based on the test methods (Magwalivha *et al.,* 2010).

The role of astrovirus in severe diarrhoea has not been well documented. However, it is considered the third causative agent after rotavirus and norovirus (Sdiri-Loulizi *et al.,* 2009). This is not quite in line with this study where astrovirus (2.91%) was the fourth

viral pathogen detected in these children with diarrhoea from Western Kenya. It however corresponded to prevalence studies reported in two African countries, Madagascarat 2.1% (Papaventsis *et al.,* 2008) and Kenyaat 6.3% (Kiulia *et al.,* 2007).

Data from this study revealed that the detection rate for sapovirus at 1.46%, was just as low as from previous studiesin Hong Kong at 1% (Li *et al.,* 2009),in Japan at 1.2% (Nguyen *et al.,* 2008), in Kenya at 2.2% (Nakata *et al.,* 1998), in Bangladesh at 2.7% (Dey *et al.,* 2007) andin Thailand at 3.4% (Malasao *et al.,* 2008).Globally, sapovirus causes sporadic cases and diarrhoea outbreaks in children with a prevalence ranging from 0.3% to 9.3% (Dey *et al.,* 2007). It is of note that detection of sapovirus in this study in comparison to the low detection rates worldwide, confirms its importance as a causative agent of diarrhoea in Kenyan children.

Co-infections were found in five (2.4%) samples, four of which were combinations of rotavirus and one of the other viruses. This is similar to already published data from previous studies where mixed rotavirus infections range between 1% and 44.6% of the samples in most cases (Roman *et al.,* 2003; Moyo *et al.,* 2007; Victoria *et al.,* 2007). There are suggestions that these co-infections may lead to long term viral shedding after clinical recovery thereby, when the child is infected again, another different virus maybe detected (Victoria *et al.,* 2007).During such incidences, one virus may be solely causing the disease while the other is still being shed (Oh *et al.,* 2003). This makes it difficult to establish which of the two viruses, is the most important etiologic agent. Incidentally, all

the five children had clinical symptoms recorded of either, vomit, fever or cough with diarrhoea days ranging from 1 to 5 days. This study however did not concentrate on the clinical severity scores in comparison to the co-infections, which previous studies have reported that there is no significance between the severity of diarrhoeal illness and mixed infections (Oh *et al.*, 2003).

Such cases of dual or multiple viral infections have been attributed to poor sanitation and hygiene (Victoria *et al.*, 2007), case in point, rural Western Kenya. According to a study carried out by Feikin *et al.* (2011), there is a low number of healthcare utilization by those residing in rural parts of Kenya than those from urban centers. A lack of proper healthcare results to a decrease in the general hygiene of children. There is therefore a possibility of a higher proportion of co-infections among these children residing in rural Western Kenya than what was observed. Differences found between this study and other studies may also be explained, at least in part, due to the fact that there were two different detection techniques used, EIA and RT-PCR as previously mentioned.Roman*et al.*(2003) reported high incidence of dual infections during autumn than other seasons of the year. This cannot be concluded in this study as there were no distinct seasonal differences in viral detection. Two of the dual infections were samples collected in 2007, two in 2008 and one in 2009 all in different months of the year.

Most studies show that mixed infections are less frequent than mono-infections, though the rate of co-infections varies widely in literature (Roman *et al.*, 2002; Oh *et al.*, 2003;

Moyo *et al.*, 2007; Victoria *et al.*, 2007; Barreira *et al.*, 2010). The association of co-infections in previous studies varies as there are different types including virus-virus, virus-bacteria, virus-parasite, bacteria-parasite, bacteria-bacteria and/or parasite-parasite thus making it difficult to compare incidences. Other variables such as patient age, hygiene, seasonal peaks or detection methods may also explain the differences detected.

Concerning the epidemiology of diarrhoea viruses among children from Western Kenya, the prevalence of viral diarrhoea was low in infants aged 0-6 months (2.9%), increased in infants aged 7-12 months (5.3%), peaked in infants aged 13-24 months (6.8%) and declined again among children of 25-60 months (5.3%) and furthermore, progressively till the age of 14 years of age (0.5%). The relatively low prevalence of viruses among older children could be partly due to immunity acquired through previous exposures. As for those who are younger (children less than 5 years of age), and have an enteric viral infection, adequate breast-feeding is questionable as this is their main source of maternal acquired immunity.

The prevalence of rotavirus infection was highest among children aged below two years (19.6%). According to this study's findings, infants aged below two years living within the IEIP study area frequent Lwak hospital more than older children. This could be a factor as to the high prevalence observed in this age group compared to others. A study in coastal Kenya by Nokes *et al.* (2008) reported similar findings. It is of note too that the prevalence of rotavirus infection in children aged 12 to 14 years was 17.9% slightly

lower than those aged below two years (p value = 0.1038). Rotavirus is considered to be age dependent infecting mostly infants and young childrenbelow five years of age (Bernstein, 2009). However, it is also believed to cause disease at any age including adults (Anderson and Weber, 2004).

Norovirus and adenovirus had the highest prevalence in children aged 3 to 5 years, 10.8% and 8.1% respectively. These findings differ from the Tanzanian study by Moyo *et al.* (2007) where children aged 6 to 12 years had the highest prevalence of norovirus (54.2%) and adenovirus (85.7%). There was no adenovirus detected in children aged between 3 and 5 years in Tanzanian children (Moyo *et al.*, 2007). A possible explanation to this difference is the geographical distribution and circulation ofthese viruses among these children.

Similarly, astrovirus (7.7%) and sapovirus (3.9%) both had the highest prevalence in children aged 6 to 8 years. These findings do not agree with earlier studies that have been carried out in Kenyan children (Kiulia *et al.*, 2007; Nakata *et al.*, 1998). In relation to comparing children below five years old and those above five years, the prevalence observed in this study does correlate with those from previous studies. A study on astrovirus reported a general prevalence of 5.3% in children aged ≤ 5 years which was higher than those aged ≥ 5 years (0.2%)(Kiulia *et al.*, 2007).

During the months of January to June of the present study period, the total number of enteric viruses detected was lower each year than what was detected between July and December. In 2009, the difference was much more distinct with 3.3% of all stool samples between January and June and 25% between July and December. The following year in 2010, during the first half of the year, the prevalence was at 25%. Thus if this is the trend each year, then the next half of the year and thereafter, will probably have an increase in the number of enteric viruses.However, this increase in percentage of enteric viruses each year could be explained by the increase in awareness of the importance of medical attention at Lwak hospital by the residents. This is so as the number of children who visited Lwak hospital recorded a gradual increase since the IEIP study began.

Every year, each enteric virus demonstrated different peaks therefore, there was no clear seasonality observed. This is probably typical of most tropical countries lying close to the equator where there are no distinct seasonal patterns as there are in temperate countries (Dennehy, 2008). Temperate countries have autumn and winter when most enteric viruses are bound to be prevalent or causing an outbreak. Kenya experiences cooler months during the long and short rainy seasons of the year between March and May and July and September respectively. Hence, if there is any seasonality to be observed, it is during these cooler times of the year, though this is not well documented (Bernstein, 2009). In 2007, rotavirus occurred during the months of May and June and again in September and October. This was the same again only in 2010 with peaks

between May and June. Also, there was no enteric virus outbreak recorded during this study period thus explaining the lack of seasonal peaks.

From the clinical perspective, vomiting and fever were the major clinical manifestations, observed in 30.8% and 30.3% of the children respectively. It has been clinically demonstrated that these symptoms are important manifestations of both rotavirus and norovirus infection, in addition to severe diarrhoea (Chen *et al.*, 2007; CDC, 2009; Nakanishi *et al.*, 2009; Barreira *et al.*, 2010).In this study, among children who had rotavirus, a higher percentage vomited than those who had fever whereas, among those who had norovirus, fever was higher. Incidentally, children who were infected with either sapovirus or adenovirusalso had a higher percentage of vomit, 7.7% and 2.6% respectively than other symptoms.This report is in agreement with Dey *et al.* (2007) who found vomiting as a common clinical feature in 76% of the children who had sapovirus infection. In most studies that have been done on enteric viruses, rotavirus is considered the most prevalent pathogen leading to a greater number of diarrhoeal hospitalizations in children under 5 years of age (Oh *et al.*, 2003; Moyo *et al.*, 2007; Nokes *et al.*, 2007; Li *et al.*, 2009). This corresponds to the present study where among those who were admitted, 13.6% had rotavirus, followed by norovirus (6.8%), astrovirus (6.8%) and adenovirus (3.4%). None of these children who were admitted had sapovirus detected in them. These findings also correspond to what Chen *et al.* (2007) found using a disease severity score test indicating rotavirus to be the most severe of the enteric viruses. However, considering the study was done in Taiwan, a different geographical setting and

probably different enteric virus serotypes circulating, norovirus was found to have the least disease severity score, unlike the present study where norovirus was second.

In the final part of the study, a GIS map was constructed and used to show the distribution of the enteric viruses that were detected and also the locations of all the study participants. According to these findings, the viruses detected were from children who were mostly from areas next to the rivers. This is in line to findings from other studies which have detected enteric viruses from water sources and sewages (Seigel *et al.*, 1996; Mølbak *et al.*, 1997 Yasin *et al.*, 2000; Kamel *et al.*, 2009; Scarcella *et al.*, 2009). These were mostly case control studies carried out from various regions. According to these past studies, important interventions that need to be carried out to prevent diarrhoeal diseases include amongst others improvements in water supply, hygiene and food handling (Flint *et al.*, 2005; Greening 2006). Enteric viruses are usually resistant to environmental stressors. They are able to survive by attaching themselves to sediments or particulate matter found in water (Greening, 2006).

STUDY LIMITATIONS

A major limitation was the inability to obtain all the 335 stool samples as per the calculation in the expected sample size. One possible explanation is the number of diarrhoea cases who visited Lwak hospital was lower at the beginning of the IEIP surveillance study in 2005 though each year it has been increasing gradually.

A further limitation was the use of two different viral detection techniques, EIA and RT-PCR. Therefore, comparison of these figures with previous information where most studies have incorporated the use of more advanced molecular techniques is difficult.

Amongst the stool samples from children that were randomly selected for this study, six children were already dead prior to the present study. The reason for their death is however unknown despite one of them testing positive for sapovirus.

CONCLUSIONS

1. This study has demonstrated a high prevalence of enteric viruses in the stools of children from rural Western Kenya with diarrhoea, with a considerable proportion of co-infections.

2. There was no seasonal pattern observed throughout the 3 ½ year study period and neither was there an outbreak.

3. The disease burden and severity of diarrhoea was associated with clinical symptoms of diarrhoea (vomit and fever) in children which differed from virus to virus.

RECOMMENDATIONS

1. Routine testing for all enteric viruses should be warranted especially norovirus, it being the second most frequent viral causative agent. The other three viruses should also be included in the various surveillance studies being carried out in both the rural and urban Kenyan children with diarrhoea.The presence of dual infections also warrants further epidemiological and pathogenic research especially using the more advanced molecular techniques for detection.

2. Routine surveillance of all enteric viruses will eventually give a clear picture on the seasonality. This study involved only randomly selected stool samples from children with diarrhoea within a 3 ½ year period.

3. Further studies on disease severity scores should be conducted on all the five viruses to give a clearer picture from Kenyan children both in the rural and urban areas.

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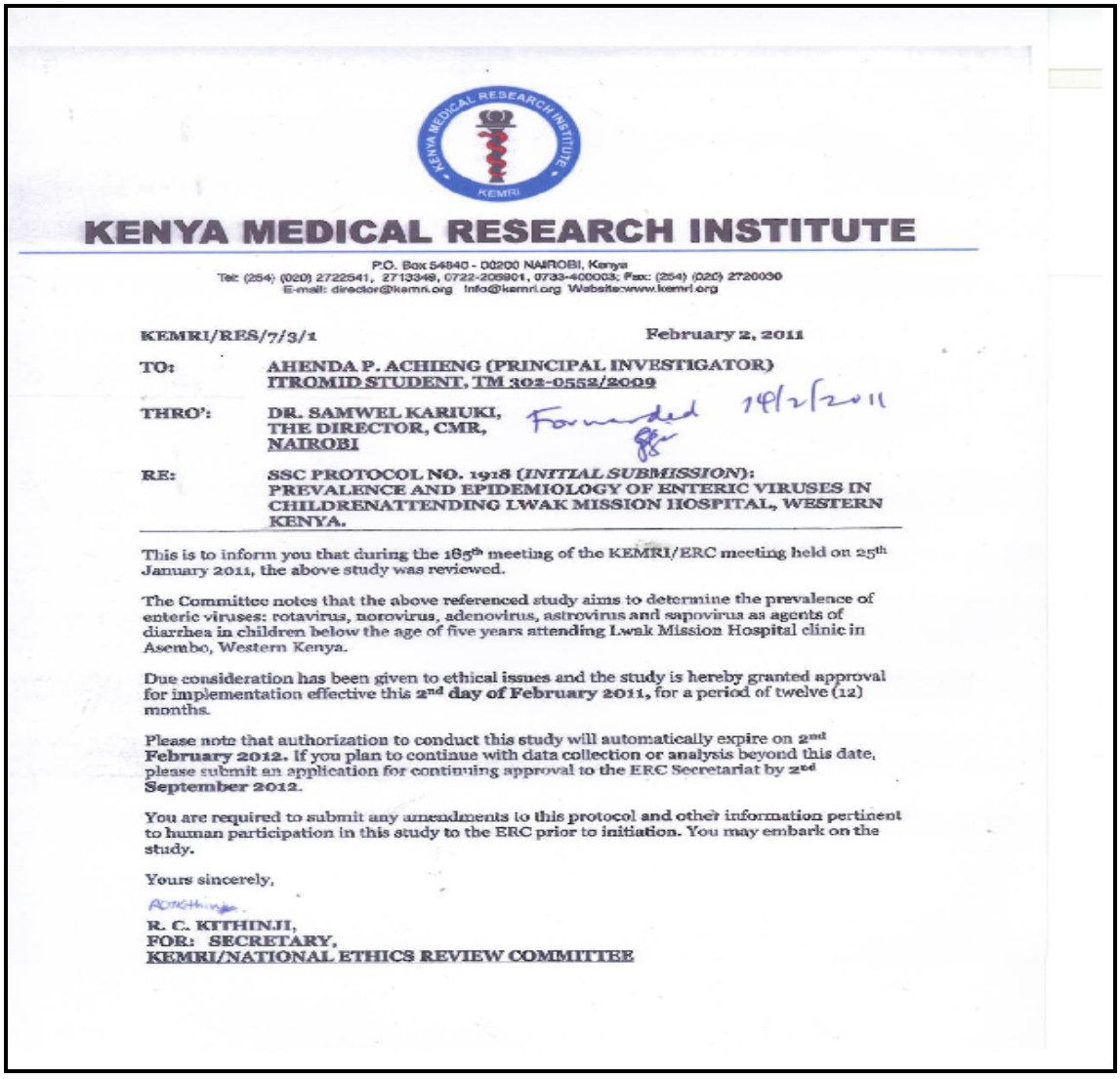
APPENDICES

APPENDIX I: Ethical Clearance

The protocol for this study wasapproved by the Scientific Steering Committee and the Ethical Review Boards of the Kenya Medical Research Institute (KEMRI) (SSC No. 1918). Below is the approval letter from the National Ethics Review Committee,

KEMRI.



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KEMRI/RES/7/3/1 February 2, 2011

TO: AHENDA P. ACHIENG (PRINCIPAL INVESTIGATOR)
ITROMID STUDENT, TM 302-0552/2009

THRO': DR. SAMWEL KARIUKI,
THE DIRECTOR, CMR,
NAIROBI

Forwarded 14/2/2011

RE: SSC PROTOCOL NO. 1918 (*INITIAL SUBMISSION*):
PREVALENCE AND EPIDEMIOLOGY OF ENTERIC VIRUSES IN CHILDRENATTENDING LWAK MISSION HOSPITAL, WESTERN KENYA.

This is to inform you that during the 185th meeting of the KEMRI/ERC meeting held on 25th January 2011, the above study was reviewed.

The Committee notes that the above referenced study aims to determine the prevalence of enteric viruses: rotavirus, norovirus, adenovirus, astrovirus and sapovirus as agents of diarrhea in children below the age of five years attending Lwak Mission Hospital clinic in Asembo, Western Kenya.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **2nd day of February 2011,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **2nd February 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 **2nd September 2012.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE