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

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Medical Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2012
DECLARATION

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

Signature………………………………… Date…………………………

Ahenda Petronella Achieng

This thesis has been submitted for examination with our approval as University Supervisors.

Signature………………………………… Date…………………………

Dr. Joseph O. Oundo,

CDC, Kenya

Signature………………………………… Date…………………………

Prof. Zipporah Ng’ang’a,

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.
ACKNOWLEDGEMENT

First and foremost, this thesis would not have been possible without the Almighty God, who gives skills, knowledge, strength and wisdom.

I owe my deepest gratitude to my supervisors, Dr. Joseph Oundo and Prof. Zipporah Ng’ang’a, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. They have both been an inspiration as I hurdle all the obstacles in the completion of this research work.

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I am indebted to the Centers for Disease Control and Prevention (CDC) at KEMRI, Nairobi and Kisumu for making the research possible through financial and material support.

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I also offer my regards and blessings to all those who supported me in any respect during the completion of this project.

Last but not least, I may not have made it without the tremendous support of my family, my father Hon. Paddy Ahenda, mother Mildred Ahenda, my sister Janet Ahenda and my brothers Dr. John Ahenda and Brian Ahenda who were an inspiration in many ways they may not know. To all of them I say thank you and God bless you.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ABI</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Adeno</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Astro</td>
<td>Astrovirus</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CA</td>
<td>California</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>C. I.</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleoside Triphosphates</td>
</tr>
<tr>
<td>DSS</td>
<td>Demographic Surveillance System</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immuno Assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>GA</td>
<td>Georgia</td>
</tr>
<tr>
<td>GIS</td>
<td>Geographic Information System</td>
</tr>
<tr>
<td>gm</td>
<td>Grams</td>
</tr>
<tr>
<td>ID</td>
<td>Identification</td>
</tr>
<tr>
<td>ICT</td>
<td>Information and Communication Technology</td>
</tr>
<tr>
<td>IEIP</td>
<td>International Emerging Infections Program</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliters</td>
</tr>
<tr>
<td>mM</td>
<td>Milli Molar</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>Noro</td>
<td>Norovirus</td>
</tr>
<tr>
<td>ORS</td>
<td>Oral rehydration solution</td>
</tr>
<tr>
<td>ORT</td>
<td>Oral rehydration therapy</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
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<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rota</td>
<td>Rotavirus</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Sapo</td>
<td>Sapovirus</td>
</tr>
<tr>
<td>SARI</td>
<td>Severe Acute Respiratory Illness</td>
</tr>
<tr>
<td>SSC</td>
<td>Scientific Steering Committee</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>USD</td>
<td>United States Dollar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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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 determine the prevalence of these five most important diarrhoeal viruses among children below 14 years of age who visited the outpatient clinic at Lwak Mission Hospital in 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 ($\leq 2$ 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 diarrhoea in 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 is a 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 movements ranging from 4 – 5 to more than 20 times per day due to the 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 of these 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, norovirus was associated with epidemic outbreaks in 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 study sought to investigate the epidemiological and clinical characteristics of specific etiologic agents of 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 diarrhoea in 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 for causing diarrhoeal diseases in children below the age of fourteen years attending LwakHospital 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

1.5.2 Specific Objectives

1. To determine the prevalence of specific viral aetiologies of diarrhoea 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
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 rotavirus and 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).

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Type of Virus</th>
<th>Nucleic Acid Type</th>
<th>Size of Virion (nm)</th>
<th>Genome Size (kb)</th>
<th>Culturable</th>
</tr>
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</table>

8
Table 2.1 Characteristics of clinically significant human enteric viruses causing diarrhoea

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Viral Type</th>
<th>Genome Size</th>
<th>Genome Length</th>
<th>Culturability</th>
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<tr>
<td>Adenoviridae</td>
<td>Mastadenovirus</td>
<td>Human adenoviruses (HAdV)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>dsDNA</td>
<td>70-90</td>
<td>28-45</td>
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<td>Mamastrovirus</td>
<td>Human astroviruses (HAstV)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ssRNA</td>
<td>28-30</td>
<td>7-8</td>
<td>Yes&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Picobirnavirus</td>
<td>Human picobirnavirus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ssRNA</td>
<td>35</td>
<td>-</td>
<td>-</td>
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<td>ssRNA</td>
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<td>Yes&lt;sup&gt;c&lt;/sup&gt;</td>
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</tbody>
</table>

Table 2.1 Characteristics of clinically significant human enteric viruses causing diarrhoea

Source: Greening, 2006

<sup>a</sup> Viruses responsible for a greater percentage of viral diarrhoea in infants and young children resulting to morbidity and mortality worldwide.

<sup>b</sup> Novel viruses that have been recently identified due to the development of new and rapid molecular methods of viral diagnosis.

<sup>c</sup> 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 it commonly 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) with 95% of childhood rotavirus diarrhoea being attributed to these strains (Bernstein, 2009). G1 is predominant in North America, Australia and Europe with a 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 which are 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 of diarrhoeal 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.

![Norovirus Diagram](image)

**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 diarrhoea in 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).
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 Kingdom 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).

![Diagram of an adenovirus virion](source.png)

**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.

![Image of an astrovirus](image.png)

**Figure 2.4:** The spiky capsid shell of the astrovirus. Source: (Dong *et al.*, 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 the community (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, are at 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 was a laboratory-based study assessing the prevalence, epidemiologic and clinical characteristics of viral diarrhoea in children below the age of fourteen years in Western Kenya between 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 Lwak Mission Hospital in Asembo, Western Kenya. This is a rural study site near Lake Victoria in Siaya District, Nyanza Province 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 km²), 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
The 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 attended Lwak Mission Hospital 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 Lwak Mission Hospital 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 Lwak Hospital clinic in Asembo, Western Kenya, with a desired 95% confidence interval and a precision of 0.05, was computed in the following formula.

\[ n = \frac{Z^2 \cdot PQ}{d^2} \]  

(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 month depending on their availability.
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 diluted (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 were then 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-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 RNA was extracted using a QIAamp Viral RNA Mini kit (Qiagen, Hilden Germany) according to the manufacturer’s protocol and as previously described (Oh et al., 2003). One hundred and forty μl of faecal supernatant was mixed with 560μl of AVL 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 RNA was mixed with 2.05 μl of 5X First strand Buffer (Invitrogen), 0.75 μl of 10 mM dNTPs (Roche), 0.75 μl of 10 mM DTT (Invitrogen), 0.5 μl of 40 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. The reverse 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 (Yanet 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

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer</th>
<th>Polarity</th>
<th>Sequence (5’ to 3’)</th>
<th>Position (nt number)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus Group I</td>
<td>GISKF*</td>
<td>+</td>
<td>CTGCCCGAATTTYGTAATGA</td>
<td>5342-5361</td>
<td>330</td>
</tr>
<tr>
<td>(NVGI)</td>
<td>GISKR*</td>
<td>-</td>
<td>CCAACCCCARCCATRTACA</td>
<td>5653-5671</td>
<td></td>
</tr>
<tr>
<td>Norovirus Group II</td>
<td>COG2F*</td>
<td>+</td>
<td>CARGARBCNATGTAGRTGGATGAG</td>
<td>5003-5028</td>
<td>387</td>
</tr>
<tr>
<td>(NVGII)</td>
<td>G2SKR*</td>
<td>-</td>
<td>CCRCNGCATRHCRTTACAT</td>
<td>5367-5389</td>
<td></td>
</tr>
<tr>
<td>Sapovirus (SV)</td>
<td>SLV5317*</td>
<td>+</td>
<td>CTCGCCACCTACRAWGCBTG</td>
<td>5083-5105</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>SLV5749*</td>
<td>-</td>
<td>CGGRCTCATAAVSTACCBCCCA</td>
<td>5494-5516</td>
<td></td>
</tr>
<tr>
<td>Human Astrovirus (HAstV)</td>
<td>PreCAP1 82b</td>
<td>+</td>
<td>GGACTGCAAGCGAGCTTGGT</td>
<td>4235-4255</td>
<td>719</td>
</tr>
</tbody>
</table>

**Source:** Yanet 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/μl Amplitaq 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 then detected 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 LwakHospital clinic in Asembo. This was done 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.
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 diarrhea attended Lwak Mission Hospital (Figure 4.1). Only 342 of these children were sampled for the IEIP population-based morbidity surveillance study.

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.

Figure 4.1: Overall coverage of study subjects
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

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Number of children with diarrhoea</th>
<th>Number of specimen tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 342</td>
<td>n/N %</td>
</tr>
<tr>
<td>0 - 2</td>
<td>174</td>
<td>50.88</td>
</tr>
<tr>
<td>3 - 5</td>
<td>67</td>
<td>19.59</td>
</tr>
<tr>
<td>6 - 8</td>
<td>35</td>
<td>10.23</td>
</tr>
<tr>
<td>9 - 11</td>
<td>26</td>
<td>7.60</td>
</tr>
<tr>
<td>12 - 14</td>
<td>40</td>
<td>11.70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number of children with diarrhoea</th>
<th>Number of specimen tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 342</td>
<td>n/N %</td>
</tr>
<tr>
<td>Males</td>
<td>174</td>
<td>50.88</td>
</tr>
<tr>
<td>Females</td>
<td>168</td>
<td>49.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Number of children with diarrhoea</th>
<th>Number of specimen tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 342</td>
<td>n/N %</td>
</tr>
<tr>
<td>Jan 07 - Jun 07</td>
<td>20</td>
<td>5.85</td>
</tr>
<tr>
<td>Jul 07 - Dec 07</td>
<td>44</td>
<td>12.87</td>
</tr>
<tr>
<td>Jan 08 - Jun 08</td>
<td>36</td>
<td>10.53</td>
</tr>
<tr>
<td>Jul 08 - Dec 08</td>
<td>62</td>
<td>18.13</td>
</tr>
<tr>
<td>Jan 09 - Jun 09</td>
<td>46</td>
<td>13.45</td>
</tr>
<tr>
<td>Jul 09 - Dec 09</td>
<td>59</td>
<td>17.25</td>
</tr>
<tr>
<td>Jan 10 - Jun 10</td>
<td>75</td>
<td>21.93</td>
</tr>
</tbody>
</table>
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).

![Pie chart showing the prevalence of different enteric viruses detected](chart.png)

- Rotavirus (13.6%)
- Norovirus (6.3%)
- Adenovirus (4.9%)
- Astrovirus (2.9%)
- Sapovirus (1.5%)
- No Enteric Virus detected (73.3%)

> Co-infection = 2.4%

**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 rotavirus whereas 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](attachment:image.png)

**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

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number of specimen tested (N)</th>
<th>Total number of viruses detected n/N %</th>
<th>Overall viruses detected in stool samples = 55 (26.70%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>111/206</td>
<td>32 (28.83)</td>
<td>15 (13.51) 9 (8.11) 7 (6.31) 3 (2.70) 1 (0.90)</td>
</tr>
<tr>
<td>Females</td>
<td>95/206</td>
<td>23 (24.21)</td>
<td>13 (13.68) 4 (4.21) 3 (3.16) 3 (3.16) 2 (2.11)</td>
</tr>
</tbody>
</table>
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.

![Distribution of enteric viruses in children with diarrhoea over time](image)

**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

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>N = 206</th>
<th>n/N %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vomit</td>
<td>78</td>
<td>37.86</td>
</tr>
<tr>
<td>Fever</td>
<td>162</td>
<td>78.64</td>
</tr>
<tr>
<td>Cough</td>
<td>106</td>
<td>51.46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of Diarrhoea Days</th>
<th>N = 206</th>
<th>n/N %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 3</td>
<td>63</td>
<td>79.13</td>
</tr>
<tr>
<td>4 to 6</td>
<td>19</td>
<td>9.22</td>
</tr>
<tr>
<td>≥ 7</td>
<td>8</td>
<td>3.88</td>
</tr>
<tr>
<td>Unknown</td>
<td>16</td>
<td>7.77</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Case Patients N (%)</th>
<th>Clinical Features n/N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vomit</td>
<td>Fever</td>
</tr>
<tr>
<td>0 – 2</td>
<td>92 (44.66)</td>
<td>42 (45.65)</td>
</tr>
<tr>
<td>3 – 5</td>
<td>37 (17.96)</td>
<td>7 (18.92)</td>
</tr>
<tr>
<td>6 – 8</td>
<td>26 (12.62)</td>
<td>12 (46.15)</td>
</tr>
<tr>
<td>9 – 11</td>
<td>23 (11.17)</td>
<td>9 (39.13)</td>
</tr>
<tr>
<td>12 – 14</td>
<td>28 (13.59)</td>
<td>8 (28.57)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Case Patients N (%)</th>
<th>Clinical Features n/N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vomit</td>
<td>Fever</td>
</tr>
<tr>
<td>Males</td>
<td>111 (53.88)</td>
<td>43 (38.74)</td>
</tr>
<tr>
<td>Females</td>
<td>95 (46.12)</td>
<td>35 (36.84)</td>
</tr>
</tbody>
</table>

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](image_url)  Proportions of children presenting with a clinical presentation accompanying an enteric virus isolation

65
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**

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

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Number of case patients N = 206</th>
<th>Total number of viruses detected n/N %</th>
<th>Rota</th>
<th>Noro</th>
<th>Adeno</th>
<th>Astro</th>
<th>Sapo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vomit + Fever</td>
<td>62 (30.10)</td>
<td>20 (32.26)</td>
<td>10 (16.13)</td>
<td>3 (4.84)</td>
<td>4 (6.45)</td>
<td>3 (4.84)</td>
<td>2 (3.23)</td>
</tr>
<tr>
<td>Vomit + Cough</td>
<td>40 (19.42)</td>
<td>13 (32.5)</td>
<td>5 (12.50)</td>
<td>2 (5.00)</td>
<td>4 (10.00)</td>
<td>2 (5.00)</td>
<td>1 (2.50)</td>
</tr>
<tr>
<td>Fever + Cough</td>
<td>90 (43.69)</td>
<td>24 (26.67)</td>
<td>10 (11.11)</td>
<td>5 (5.56)</td>
<td>6 (6.67)</td>
<td>5 (5.56)</td>
<td>1 (1.11)</td>
</tr>
<tr>
<td>Vomit + Fever + Cough</td>
<td>34 (16.50)</td>
<td>11 (32.35)</td>
<td>4 (11.76)</td>
<td>2 (5.88)</td>
<td>3 (8.82)</td>
<td>2 (5.88)</td>
<td>1 (2.94)</td>
</tr>
</tbody>
</table>
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. This is slightly lower than what was reported from a study in Tanzania where 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 recruited and 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 is inconsistent to the prevalence reported by other studies (Clark and McKendrick,
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, Madagascar at 2.1% (Papaventsis et al., 2008) and Kenya at 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 studies in 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) and in 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. Romanet 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;
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 children below 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 of these 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 adenovirus also 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.
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.
REFERENCES


The Encyclopedia of Science website:


APPENDICES

APPENDIX I: Ethical Clearance

The protocol for this study was approved 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,
KENYA MEDICAL RESEARCH INSTITUTE

February 2, 2011

TO:
AHENDA P. ACHENG (PRINCIPAL INVESTIGATOR)
ITROMID STUDENT.  TM. 2008-2009

THRO:
DR. SAMUEL KARIUKI,
THE DIRECTOR, CMIR,
NAIROBI

RE:
IRC PROTOCOL NO. 1058 (INITIAL SUBMISSION):
PREVALENCE AND EPIDEMIOLOGY OF ENTERIC VIRUSES IN
CHILDREN ATTENDING IWAK MISSION HOSPITAL, WESTERN
KENYA.

This is to inform you that during the 18th meeting of the KEMRI/IRC meeting held on 29th
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, norovirus and sapovirus in children of
diarrhea in children below the age of five years attending Iwak 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 3rd 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 2013. If you plan to continue with data collection or analysis beyond this date,
please submit an application for continuing approval to the IRC Secretary by 2nd
September 2012.

You are required to submit any amendments to this protocol and other informations pertinent
to human participation in this study to the IRC prior to initiation. You may consult on the
study.

Yours sincerely,

R. K. KITHEJI,
SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE