IDENTIFICATION OF DIARRHOEA CAUSING VIRAL AGENTS AND MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES IN CHILDREN BELOW FIVE YEARS FROM MUKURU SLUMS, NAIROBI COUNTY

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I56/CE/11243/2008

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Medical Biochemistry) in the School of Pure and Applied Sciences, Kenyatta University.

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DECLARATION

I GIKONYO JOSHUA NDUNG’U declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

Signature .................................. Date.............

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

To my loving, caring, supportive and encouraging fiancee Jackline Mumbi Githinji.
ACKNOWLEDGEMENT

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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Enzyme-linked immunosorbent assay</td>
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<td>WHO-</td>
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ABSTRACT

Enteric viruses have been recognized as the most important etiologic agents of gastroenteritis, the root cause of diarrhoea and a major worldwide menace responsible for childhood morbidity and mortality. In Kenya, there is no evidence of studies carried out on outpatient populations to assess the relative importance of enteric diarrhoea-causing viruses such as noroviruses, astroviruses, rotaviruses and enteric adenoviruses. The aim of this study was to investigate the prevalence of these enteric viruses as agents of diarrhoea in children less than five years of age, in and around Mukuru slums in Nairobi County, and to genotype group A rotaviruses. Stool samples were collected at two medical laboratories; one in Reuben medical center and the other one in St. Mary’s health center, between January 2010 and March 2011 from 340 children less than five years of age seeking treatment for diarrhoea. The samples were screened for rotavirus (RVs), enteric adenovirus (EAdVs), astrovirus (AstVs) and noroviruses (NVs) Genotype I and II using Reverse transcriptase polymerase chain reaction. Shedding of RV was detected in 24%, EAdV in 2.7%, NV in 1.7%, and AsV in 1.5% of the samples analyzed. Rotaviruses and adenoviruses were found to be in circulation throughout the period of study, while astroviruses and noroviruses were absent in different months of the study. However, an increased shedding of all the viruses under study was noted in the wet and cold months of July and August. The average age of children infected with these viruses was less than 2 years. The incidence and diversity of the main human rotavirus genotypes (G1, G2, G3, G4 and G9 and P[8], P[4], P[6], and P[9]) were determined by using established and adapted reverse transcriptase PCR-based genotyping methods. The P type (VP4 associated) was successfully determined in 35 (87.5%) of the 40 page positive samples, while the G type (VP7 associated) was successfully determined in 37 (92.5%). Only five samples could not be assigned a G or P type. Three P types existed in Mukuru slums, P[8] (60%), P[6] (22.9%), P[4] (11.4) and their relative incidence varied over the 15 months of this study. No P[9] or P[10] types were detected. Type G1 was the most predominant of the G types (40.5 %), and the incidences of G3 and G9 were 21.6 and 32.4% respectively. Mixed types G3/G9 were detected at 5.4%, while G2, G4 and G8-type viruses were not detected. These findings indicate that rotaviruses, noroviruses, enteric adenoviruses and astroviruses are important causes of acute gastroenteritis in Mukuru slums Nairobi Kenya, and rotaviruses are the leading cause of viral gastrointestinal infections followed by adenoviruses. An indication that the prevalence of certain genotypes may change over a rotavirus season is significant and mirrors observations from studies in other tropical climates. Thus monitoring of the genotypic changes among circulating viruses should be encouraged over the coming years.
CHAPTER ONE
INTRODUCTION

1.1 Background information

Diarrhoea is a condition of having three or more loose or liquid bowel movements per day. It is a common cause of death in developing countries and the second most common cause of infant deaths worldwide (Navaneethan and Giannella, 2008). In 2009, WHO estimated diarrhoea to have caused 1.1 million deaths in people aged 5 and over, and 1.5 million deaths in children under the age of 5 years and therefore remains the second leading cause of death (16%) after pneumonia (17%) in this age group.

The loss of fluids through diarrhoea causes dehydration and electrolyte imbalances, which if not treated leads to death (Elliott, 2007). Oral rehydration salts (ORS) and zinc tablets are the treatment of choice and have been estimated to have saved 50 million children in the last 25 years (WHO, 2009). There are many causes of infectious diarrhoea which include viruses, bacteria and parasites (Navaneethan and Giannella, 2008). These agents infect the host's gastrointestinal tract (GIT) and cause gastroenteritis (GE), an inflammation of the gastrointestinal tract, involving both the stomach and the small intestine and resulting in acute diarrhoea. Viruses are the leading cause of gastroenteritis contributing 50-70%, while bacterial and parasitic gastroenteritis constitutes 15-20% and 10-15%, respectively (Arthur, 2009).

Many different enteric viruses, including rotaviruses, noroviruses, adenoviruses type 40 and 41, sapoviruses and astroviruses can cause gastroenteritis (Patel et al., 2009). At least 50% of cases of GE due to food borne illness are caused by noroviruses in adults, while 20% of cases and the
majority of severe cases in children are due to rotavirus (Bresee, 2003). Norovirus is the most common cause of viral diarrhoea in adults, while rotavirus is the most common cause in children under five years old (Greenberg, 2009)

Figure 1.0 Electron micrographs of viruses that cause gastroenteritis in humans. A = rotavirus, B = adenovirus, C = norovirus and D = astrovirus. Magnification = x 200,000. (Source, Koopmans and Duizer, 2004)

Gastroenteritis often involves stomach pain or spasms, diarrhoea and/or vomiting, with non-inflammatory infection of the upper small bowel, or inflammatory infections of the colon (Gerald et al., 2004). Diarrhoea can be acute (sudden onset and lasts less than two weeks) or chronic (persistent). Acute diarrhoea is more common in children and in most cases, it eases and goes within several days, but sometimes takes longer. The main risk is dehydration (Ehiri, 2009). Mild dehydration is common and is usually easily and quickly reversed by drinking lots of fluids. Severe dehydration can be fatal unless quickly treated because the organs of the body need a certain amount of fluid to function normally (Ehiri, 2009).

Symptoms of acute dehydration in children include: passing little urine, dry mouth, dry tongue and lips, fewer or no tears when crying, sunken eyes, weakness, being irritable or lethargic.

Symptoms of severe dehydration in children include: weight loss, drowsiness, pale or mottled skin, cold hands or feet, decreased skin elasticity, very few wet nappies fast (but often shallow)
breathing (Khanna et al., 2009). This is a medical emergency and immediate medical attention is needed. Every child experiences several episodes of diarrhoea during their first years of life, approximately three episodes per year (Elliott, 2007). This also applies and is common to the Kenyan situation, but little information exists on the causes, prevalence and epidemiology of diarrhoea, as well as the molecular characteristics of rotaviruses circulating in Kenya.

1.2 Problem statement and justification

In Kenya, data on viral gastroenteritis in children below the age of five years is mostly on rotavirus and from hospitalized patients detected using a sentinel surveillance system. There is no evidence of studies carried out on outpatient populations in Kenya to assess the relative importance of other diarrhoea viruses such as noroviruses, adenoviruses, astroviruses and sapoviruses. Hence the exact epidemiology of gastroenteritis in outpatient children remains unknown. Moreover, no study in Kenya has been undertaken to determine the types and distribution of viruses responsible for diarrhoea in infants and young children in Mukuru slums.

Viral and epidemiologic data from outpatient population is required to study the association between these viruses from the environment and the children. Obtaining local baseline information regarding these viruses is important in developing and evaluating preventive strategies of enteric viruses in children. It is against this background that this study was instituted to collect and analyze the information of diarrhoea causing viruses in Mukuru slums. This information will be useful for the development of safe and highly effective vaccines, which could prevent at least cases of severe diarrhoea, and reduce morbidity and mortality from gastroenteritis.
1.3 Research questions

i. Which enteric viruses are responsible for diarrhoea in children aged five years and below in Mukuru Slums Nairobi.

ii. What is the prevalence of diarrhoea causing viruses in Mukuru slums Nairobi?

iii. What is the genetic variability of group A rotaviruses circulating among these children?

1.4 Objectives

1.4.1 Main objective

To identify diarrhoea causing viruses, determine their prevalences, and characterize rotavirus group A in children of 5 years and below in Mukuru slums, Nairobi county.

1.4.2 Specific objectives

i. To detect the presence of diarrhoea causing viruses in stool samples from children below five years of age in Mukuru slums Nairobi.

ii. To determine the prevalence of diarrhoea causing viruses in children from Mukuru slums.

iii. To determine the genotypes of group A rotaviruses present in children aged five years and below with diarrhoea in Mukuru slums.
CHAPTER TWO
LITERATURE REVIEW

2.1 Diarrhoea

Diarrhoea is the condition of having three or more loose or liquid bowel movements per day (WHO, 2009). It is a common cause of death in developing countries and the second most common cause of infant deaths worldwide (Navaneethan and Giannella, 2008). The loss of fluids through diarrhoea can cause dehydration and electrolyte imbalances (Elliott, 2007). Diarrhoea can be acute (sudden onset and lasts less than two weeks) or chronic (persistent).

Acute diarrhoea is more common in children and in most cases it eases and goes within a few days (Ehiri, 2009). Mild dehydration is common and is usually easily and quickly reversed by drinking lots of fluids. Severe dehydration can be fatal unless quickly treated because the organs of the body need a certain amount of fluid to function normally. The main risk is dehydration (Gerald et al., 2004).

Symptoms of acute dehydration in children include: passing little urine, a dry mouth, a dry tongue and lips, fewer or no tears when crying, sunken eyes, weakness, being irritable or lethargic. Symptoms of severe dehydration in children include: weight loss, drowsiness, pale or mottled skin, cold hands or feet, decreased skin elasticity, very few wet nappies and fast (but often shallow) breathing (Khanna et al., 2009).

Oral rehydration salts (ORS) and zinc tablets are the treatment of choice and have been estimated to have saved 50 million children in the last 25 years (WHO, 2009). There are many causes of infectious diarrhoea, which include viruses, bacteria and parasites. These agents infect the host's
GIT and cause gastroenteritis, an inflammation of the gastrointestinal tract, involving both the stomach and the small intestine and resulting in acute diarrhoea (Navaneethan and Giannella, 2008).

2.2 Gastroenteritis (GE)

Gastroenteritis is an inflammation of the stomach, small and large intestines (Gerald et al., 2004). The most common causes of gastroenteritis are microbes, toxins and parasites, which irritate the lining of the gastrointestinal tract and affect nutrient absorption, trigger the process of inflammation and may lead to ulceration of the lining. A rare case of gastroenteritis known as eosinophilic gastroenteritis is associated with allergies, and also causes inflammation of the stomach and small intestines, resulting in the typical symptoms of nausea, vomiting, diarrhoea and abdominal pain (Gerald et al., 2004).

2.2.1 Viral gastroenteritis

Viral gastroenteritis is an infection caused by a variety of enteric viruses resulting in diarrhoea and/or vomiting. It is often called the "stomach flu," although it is not caused by the influenza viruses (Zulfigar, 2007). Many different enteric viruses, including rotaviruses, noroviruses, adenoviruses types 40 and 41, sapoviruses and astroviruses can cause gastroenteritis. At least 50% of cases of gastroenteritis due to food borne illness in adults are caused by norovirus, whereas 20% of severe cases in children are due to rotavirus (Bresee, 2003). The main symptoms of viral gastroenteritis are watery diarrhoea and vomiting. The affected person may also have headache, fever, and abdominal cramps "stomach ache" (Elliott, 2007).
2.2.1.1 Acute viral gastroenteritis

Most cases of gastroenteritis are due to infections which are often acute in nature and will resolve spontaneously without treatment (Chris, 2010). The incubation period may vary from a few hours to a few days in viral gastroenteritis. The symptoms of acute gastroenteritis are very intense with extreme nausea, violent vomiting and explosive diarrhoea accompanied by severe abdominal pain being a typical presentation of infectious gastroenteritis (King et al., 2003).

2.2.1.2 Chronic viral gastroenteritis

Less frequently, a case of infectious gastroenteritis can be persistent – chronic in nature. A chronic case of infectious gastroenteritis is more likely to occur in an immune-compromised patient as is the case in HIV/AIDS (an opportunistic infection) or a poorly managed chronic condition like diabetes. A mal-absorption syndrome is often a result of chronic gastroenteritis, irrespective of the cause, and leads to mineral and vitamin deficiencies (Bards, 1988).

2.2.1.3 Viral gastroenteritis in immune-compromised patient

The main viral causes of severe gastroenteritis in immune-suppressed patients are cytomegalovirus (CMV) and Epstein–Barr virus (EBV), which mainly affect patients with AIDS and transplant recipients (Pirsch, 1999). CMV is a frequent pathogen in diarrhoea associated with AIDS with CD4 counts below 100 cells/mm³ (Weber et al., 1999). However, the introduction of antiretroviral therapy has drastically reduced its frequency in this group of patients (Goodgame, 2001). Other viruses that produce HIV-associated gastroenteritis include astrovirus, picobirnavirus, calicivirus and adenovirus (Grohmann et al., 1993).
2.2.2 Transmission and pathogenesis of viral gastroenteritis

Viral gastroenteritis is highly contagious and the viruses that cause the infection are often passed from person to person by contact (Bresee, 2003). Small children are the most susceptible because of their close contact with other children, and their less than optimal hygienic habits. Adults are generally less subject to infection because of immunity acquired by previous exposure to the virus. People who have had the infection can be contagious even after they feel better, while some people are infected but never show symptoms (Arthur, 2009).

Individuals may also become infected by eating or drinking contaminated foods, beverages or water. Because of this, outbreaks of gastroenteritis are common in childcare and other group settings. To date, the molecular mechanisms by which most of the viruses cause diarrhoea has not been clearly understood, except for the rotaviruses that have well been studied (Raming, 2004). Rotaviruses may cause diarrhoea by several mechanisms including (i) mal-absorption that occurs secondary to the destruction of enterocytes, (ii) villus ischaemia and activation of the enteric nervous system that may be evoked by release of a vasoactive agent from infected epithelial cells in the absence of significant pathologic lesions or enterocyte damage, and (iii) intestinal secretion stimulated by the intracellular or extracellular action of the rotavirus non-structural protein, NSP4, a novel enterotoxin and secretory agonist with pleiotropic properties (Estates et al., 2001).

Non-structural protein 4 also affects epithelial cell function and interactions; (i) induces an age- and dose-dependent diarrhoeal response that is similar to virus-induced disease, (ii) stimulates a Ca\(^{2+}\) dependent cell permeability where the secretory response is age-dependent, and (iii) alters
epithelial cell integrity (Estates et al., 2001). Enteric viruses associated with gastroenteritis cause diarrhoea, usually mild and self-limiting, although some members of the group cause asymptomatic infections. Some of the most important enteric viruses that cause diarrhoea include: Noroviruses, Adenoviruses, Rotaviruses, Calisiviruses and Astroviruses.

2.3 Noroviruses (NoV)

Noroviruses are members of the family Caliciviridae (Lopman et al., 2002). Noroviruses are RNA non-enveloped viruses, with a single stranded poly-adenylated genome size of approximately 7.6 kb, and a diameter of approximately 27–35 nm. These genetically diverse viruses can be segregated into five separate genogroups (GI, GII, GIII, GIV and GV) based on sequence comparison of regions of the genome (Pang et al., 2000). Three genogroups, GI, GII and GIV, are known to infect humans causing winter-vomiting disease and are recognized as an important cause of acute non-bacterial gastroenteritis worldwide (Phan et al., 2006).

The norovirus genome is organized into three open reading frames (ORF): ORF1 encodes six non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor structural protein VP2 (Greenberg, 2009). Conserved regions within either the RdRp gene (region A), the 30-end of ORF1 (region B) or the capsid sequence (region C, close to the 50-end of ORF2, and D, at the 30-end of ORF2) are the main targets for diagnostic RT-PCR assays (Vinje et al., 1997). Diagnosis can be done by electron microscopy, antigen detection through enzyme immunoassay, or by molecular methods aiming to amplify regions of norovirus genome (Lopman et al., 2003). Human noroviruses (NoVs) are the leading cause of acute gastroenteritis outbreaks and are also recognized as a
frequent agent of sporadic gastroenteritis in all age groups worldwide (Fankhauser et al., 1998). Noroviruses are generally responsible for epidemics of stomach flu and occur frequently between the months of October and April. It is believed that at least 50 percent of all food borne outbreaks of gastroenteritis can be attributed to noroviruses. The low infectious dose of noroviruses (10–100 virus particles) and its high stability in the environment favor transmission of these viruses by droplets or objects that may help spread infection to family members and people in semi-closed environments such as schools and military camps.

An investigation of the prevalence of norovirus infections among 10 European countries from 1995 to 2000 indicated that 85–90% of all cases of non-bacterial epidemic gastroenteritis are associated with noroviruses (Lopman et al., 2003). Noroviruses have been identified in 3.5–20% of sporadic cases of gastroenteritis mostly in infants and children (Bon et al., 1999). However, outbreaks of noroviruses are frequent in semi-closed environments and many have been reported in hospitals, nursery homes, cruise ships, schools and kindergartens, and in the military camps. Food borne transmission either by food contaminated at source or by food handlers has also been reported.

Outbreaks of norovirus gastroenteritis pose a major economic burden to healthcare systems and the tourism and catering industry. Lopman et al., (2004) estimated that nosocomial norovirus outbreaks cost over £70 million (140 million US dollars) to the healthcare system in England and Wales during 2002–2003 years. Like other RNA viruses, the broad genetic diversity and evolution of noroviruses is due mainly to the accumulation of point mutations (antigenic drift), but recombination between different genotypes during co-infections has also been reported.
(Hansman et al., 2004). Norovirus GII/4 strains are endemic in hospitals and long-term care facilities and recent reports suggest the epidemic spread of newly emerging GII/4 norovirus variants (Kirkwood, 2001). Such epidemic variants have been identified on several occasions. A variant that emerged in the mid-1990s was responsible for the epidemic described between 1995 and 2001 (Noel et al., 1999). A second epidemic was described in 2002–2003 (Widdowson et al., 2005) which was caused by a newly emerged GII/4 variant, and two more epidemics were described in 2004 and 2006, both originating through the emergence of two different variants (Allen et al., 2008).

2.4 Human Astroviruses (HAstV)

Human astroviruses (HAstV) are recognized as a common cause of infantile gastroenteritis worldwide (Walter et al., 2000). Astroviruses occur primarily in infants, children under the age of five, and the elderly. It is the most active during the cold months (Herrmann et al., 1991). Epidemiological studies carried out in different locations in the world have reported astrovirus prevalence rates of 2 to 16% among children hospitalized with diarrhoea and 1 to 17% among children with diarrhoea in community-based studies (Walter et al., 2000). These studies reveal an incidence of astrovirus infection in children with GE of 2% in India (Shetty et al., 1995), 3% in Brazil (Stewien et al., 1993) and 4.2% in Australia (Palombo et al., 1996).

However, in some studies, higher rates of astrovirus infection have also been reported. These include 6.3% in France (Bon et al., 1999), 6.8% in USA (Dennehy et al., 2001) and 7% in South Africa (Steele et al., 1998). In this regard, seroprevalence studies in England reported that by the age of 5 to 10 years, astrovirus antibodies are found in 75% of the children (Lee et al., 1994).
This suggests that older children may have acquired antibodies against astroviruses early in life due to natural exposure to these viruses.

Astroviruses were first observed by electron microscopy (EM) in stool specimens from infants with gastroenteritis in 1975 (Appleton et al., 1975; Madeley et al., 1975). Human astroviruses belong to the family Astroviridae, which contains a single genus; Astrovirus (Van et al., 2000). Astroviruses are small, non-enveloped icosahedral viruses, measuring about 28-30 nm in diameter with a smooth margin and a star-like EM appearance. The genome consists of plus-sense, single-stranded RNA that is approximately 6,800 nucleotides in length excluding the 3' poly (A) tail. To date, eight serotypes of human astrovirus have been identified (Matsui et al., 1996). The astrovirus genome is organized in three open reading frames (ORFs): ORF1a and ORF1b at the 5' end of the genome code for the nonstructural viral proteins, while ORF2 at the 3' end encodes the capsid proteins (Jiang et al., 1993).

Based on the nucleotide and encoded amino acid sequences of the amino-terminal or carboxyl-terminal region of ORF2, these viruses have been grouped into eight genotypes which have been shown to completely correlate with the eight established astrovirus serotypes (Martha et al., 2004). Typing surveys indicate that Human astrovirus type 1 (HAstV-1) is the most prevalent, types 2 to 4 are common, and types 5 to 7 are less common, while type 8 has only recently been identified. These studies have shown that it is not uncommon to find two or more astrovirus types circulating in one region during a given period of time, and they have also described variations in the prevalent astrovirus type with time (Martha et al., 2004).
The prevalence of Human astrovirus in children with no diarrheic symptoms has also been determined, although in a more limited number of studies. The viruses have been found in ~2.0% of the children analyzed; however, the astrovirus types associated with the asymptomatic infections have not been characterized (Herrmann et al., 1991). Human astroviruses are known to cause a less severe form of viral gastroenteritis than that caused by other enteric pathogens. Infection occurs as both sporadic cases and in outbreaks, predominantly affecting young children, although infection has been documented in all age groups (McIver et al., 2000).

Astroviruses are reportedly the most common viral agent associated with diarrhoea in immune-suppressed adults (Guix et al., 2002). Following a 1-4 day incubation period, astrovirus infection typically presents as watery diarrhoea that resembles a mild form of rotavirus gastroenteritis. Astrovirus diarrhoea is principally seen in young children 6 months to 2 years of age and may be associated with anorexia, fever, vomiting and abdominal pain. Although astrovirus diarrhoea does not normally result in significant dehydration or hospitalization, individuals suffering from poor nutritional status, immunodeficiency, severe mixed infection, or underlying gastrointestinal disease are at risk for the development of complications (Blacklow et al., 1991; Greenberg et al., 1992).

Immunity to astrovirus infection is not well understood. Young children and the institutionalized elderly are usually the populations that develop symptomatic infection, suggesting that antibodies are acquired early in childhood, provide protection through adult life, and wanes late in life (Glass et al., 1996). A recent study by Koopmans and colleagues (1998) determined that
there is no heterologous protection between the seven human astrovirus serotypes. In temperate regions, most astrovirus infections are detected in the winter while in tropical climates, most astrovirus infections are noted in during the rainy season (Matsui et al., 1996). This temporal pattern of infection is similar to that of rotavirus infection (Maldonado, 1996).

2.5 Rotaviruses (RVs)

2.5.1 Classification and occurrence

Rotaviruses are members of the Reoviridae family (Matthews, 1979) and are characterized by their non-enveloped icosahedral structure of 70nm in diameter. It derives its name from the wheel like appearance it has when viewed under an electron microscope (rota is latin for wheel) (Anderson et al., 2004).

![Figure 2.0 Computer rendering of rotaviral particle (Umash et al., 2003)](image)

Rotavirus is a segmented double stranded RNA (dsRNA) virus, and presents a triple concentric layer of proteins. Its genome is made up of 11 segments of double stranded RNA held in the
inner core of the three-layered virus (Varani et al., 2002). The genome codes for 6 viral structural proteins (VP1, 2, 3, 4, 6, 7) and 6 non-structural proteins (NSP1-6) (Graff et al., 2002). The inner layer is formed by: virus protein 2 (VP2) which is the main structural component of the innermost layer involving the genome, the virus protein 1 (VP1) which is the RNA-dependent RNA polymerase for rotavirus, and viral protein 3 (VP3) which acts as the mRNA capping enzyme (guanilyltransferase and methylase) and is a replication intermediate (Vende et al., 2002).

The intermediate layer is made up of the structural viral protein 6 (VP6) associated with VP2 and confers to the structure of the so-called double-layered particles (DLP). The outer layer is constituted by trimeric structures of VP7 glycoprotein and the dimeric spikes of VP4 forming the triple-layered particles (TLP); the infectious form of the virus (Yeager et al., 1990). These two structural proteins, VP7 (the glycoprotein or G protein) and VP4 (the protease-cleaved protein or P protein), making up the outer shell are considered important for vaccine development since they define the genotype of the virus and are the major antigens involved in virus neutralization (Prasad et al., 1999).

Because the genes encoding these proteins segregate independently of each other during reassortment, a dual-serotyping system to account for the specificities of both VP7 and VP4 has been adopted. Thus the classification of rotaviruses is based on differences in the VP7 (G) and VP4 (P) capsid proteins, where the G serotypes 1–4, and P genotypes P[8] and P[4] predominate worldwide (Hoshino et al., 1985).
Viral protein 7 is a 37 kD glycoprotein that makes up the smooth portion of the outer capsid. It can induce neutralizing antibodies and determines the G serotype. It is also a highly variable portion of the virus capable of re-assortment and possible crossover with animal strains of the virus (Maunula et al., 2002).

![Figure 3.0 Structural diagram of Rotavirus (Umash et al., 2003).]

The VP7 also has associations with heat shock cognate protein (hsc 70) and some integrins, both related to viral entry of the cell (Guerrero et al., 2002). VP4 is an 88 kDa protein that dimerizes to create 60 spikes on virus surface (Golantsova et al., 2004), and it is cleaved by the pancreatic enzyme trypsin to form VP5 and VP8. VP4 and its cleavage products are associated with cell attachment and invasion and cleavage is necessary for infectivity. The VP4 is antigenic and induces neutralizing antibodies. The specific structure of this protein is used to determine the rotavirus P serotype, as well as host specificity, virulence and protective immunity. It has also
been associated with heat shock cognate protein (hsc70) during cell entry (Golantsova et al., 2004).

2.5.1 Rotavirus Non-Structural Proteins

**NSP1**—Binds Interferon Regulatory Factor 3 and may inhibit interferon response during rotavirus infection.

**NSP2**—In conjunction with NSP5, NSP2 is involved in the synthesis and packaging of viral RNA and creation of viroplasms. The protein is a replication intermediate.

**NSP3**—Is a 36kD protein that binds viral mRNA at the 3' end and promotes viral protein synthesis. It also represses host cell protein synthesis. This protein is a possible target for a new class of antivirals.

**NSP4**—Has been shown to act as an enterotoxin and cause diarrhoea during infection. There is also correlation between VP6 virus subgroup and NSP4 genotype.

**NSP5**—This phosphoprotein works with NSP2 in RNA synthesis and packaging, and to induce viroplasms. It is also a replication intermediate.

**NSP6**—Little information is available on NSP6, but it is associated with NSP5 and its function (Mandell et al., 2000).

Based on antigenic specificity of VP6, RVs are classified into seven groups (A-G), prevailing the infections by group A strains (RV-A). Moreover, VP6 epitopes allow the differentiation of group A strains into sub-groups (SG-I, SG-II, SG-I/II, and non-SG-I/II), prevailing SG-II in human infections (Estes et al., 2007). These strains are molecularly distinguished into genogroup I (SG-I) and genogroup II (SG-II, SG-I/II, and SG non-I, non II) (Iturriza et al. 2002).
G and P genotyping is attributed respectively to VP7 and VP4, where twenty-five G and 31 P genotypes have been described (Abes et al., 2009). The most common G serotypes currently are G1, G2, G3, G4, and G9, with G1 being most prevalent and G9 the fastest emerging worldwide (Laird et al., 2003).

![Pie chart showing the prevalence of VP7 serotypes](image)

**Figure 4.0** Prevalence of VP7 serotypes (Laird et al., 2003).

Globally, viruses carrying the genotype pairs G1 P[8], G2 P[4], G3 P[8], and G4 P[8] are important causes of diarrhoea among infants worldwide, accounting for 95.9% of all typeable strains (Gentsch et al., 2005).

Polyacrylamide gel electrophoresis (PAGE) has high sensitivity and specificity for determination of the prevalent rotavirus strains in a community (Argüelles et al., 2000). Electrophoresis of the rotavirus RNA genome allows detection and classification of the viruses into two major groups; the long (L) and the short (S) electrophoretic profiles based on the migration patterns of gene
segments 10 and 11 on polyacrylamide gel (figure 5.0). The variations in the electrophoretic mobility of one or more RNA segments allow different rotavirus strains to be further classified into seven electropherotypes (e-type) from A-G, according to the migration pattern of the 11 RNA segments (Matsuno et al., 1985). Electrophoresis of the rotavirus RNA genome has often been used as a useful indicator of the genomic diversity of rotavirus isolates in populations over a certain period (Cunliffe et al., 2001).

![Figure 5.0](image)

**Figure 5.0** (a) Representative electropherotype variants detected in Irish children (Matsuno et al., 1985). Lanes: 1 through 4, long electropherotype patterns; 5 through 7, short electropherotype patterns. (b) Mixed infection identified by polyacrylamide gel electrophoresis (the segments are 12 and appearing 4-2-3-2-1 style, instead of 11 and a style of 4-2-3-2).

Rotavirus is the leading cause of severe dehydrating diarrhoea in children under five years of age worldwide, and accounts for around half a million deaths in infants in developing countries per year. Even in advanced countries such as the US, it accounts for up to 500 deaths per annum (Widdowson et al., 2005). Rotaviruses are important agents of acute infantile gastroenteritis in
infants worldwide (Kapikian et al., 1996), and have been reported to cause a high degree of morbidity and mortality in developing countries. They are the leading cause of stomach flu among children between 3 months and 15 months of age and are associated with more than half a million deaths per year in Africa and Asia (Parashar et al., 2006). Worldwide, RVs cause approximately 112 millions of domestic episodes of diarrhoea, 25millions of clinic visits, 2 millions of hospitalizations and about 800,000 deaths of children below five years of age annually (Angel et al., 2007).

Previous studies in developed and developing countries have similarly shown that rotavirus is an important pathogen, responsible for 55% of all the gastroenteritis in Australia (Barnes et al., 1998), 79% in Germany (Oh DY et al., 2003), 42% in Indonesia (Subekti et al., 2002), 14.1% in Saudi Arabia (Akhter et al., 1999), 32.5% in Northern Jordan (Youssef et al., 2000), 55.9% in China (Qiao et al., 1999), and 61% in France (Bon et al., 1999). The prevalence of rotavirus disease is similar in children in both developed and developing countries. However, children in developing countries die more frequently, possibly due to poorer access to rehydration therapy and prevalence of malnutrition (Parashar et al., 2003).

Published data indicated that the highest rotavirus prevalence rates occurred in children aged 6 to 18 months with severe infections occurring more frequently in younger children aged 6 to 12 months in developing countries (Bishop et al., 2004). In Central Africa, rotaviruses were found most frequently among children less than 1 year-old (Georges et al., 1984). Another study in Bahrain showed that rotavirus was detected most frequently among the age group of 6-11 months (Dutta et al., 1990).
Rotavirus infection rates vary seasonally with the majority of cases in temperate climates occurring in the winter months between November and February. In tropical and developing countries, this seasonality is less marked, and infections emerge year-round (Gleizes et al., 2006). The incidence of infection with particular rotavirus genotypes varies between geographical areas during a rotavirus season and from one season to the next (Iturriza et al., 2002). The world distribution of rotavirus infection is illustrated in figure 6.0.

Transmitted by the fecal-oral route, they infect the mature enterocytes on the tips of small intestine villi and lead to villous epithelium atrophy and compensatory repopulation of the epithelium by immature secretor cells, with secondary hyperplasia of the crypts (Salim et
al., 1995). The mechanism that induces diarrhoea include; destruction of enterocytes, villus ischemia and activation of the enteric nervous system and intestinal secretion stimulated by the intracellular or extracellular action of the rotavirus non-structural protein, NSP4 (Estates et al., 2001). Symptoms include a profuse watery diarrhoea, vomiting, abdominal pain, and possibly fever. Severe cases may lead to death; mainly through acute dehydration (Diggle, 2007)

Infection rates for rotavirus are highest in the under 5-year old age group and decrease progressively towards adulthood, as immunity acquired in childhood protects most adults (Estates et al., 2001).

Often, children suffering from rotavirus gastroenteritis (RVGE) require only outpatient care (medical consultation). However, in the presence of dehydration, emergency care or hospitalization and intravenous hydration are necessary. By the age 5 years, 4 out of 5 children will have an episode of rotavirus gastroenteritis, and while not every rotavirus infection (including the first infection) is symptomatic, 1 in 5 will visit a clinic and 1 in 65 will be hospitalized (Parashar et al., 2003). Rotavirus gastroenteritis imposes a heavy economic burden, by incurring direct (consultation, emergency, hospitalization, medication) and indirect costs (parent work days lost, baby-sitting, additional diapers) hence affecting both individual and the country’s economy (Verstraeten, 2006).

Global rotavirus surveillance activities are ongoing to evaluate the burden of disease and epidemiology of the infection in developing countries (Armah et al., 1995). The surveillance methods employed to identify serotypes can vary from country to country, which can have an impact on the detection of emerging serotypes. Hence, the development of surveillance networks
such as the Euro Rota Net, which now covers many Central and Eastern European countries, is an important improvement in the reporting of rotaviral serotypes (Iturriza et al., 2009).

2.5.2 Molecular characterization of group A rotaviruses

In many countries the molecular characterization of the two important neutralizing antigens (VP4 and VP7) is performed by reverse-transcriptase polymerase chain reaction (RT-PCR) techniques. The VP7 types are considered important for vaccine development and have been the target of surveillance efforts to determine the circulating types globally (Kapikian et al., 1996). In West Africa, the VP4 and/or VP7 types of rotavirus strains have been characterized only in a limited number of studies in a few countries, including Ghana, Guinea-Bissau, and Nigeria (Aminu et al., 2008).

In an early study in Gambia, VP7 serotype G1 and G2 viruses detected by monoclonal antibodies circulated at similar levels, and G3 strains were also identified in this small study (Williams et al., 1985). In Ghana, the VP6 subgroup and VP7 serotype were also determined by monoclonal antibody enzyme immunoassay (Adah et al., 1997). The VP6 subgroup II strains predominated, and VP7 serotype G1 strains were most often detected followed by G4 strains; G2 and G3 strains were present at lower levels. Investigators in the other West African studies using molecular techniques similar to those described in this study, have reported the wide and unusual diversity of rotavirus strains circulating in the region.

In Nigeria and Guinea-Bissau for instance, G1 and G3 strains were detected by similar RT-PCR methods for the VP7 genotype determination (Aminu et al., 2008), and circulation of the so-
called "mosaic" G1/G3 viruses was observed. Furthermore, many "untypeable" VP7 and unusual rotavirus strains were observed commonly in this region (Fischer et al., 2002). The VP4-P[6] genotype has been reported to be widely circulating in West Africa (Aminu et al., 2008) and, although unusual in other regions, appears to occur commonly in virulent strains associated with disease in young children.

2.5.3 Vaccines for rotavirus diarrhoea

Currently, rotavirus vaccine strategies are based on the need for a polyvalent vaccine candidate encompassing the epidemiologically important group A rotavirus VP7 serotypes (Gouvea et al., 1990). At present, this has included the 4 most commonly occurring VP7 rotavirus serotypes (G1–G4), which are considered to be critical for inclusion in a vaccine candidate, and also contain the most prevalent VP4 type found in human rotaviruses (P8). Two live oral vaccines, monovalent Rotarix vaccine (GlaxoSmithKline, Research Triangle Park, NC, USA) and pentavalent Rotatek vaccine (Merck, Rahway, NJ, USA) has effectively reduced hospitalizations for subsequent infections with G1P[8], G2P[4], G3P[8], and G4P[8] rotaviruses by >85% (Iturriza et al., 2009). Both vaccines are being used and have demonstrated good safety, efficacy, and effectiveness (Gouvea et al., 1990).

Protection from rotaviral infection and disease is believed to be type specific as VP7 and VP4 proteins elicit neutralizing antibody responses (Iturriza et al., 2009). Since rotaviruses are ubiquitous in the animal kingdom; interspecies transmission and the exchange of genetic material between animal and human strains through re-assortment can lead to the emergence of novel rotavirus strains of epidemiological significance (Iturriza et al., 2002). Also, bearing in mind the
genetic variability of the virus, either vaccine or host natural immunity to the virus or both may pose a selective pressure that may result in emergence of unusual genotypes. In fact, hitherto undescribed genotypes have been found (Solberg et al., 2009). These selective events may represent a significant antigenic “shift” or “drift”, as has been shown for influenza virus with a real impact in the epidemiology of the disease. Therefore, it is important to monitor wild strains of the virus in order to evaluate all these consequences, and to accompany the evolution of the infection.

Little is known about the distribution and diversity of the rotavirus VP7 genotypes and VP4 genotypes in West and East Africa, and there have been no reports of the VP7 types in Kenya. In this study, the presence of the VP4 and VP7 genotype of human rotaviruses as well as other viral agents that cause GE in Mukuru slums Nairobi Kenya was investigated.

2.6 Enteric adenoviruses (EAdV)

Human adenoviruses belong to the family Adenoviridae. They are DNA viruses that are non-enveloped, icosahedral, with a linear dsDNA genome, and 70 nm in diameter. Adenoviruses were first isolated in the 1950s in adenoid tissue-derived cell cultures, hence the name (Jalal et al., 2005). These primary cell cultures were often noted to spontaneously degenerate over time, and adenoviruses are now known to be a common cause of asymptomatic respiratory tract infection that produces in vitro cytolysis in these tissues.

In 1975, previously unrecognized adenoviruses were detected by electron microscopy (EM) in stool specimens from infants with diarrhoea (Ingrid et al., 1984). These adenovirus species have
been designated enteric adenoviruses (EAds). They are fastidious and cannot be cultivated in cell cultures by conventional means.

![Figure 7.0 Enteric Adenoviruses as seen under Electron microscope](International Committee on Taxonomy of Viruses, 2002)

An extremely hardy virus, adenovirus is ubiquitous in human and animal populations, survives long periods outside a host, and is endemic throughout the year. It is transmitted via direct inoculation to the conjunctiva, a fecal-oral route, aerosolized droplets, or exposure to infected tissue or blood. The site of entry generally determines the site of infection; respiratory tract infections result from droplet inhalation, while gastrointestinal tract involvement results from fecal-oral transmission (Echavarria et al., 2003).

Based on hemagglutination inhibition (HI) and neutralization tests, six groups (A-F) and 52 serotypes of adenoviruses have been determined. Group F of adenovirus including types 40 and 41 are the main etiologies of 1-20% of acute gastroenteritis (Samarbaf-Zadeh et al., 2010), and 50% of all adenoviruses found in stool specimens are types 40 and 41 (Baum, 2000). Adenovirus generally occurs in children under the age of 2 (Wadell et al., 1999). After rotavirus infection, enteric adenoviruses are the second most common cause of severe and
acute gastroenteritis in children under five years (Samarbaf-Zadeh et al., 2010). Enteric adenoviruses (EAds) replicate readily in the human intestine and may be cultured from asymptomatic individuals; thus, their presence in the setting of a diarrhoeal syndrome may be incidental. Many serotypes are fastidious in culture. Serotypes 40 and 41 had been termed "non-cultivatable", however, they have been cultured in the setting of diarrhoeal syndromes using newer cell lines (Kidd et al., 2003). Monoclonal antibody assays, enzyme-linked immunosorbent assay, and electron microscopy support the association of these strains with enteric disease. However, one cannot assume that enteric disease is limited to these strains. In fact, various serotypes of adenovirus have been associated with infectious diarrhoeal syndromes in recipients of hematopoietic stem cell transplants (Samarbaf-Zadeh et al., 2010).

The lesions produced by serotypes 40 and 41 in the enterocytes lead to atrophy of the villi and compensatory hyperplasia in the crypts, with subsequent mal-absorption and loss of fluids (Wadell et al., 1999). After the infection, specific antibodies develop in most cases, and non-neutralizing antibodies are useful for measuring the immune response. The symptoms usually appear one week after exposure, where fever and watery diarrhoea are usually limited to 1-2 weeks. Adenovirus infections occur year-round and no racial predilection has been described (Wadell et al., 1999). In several studies EAds has been found in 2.9 - 13% of the stool specimens from infants and young children with acute gastroenteritis (de Jong et al., 2003). The incidence of enteric adenoviruses was reported as 6.7% in Iran (Saderi et al., 2003), and 13% in Mexico (Maldonado et al., 1998).
These figures are high in comparison to other reports from Brazil at 1.55% (Soares et al., 2002), 3.1% in Australia (Grimwood et al., 1995), 3% in South Africa (Kidd et al., 1996), and 3% in France (Bon et al., 1999). Seroprevalence studies carried out in South Africa have shown that 50% of children less than four years of age have antibodies to enteric adenoviruses (Soares et al., 2002). This indicates that there is an initial exposure to adenovirus infection in infants and in toddlers. The importance of enteric adenoviruses in infantile diarrhoea in developing countries has been less well studied, but antibodies against enteric adenoviruses have recently been shown to be common and widespread throughout the world (Kidd et al., 2003).

2.7 Human caliciviruses (HCvs)

Human caliciviruses are members of the family Caliciviridae, and its two genera include; the Norwalk-like viruses (NLVs) and Sapporo-like viruses (SLVs) (Berke et al., 2007). Human caliciviruses contain a single positive stranded RNA genome and are about 33nm in diameter (Prasad et al., 1999). Infection by calicivirus produces an expansion of the villi of the proximal small intestine. The epithelial cells remain intact, and there is shortening of the microvilli (Treanor, 2000). The mechanism by which diarrhoea is produced is unknown, although it has been suggested that the delay in gastric emptying observed in Norwalk virus gastroenteritis may play a role (Meeroff et al., 1980).

2.8 Diagnosis and control of viral gastroenteritis

2.8.1 Antigen detection techniques

These include; Enzyme Immunoassay (EIA), agglutination with latex particles (LA) and more recently immunochromatography (ICG), all of which are available commercially for group A
rotavirus, adenovirus and astrovirus. The EIA has proven to be more sensitive than direct visualization by electron microscopy and also has a high specificity in the detection of group A rotavirus, especially when monoclonal antibodies are used. The LA technique has lower sensitivity than EIA. ICG shows high sensitivity and results comparable to those achieved with EIA, and is rapid and technically very simple (Sherlock et al., 1989).

2.8.2 Molecular biology techniques
These techniques are more sensitive than immunoassay methods. They are useful for the confirmation of the results of other techniques, and for the genotyping of these viruses. The RT-PCR techniques have been developed for group A rotavirus, adenovirus, astrovirus and norovirus group I and II (Cubitt et al., 1999).

2.9 Prevention and treatment of viral gastroenteritis
2.9.1 Treatment
Most cases of diarrhoea are caused by viruses and do not respond to antibiotics. Hence, treatment of viral gastroenteritis is symptomatic and it aims at preventing or treating the dehydration induced in the disease. Therefore, it is important to start liquid intake early, in order to correct the water deficit and combat the losses due to vomiting and diarrhoea (Alam et al., 2003). Rehydration is achieved by giving the person oral rehydration therapy (ORT) although intravenous delivery may be required if a decreased level of consciousness or an ileus is present. Oral rehydration solutions such as Gastrolyte, Repalyte or Hydrolyte, which are commercially available, are the best option. Antiemetic drugs may also be helpful for vomiting in children (Haffejee, 1991).
2.9.2 Prevention of viral gastroenteritis

Persons can reduce their chance of getting infected by frequent hand washing, prompt disinfection of contaminated surfaces with household chlorine bleach-based cleaners and prompt washing of soiled articles of clothing. If food or water is thought to be contaminated, it should be avoided. Rotavirus gastroenteritis can also be prevented by vaccines. Currently there are two licensed rotavirus vaccines (Rotateq and Rotarix) that protect against severe diarrhoea from rotavirus infection in infants and young children. These vaccines are given to children in their first year of life with other childhood vaccines (Unicomb et al., 2001).
3.1 Study site

The study was carried out at Mukuru slums Nairobi Kenya. The samples were collected at St. Mary's health centre and Reuben medical centre, both located in different regions of Mukuru slums. These are community-based health centres that provide primary healthcare to the area residents, especially those from within and around Mukuru slums. Mukuru slums is an especially good target to capture all the children who are suffering from acute or chronic gastroenteritis, direct from their residential area before they are referred to general hospitals for possible hospitalization if the diarrhoea persists.

Plate 1.0 The study site; Mukuru slums. The plate shows open sewers and waste disposal that may increase the level of contamination in Mukuru slums, thus posing a major threat to public health. A - an open sewerage system, B - a child seated besides the flowing sewer, C - butchery located besides an open sewerage system and D - men eating and drinking in the same environment.
3.2 Ethical clearance
The study was approved by Ethical Review Committee (ERC) of Kenya Medical Research Institute (KEMRI). It was voluntary and parents or guardians signed a consent document to allow participation of their children. All personal identification was removed from the fecal samples sent to KEMRI for viral gastroenteritis analysis, and the samples were assigned new laboratory numbers. Viral RNA/DNA used for this study was obtained from these anonymous samples.

3.3 Study population
The study population consisted of children aged five years and below who attended St. Mary's health centre and Reuben medical centre in Mukuru slums, presenting with symptoms suggesting acute or chronic gastroenteritis such as vomiting, diarrhoea, fever and abdominal cramps.

3.3.1 Inclusion criteria
- All children of five years and below with diarrhoea not exceeding 7 days.
- Consent of parents or caregivers

3.3.2 Exclusion criteria
- All children above the age of five years with diarrhoea
- All children of five years and below with diarrhoea exceeding 7 days.
- Non-consent of parents or caregivers
3.4 Study design

A purposive cross sectional sampling method was applied by taking every child with acute or chronic diarrhoea seeking treatment at St. Mary's and Reuben medical centre in Mukuru Slums, and falling in the selection criteria.

3.5 Fecal specimen collection and handling

Fecal samples were collected from 340 outpatients (children below five years of age) who visited Reuben medical center and St. Mary's health center in Mukuru slums with gastroenteritis symptoms, from Jan 2010 to March 2011. Specimens were transported to the Virology Laboratory in Kenya Medical Research Institute in sterile cryovials placed in a cooler and stored at -20 °C. The demographic characteristics of the patient and a clinical history for each patient were collected by a nurse or attending physician, and the following signs and symptoms were noted: diarrhoea, vomiting, abdominal pain, dehydration and fever.

3.6 Methodology

3.6.1 Virus Detection

The presence of viruses in stool samples was determined using Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique.

3.6.1.1 Viral DNA/RNA extraction

Extraction of RV, AstV and NorV RNA was done using ZR Soil/Fecal RNA MicroPrep™ extraction kit obtained from Seegen South Africa, as illustrated below.
Protocol for extraction of viral RNA

About 250 mg of the sample was put into a ZR bashing bead™ Lysis Tube, and 0.8–1.0 ml S/F RNA Lysis Buffer was added. The tube was then secured in a bead beater fitted with a 2 ml tube holder and processed. This was followed by centrifugation in a microcentrifuge at ≥12,000 xg for 1 minute, after which 400μl of the supernatant were transferred into an RNase-free tube and 1 volume of RNA binding buffer added to the supernatant and mixed well by pipetting. The mixture from the above was transferred into a Zymo-Spin™ IIIC column in a collection tube and centrifuged at ≥3,000 xg for 30 seconds. The flow-through was saved.

One volume of ethanol (95-100%) was added to the flow-through from the step above and mixed well by pipetting, then the mixture was transferred into a new Zymo-Spin™ IIIC column in a collection tube and centrifuged at ≥12,000 xg for 30 seconds. The flow-through was discarded and the step repeated as needed. A 400μl aliquote of RNA prep buffer was added to the column and centrifuged at ≥12,000 xg for 1 minute and the column transferred into an RNase-free tube. A 100μl aliquote of DNAse/RNAse-Free Water was added directly to the column matrix and left to stand at room temperature for 1 minute, after which it was centrifuged at ≥12,000 xg for 30 seconds. The eluted RNA was transferred into a prepared Zymo-Spin™ IV-HRC spin filter in an Rnase-free tube and centrifuged at 8,000 xg for 1 minute.

Thereafter, 200 μl of RNA binding buffer were added to the filtrate followed by 300 μl ethanol (95-100%) and mixed well by pipetting. The mixture was then transferred into a Zymo-Spin™ IC column in a collection tube and centrifuged at ≥12,000 xg for 30 seconds. The flow-through was discarded. 400μl of RNA prep buffer was added to the column and centrifuged at ≥12,000
xg for 1 minute and the flow-through was discarded. Then 700\(\mu\)l of RNA wash buffer was added to the column and centrifuged at \(\geq 12,000\) xg for 30 seconds, the flow-through was discarded, and the step repeated with 400 \(\mu\)l RNA Wash Buffer.

The columns were then centrifuged at \(\geq 12,000\) xg for 2 minutes in the emptied collection tube to ensure complete removal of the wash buffer after which they were then carefully removed and placed into an RNase-free tube. After this, 6 – 8 \(\mu\)l of DNase/RNase-free water was added directly to the column matrix and let to stand for 1 minute at room temperature. The column was then centrifuged at top speed for 30 seconds. The eluted/total RNA was used immediately or stored at \(\leq -70^\circ\text{C}\).

Adenovirus DNA extraction was done using QIAamp MiniElute\textsuperscript{TM} Virus Kit from QIAGEN USA, as per the manufacturer’s protocol (Appendix 1.1).

### 3.6.1.2 Reverse transcription

Reverse transcription was done using RevertAid\textsuperscript{TM} First Strand cDNA Synthesis kit from Seegen South Africa, following the manufacturer’s protocol below.

#### Protocol for reverse transcription

The following reagents were added to an RT tube on ice; 8\(\mu\)l of total RNA, 1\(\mu\)l of random hexamer (0.2\(\mu\)g/ \(\mu\)l) and 3\(\mu\)l of DEPC–treated water, adding up to a total volume of 12\(\mu\)l per tube. The tubes were then incubated at 80\(^\circ\text{C}\) for 3 minutes and latter chilled on ice for 2 minutes after which they were span briefly.
The following reagents were then added to the tubes; 4μl of 5x RT buffer, 2μl of 10mM dNTP, 1μl of RNase inhibitor (20 μ/μl) and 1μl of reverse transcriptase (200 μ/μl) adding up to a volume of 20μl per tube. The tubes were then incubated at 37°C for 90 minutes and then heated to 94°C for 2 minutes, after which they were chilled on ice for 2 minutes and later span briefly. All the cDNA samples were stored at -20°C until ready for use.

3.6.1.3 Amplification of cDNA

Amplification of cDNA was done using Seeplex® Diarrhoea ACE Detection kit (Seegen, South Africa) according to the manufacturer’s protocol illustrated below. This kit allowed a multiplex assay that permitted the simultaneous amplification of target DNA/cDNA of human enteric adenoviruses type 40 and 41, group A rotaviruses, Norovirus GI and II, Astroviruses and an internal control (IC).

Protocol for PCR

A reaction mix was prepared by adding the following reagents into a 0.2ml PCR tubes; 4μl of 5x DV PM, 3μl of 8-mop solution and 10μl of 2x multiplex master mix, adding up to a total volume of 17μl of PCR master mix. NB: the amount of each reagent needed was calculated based on the total number of reactions (samples + controls). To the master mix, 3μl of the sample’s nucleic acid were added. For the negative control, 3μl of the DV/DB ACE NC* were used, while DV/DB ACE PC* was used for the positive control. The tubes were then placed in a preheated (94°C) thermocycler and ran using the following program (Table 1.0).
### Table 1.0 Thermocycler program for amplification of cDNA

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Duration (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94°C</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>94°C</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>10</td>
</tr>
</tbody>
</table>

After amplification, the samples were resolved in a 1% agarose gel alongside a 1000 bp ladder and then viewed under UV light.

#### 3.6.2 Genotyping rotavirus RNA

An established WHO Rotavirus Reference Laboratory Manual 2002 (South Africa) was used. Genotyping involved polyacrylamide gel electrophoresis of rotavirus genomic RNA, reverse transcription of purified RNA, first round polymerase chain reaction of cDNA, and a nested PCR of the amplified VP4 and VP7 genes.

#### 3.6.2.1 Polyacrylamide gel electrophoresis

**3.6.2.1.1 Extraction of rotavirus dsRNA from stool for PAGE**

Stool suspensions were made by adding a pea-sized amount of fecal material to approximately 5ml of distilled water and mixed well. From this 450μl were put into eppendorf tubes to which 50μl of pre-wormed 1M sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS) (pH 5.0) was added. The tubes were then incubated at 37°C in a water bath for 15 minutes and then 500μl of phenol /chloroform (1:1) were added to the eppendorf tubes and vortexed for 1
minute. They were then incubated for further 15 minutes in the 56°C water bath. The tubes were opened and resealed prior to vortexing to reduce the air pressure within the tubes, and prevent them from popping open during mixing. The tubes were then vortexed for one minute and centrifuged for 3 minutes at 12000 rpm. The upper aqueous phase containing the dsRNA was carefully removed and placed in a clean eppendorf tube. The interface material was highly avoided as it contained proteins and DNA that could contaminate the extract and potentially degrade the RNA.

Forty µl of 3M sodium acetate were added followed by 1ml of ice-cold absolute ethanol and mixed gently by turning the tubes over 4-6 times. The tubes were then incubated at -20°C overnight to allow the dsRNA to precipitate. For 15 minutes at 12000 rpm, the tubes were centrifuged to pellet the dsRNA and the supernatant was discarded and the samples air dried. The pellets were then re-suspended in 30µl PAGE sample dye before loading on a PAGE gel.

### 3.6.2.1.2 Gel casting

The glass plates were carefully cleaned with ethanol and assembled for gel casting. 10% resolving gel was prepared by adding 10 ml of 30% Acrylamide stock, 3.75 ml resolving buffer (pH 8.9), 15µl TEMED and 450µl of 10% Ammonium persulphate to 15.8 ml of distilled water in a trough respectfully. The mixture (resolving gel) was then poured in to the assembled glass plates and allowed to polymerize for one hour. A 3% spacer gel was then prepared by adding 1.6 ml of 30% Acrylamide stock, 1.25 ml spacer buffer (pH 8.9), 5µl TEMED and 150µl of 10% Ammonium persulphate to 6.8 ml of distilled water in a trough. The mixture (spacer gel) was then poured on top of the polymerized 10% resolving gel and allowed to polymerize for 45
minutes with a comb inserted to form the wells. The comb was removed and 40 µl of each sample were loaded into the wells of the gel and ran for 17-21 hours under a current of 90 volts.

3.6.2.1.3 Silver staining of the gel

The gel was removed from the glass plates and cut at the bottom right hand corner to orientate it, and then put on a trough. Fixing solution one (which contained 80 ml Ethanol, 110 ml dH₂O, and 10 ml Acetic acid) was added to the gel and incubated for 30 minutes on an orbital shaker. This solution was drained off and replaced with fixing solution two (which contained 20 ml Ethanol, 180 ml dH₂O and 1 ml Acetic acid) and incubated for 30 minutes on an orbital shaker, after which the fixing solution was drained off and replaced with silver nitrate staining solution which had been prepared just before use by adding 200 ml of distilled water to 0.37 g of AgNO₃.

The gel was then incubated for 30 minutes on an orbital shaker. The silver Nitrate staining solution was drained and the gel was washed twice with distilled water for two minutes each time. Fifty milliliters of developing solution (which contained 7.5 g NaOH, 2ml of 36% Formaldehyde and 250 ml of dH₂O) were added to the gel and agitated for 30 seconds to remove any black precipitate and then drained off. The remaining 200ml of developing solution were added on to the gel and incubated on the orbital shaker for five minutes or until RNA bands could be seen.

The developing solution was drained off and a stopping solution (which had been prepared by adding 10 ml of Acetic Acid to 200 dH₂O) was added to prevent further coloring. The gel was incubated for five minutes before rinsing it in distilled water, after which it was visualized over
an illuminator. The gel was then dried overnight on the easy breeze gel Dryer for long time storage.

3.6.2.2 RT-PCR of Rotavirus dsRNA (VP4 and VP7 cDNA synthesis)

Purified rotavirus double stranded RNA was extracted using Trizol method in the WHO rotavirus reference laboratory manual 2002 (South Africa). Stool suspensions were made by adding a pea-sized amount of fecal material to approximately 5ml of distilled water and mixed well. The suspensions were then centrifuged at 5000rpm for 5 minutes at room temperature after which 200μl of the supernatant was transferred to a clean eppendorf tube. 500μl of Trizol were added to the tubes and vortexed for 30 seconds, and then incubated at room temperature for 5 minutes. To the tubes, a 100μl of chloroform was added and vortexed for 30 seconds and then incubated at room temperature for 3 minutes. The tubes were centrifuged at 12000 rpm for 15 minutes at 4°C to separate the phases.

The clear aqueous phases were transferred to clean eppendorf tubes avoiding the white interface and pink organic phase. 700μl of ice-cold isopropyl alcohol were added and mixed gently by turning the tubes 4-6 times. They were then incubated at room temperature for 20 minutes. The tubes were centrifuged at 12000 rpm for 15 minutes at 4°C to pellet the dsRNA. The supernatant was discarded and the pellets allowed to air dry. The pellets were then resuspended in 15μl of de-ionized water awaiting reverse transcription.

After extraction, 8μl of each purified dsRNA were put in a clean PCR eppendorf tube and 1μl of each transcription primers added (sBeg and End9 for VP7, and Con2 Con3 for VP4). The
mixture was then boiled for 5 minutes and immediately chilled in an ice bath. A master mix
(which constituted 0.8μl of 10mM dNTP’s, 0.4μ RNase (AMV) and 2.0μl 5x AMV buffer) was
prepared whereby, the volume of each reagent was multiplied by the number of samples. 3.2μl of
the master mix were then put into each tube containing denatured RNA and incubated in a water
bath at 42°C for 26 minutes.

3.6.2.3 Amplification of cDNA by multiplex PCR
A master mix which comprised of 4μl 10mM dNTP’s, 0.3μl Taq Polymerase, 4μl x10 Taq Buffer,
2.4μl 25mM MgCl₂ and 30μl dH₂O was prepared whereby, the volume of each reagent was
multiplied by the number of samples. 40μl of the master mix were then put into each tube
containing cDNA and run for 30 cycles in a thermo cycler. The samples were then resolved in a
1% agarose gel and viewed under UV light.

3.6.2.4 Genotyping (nested PCR) of the amplified VP7 and VP4 genes
3.6.2.4.1 VP7 nested PCR
A master mix was prepared by adding 10mM dNTP’s, 25mM MgCl₂, x10 Taq Buffer, Taq
Polymerase, dH₂O, primers of each VP7 genotype (G₁, G₂, G₃, G₄, G₅, G₆) and primer End9 to a
clean eppendorf tube, while multiplying the volume of each reagent by the number of the
samples. 40μl of the master mix were put into each tube containing the VP7 cDNA and ran for
thirty cycles in a thermo cycler. The samples were then ran in 1% agarose gel and viewed under
UV light.
3.6.2.4.2 Genotyping of the VP4 gene

A master mix was prepared by adding 10mM dNTP’s, 25mM MgCL₂, x10 Taq Buffer, Taq Polymerase, dH₂O, primers of each VP4 genotype (P₄, P₆, P₈, P₉, P₁₀) and the Con₃ primer to a clean PCR eppendorf tube, while multiplying the volume of each reagent by the number of the 'samples. 40µl of the master mix were then put into each tube containing the first time amplified VP4 cDNA and then transferred to a thermo cycler that had been set to run 30 cycles. The samples were then resolved in a 1% agarose gel and viewed under UV light.

3.7 Data analysis

The data collected was routinely entered into a database created in Microsoft excel for analysis, while statistical analysis were done using the chi squire. The prevalence and seasonal distribution of different enteric viruses, age and gender of the child were presented as proportions of the total population.
CHAPTER FOUR
RESULTS

4.1 Distribution and prevalence of diarrhoea causing viruses

A total of 340 patients (children below five years of age) were screened for viral gastroenteritis in the period between January 2010 and March 2011. Their stool samples were analysed for rotaviruses, enteric adenoviruses types 40 and 41, astroviruses, and norovirus GI and GII by RT-PCR method. Among the 340 stool specimens, 102 (30%) contained at least one of the above named viruses except noroviruses GI, whereas no virus was detected in 238 of the remaining samples (70%). As shown in Figure 4.1, the prevalence of rotavirus infection was the highest in 82 patients (24%). Enteric adenoviruses were detected in 9 patients (2.7%), noroviruses GII in 6 patients (1.7%), while astroviruses were detected in 5 patients (1.5%) of the stool samples. There was no noroviruses GI detected from the samples.

![Figure 4.1 Prevalence of rotavirus, enteric adenoviruses type 40 and 41, astrovirus and norovirus GI and GII, in children aged five years and below in Mukuru slums Nairobi.](image)
Of the 102 positive samples, rotaviruses were the most abundant (80%), followed by enteric adenoviruses which were in 9% of the positive samples. Noroviruses GII and astroviruses were found in 6 and 5% of the positive samples, respectively. A comparative distribution of the four viruses detected in the 102 positive samples is illustrated in Figure 9.0.

Figure 9.0 Percentage distribution of rotavirus, enteric adenovirus 40 and 41, norovirus GII and astrovirus, in the 102 positive samples from children aged five years and below in Mukuru slums Nairobi.

Dual infections were found in 8 of the 102 positive samples (10.78%). Majority of these (87.5%) were combinations of rotavirus with one of the other three viruses. The most frequent dual gastrointestinal infections were rotavirus and adenovirus (4/8; 50%). In addition, 2/8 (25%) samples were positive for both rotaviruses and noroviruses, while only 1/8 (12.5%) sample tested positive for both rotaviruses and astroviruses. Another 1/8 (12.5%) had a combination of both
enteric adenoviruses and noroviruses GII. Dual infections appeared to be more significantly detected during cold seasons when viral gastroenteritis was at its peak. No specific clinical severity of the gastroenteritis was significantly related to the detection of dual infection diseases. Moreover, it was observed that dual infections were not specific to certain children age groups.

The proportional age distribution of children with acute viral diarrhoea as diagnosed by RT-PCR is depicted in figure 10.0. Rotaviruses were the most common cause of acute gastroenteritis and occurred most frequently with 73% in the <1-yr old age group, while the least common rotavirus infection was 12% among 3 yrs and above age group.

![Figure 10.0](image-url)  
Figure 10.0 Comparative age distribution and detection rates of rotavirus, enteric adenoviruses, norovirus GII and astroviruses in children aged five years and below in Mukuru slums Nairobi.
Also as shown in Figure 10.0 above, enteric adenoviruses were detected in children of up to two years of age and none was detected in children between three to five years. Adenoviruses infections were however found to be significantly higher in children aged between 7 to 12 months (44.44%; 4/9) compared to any other age group, followed by children between 1 to 2 years (33.33%; 3/9). Noroviruses were detected in children whose ages were between 4 months to five years of age, with the highest detection observed in the 7 – 24 months old age group.

Astroviruses were detected in all age groups, except in the age group between 4 to 6 months.

The major clinical symptoms related to viral gastroenteritis in this study were as shown in Table 2.0. Symptoms associated with rotavirus infections were mainly watery stool, vomiting, abdominal cramps, and fever. Most patients with enteric adenovirus infections frequently displayed symptoms associated with watery stool and abdominal cramps, while vomiting, and fever were displayed at low rates in all the studied cases.

Patients with astrovirus infection displayed symptoms including watery stool, vomiting, abdominal cramps, and fever. In norovirus infected patients, watery stool and vomiting were the most common. The symptoms of rotavirus infection were found to be more severe compared to the other three viruses, the leading symptoms being watery stool and fever.
Table 2.0 Percentage distribution of clinical symptoms associated with viral acute gastroenteritis in children from Mukuru slums.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Watery/loose tool</th>
<th>Vomiting</th>
<th>Abdominal crump</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>96</td>
<td>62</td>
<td>98</td>
<td>68</td>
</tr>
<tr>
<td>Norovirus (Gii)</td>
<td>92</td>
<td>90</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>87</td>
<td>85</td>
<td>75</td>
<td>86</td>
</tr>
</tbody>
</table>

The distribution of the four gastroenteritis viral causing agents according to gender was as illustrated in table 3.0. Overall, 47.6% (39/82) of the rotavirus infections were identified in female children, while 52.4% (43/82) were found in male children. For the adenovirus positive samples, 5/9 (55.6%) were detected in female children and the remaining 4/9 (44.4%) were detected in male children.

Table 3.0 Distribution of diarrhoea viruses among the male and female children aged five years and below from Mukuru slums.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. of cases in Female children (%)</th>
<th>No. of cases in Male children (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus</td>
<td>39 (47.6)</td>
<td>43 (52.4)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>5 (55.6)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Norovirus G(ii)</td>
<td>3 (50)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>3 (60)</td>
<td>2 (40)</td>
</tr>
</tbody>
</table>

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Norovirus positive samples were equally distributed among the male and female children, with 3/6 (50%) on both sides. Of the astrovirus positive samples, 60% (3/5) were detected in female children, while 2/5 (40%) were from male children. The Chi square test showed that the difference between gender infections was not statistically significant. Using a P value < 0.05 and 3 degrees of freedom, the critical value was 7.815 while the tabulated chi square value was 0.479. This showed that viral infections were equally distributed between male and female children.

During the 15 months of study, a distinct seasonality of viral diarrhoea was identified. The monthly distribution of rotavirus, adenovirus types 40 and 41, Astrovirus and Norovirus GII infections in percentage of each pathogen detected is displayed in Figure 11. All viral gastroenteritis peaked in the cold months of July and August, with minimum infections in November and December for astrovirus, adenovirus and norovirus, while rotavirus infection was minimal in April. Rotavirus and enteric adenovirus infections were found to occur all year round, while astrovirus and norovirus infections were absent in some months of study.

![Figure 11.0 Distribution of viral agents in acute gastroenteritis patients by season in Mukuru slums Nairobi.](image-url)
4.2 Genotyping group A rotavirus

4.2.1 Rotavirus RNA electropherotypes

Of the 82 rotavirus-positive specimens (by RT-PCR), 40 were ran through PAGE and yielded rotavirus RNA electrophoretic patterns. Thirty two (80%) of the isolated strains were long electropherotypes while eight (20%) of the strains were short electropherotypes. There were no profiles of mixed infections detected in the electropherotypes (Plate 2.0). All along the 15 months of this study, two different rotavirus strains as defined by PAGE-RNA electropherotypes co-circulated in the Mukuru slums, with the long strain being predominant and persistent during the period of this survey (Figure 12.0).

Throughout the study, the long electropherotypes were always associated with G1 and G9 genotypes, whereas the short electropherotypes were associated with genotype G3. However, it was noted that the pattern of the electropherotype suggested but did not confirm a particular genotype, as described by Sethi et al. (2006).

Plate 2.0 Genomic RNA electrophoresis of representative strains from rotavirus electropherotypes identified in Mukuru slums. Letter A indicates a short electropherotype while B indicates a long electropherotype. RNA migration was from the top to the bottom, and Lanes 2, 3, 4, 5, 6, 8, 9, and 10 represents long electropherotypes, while lanes 1 and 7 represent the short electropherotypes detected.
The monthly distribution of the long electropherotypes appeared to be random and no apparent seasonal variations could be detected. The short profiles were more prevalent in cold and wet months and were not observed in dry months of the study period (Figure 12.0).

**Figure 12.0** Temporal distribution of rotavirus electropherotypes in children with acute gastroenteritis during January 2010 through March 2011 in Mukuru slums Nairobi.

The distribution of long RNA electropherotypes did not appear to vary with age and it occurred in all ages under study (one month to five years old), while the short RNA profiles were identified among children aged up to two years old (Table 4.0).
Table 4.0 Distribution of rotavirus RNA electropherotypes among different age groups of outpatient children with acute gastroenteritis in Mukuru slums Nairobi.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Electropherotypes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long patterns</td>
<td>Short patterns</td>
</tr>
<tr>
<td>0-3 Months</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>4-6 Months</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>7-12 Months</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>13-24 Months</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>25-60 Months</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

4.2.2 VP7 genotypes

The G types (VP7 associated) were successfully determined in 37 (92.5%) of the 40 page positive samples. A G type was assigned after a PCR with G1, G2, G3, G4, G8 and G9 – specific primers was carried out on rotavirus cDNA template.

Plate 3.0 VP7 genotypes as seen under UV light in agarose gel. Lane 1 – 1000bp marker, Lane 2 – G1 (749 bp), Lane 3 – G3 (374 bp), Lane 4 – G9 (306 bp), Lane 5 – G1 (749bp) and Lane 6 G9(306bps).
The overall incidence for G typing was G1, 40.5% (15), followed by G3, 21.6% (8), and G9, 32.4% (12) (Table 5.0). Mixed types (G3/G9) were detected at 5.4% (2), while G2, G4 and G8-type viruses were not detected. The remaining three (7.5%) positive samples although positive for rotavirus, remained untypable. (Table5.0). The incidence of each type was seen to vary from month to month. G1 and G9 occurred most frequently in February and August, while G4 was high in November and December (Table 5.0).

4.2.3 VP4 genotypes

The P types (VP4 associated) were successfully determined in 35 (87.5%) of the 40 page positive samples (Table 5.0). A P type was assigned after a PCR with P[4], P[6], P[8], P[9] and P[10]-specific primers was carried out on rotavirus cDNA template.

Only three P types were shown to be prevalent in Mukuru slums: P[4]- 4 (11.4%), P[6]- 8 (22.9%), and P[8]- 21 (60%). P[6]/P[8] mixed types were detected in 2 samples (5.7%) (Table 5.0), while no P[9] or P[10]-type viruses were detected during the survey. Two samples (5.4%) were untypeable. The incidence of each type was seen to vary from month to month, with P[6] occurring most frequently in January, while an increase in P[8] was noticed in August and February. The incidence of P[4] was high in January (Table 5.0).
Table 5.0 Distribution of rotavirus G and P types over 15 months in Mukuru slums.

<table>
<thead>
<tr>
<th></th>
<th>No. and % (in parenthesis) of G types</th>
<th>No. and % (in parenthesis) of P types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>G3</td>
</tr>
<tr>
<td>Jan-March 2010</td>
<td>3 (30)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>April-June 2010</td>
<td>2 (33.33)</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>July-Sept 2010</td>
<td>6 (54.6)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>Oct-Dec 2010</td>
<td>2 (40)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Jan-March 2011</td>
<td>2 (25)</td>
<td>2 (25)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>8</td>
</tr>
</tbody>
</table>

Key = Mix$^a$— mixed G or P types; NT - not typeable; in brackets - %.
4.2.4 Combination of G and P types

During the typing assays it was observed that a specific G type could always coexist with a certain P type; namely, G1 and G3 always coexisted with P[8], while G9 was found to associate with P[6] or P[4]. The incidence of each genotype varied considerably for the 15 months (Fig. 13.0). Overall, G1 P[8] was recorded as the most common (41.2% of all doubly typed viruses). The other types, namely, G3 P[8], G9 P[4], G9 P[6], and mixed types (G3/G9 P[8]) were less frequent (20.3, 11.8, 20.3 and 5.9%, respectively).

It was noted that G1 P[8] was the most predominant type collected from both the male and female children. A marked increase in the number of rotavirus positive samples was observed in July and August. This went along noticeable drop in genotype G9 P[6] and an increase in G1P[8] as confirmed by data shown in Fig. 13.0. 35.7% of G1 P[8] types were collected during the cold season in this study.
Figure 13.0 Graphic illustration showing the incidence and diversity of rotavirus genotypes for a period of 15 months in Mukuru slums Nairobi county.
5.1 Discussion

This is the first study evaluating the incidence and genetic diversity of diarrhoea viruses in Mukuru slums Nairobi. The study demonstrated that rotaviruses, adenoviruses, noroviruses GII and astroviruses were important etiologic agents of acute gastroenteritis among children below five years of age in Mukuru slums. Analysis of the main epidemiological incidence of acute infectious diseases in this study revealed that gastroenteritis played a major part in the overall morbidity for acute infectious diseases in the region.

Group A rotaviruses were detected in 24% of the gastroenteritic stool samples, enteric adenoviruses in 2.7%, norovirus GI in 1.7% and astroviruses in 1.5%. For rotaviruses, this figure corresponds to the published median rate of 24% reviewed in 43 published studies of rotavirus epidemiology in Africa (Cunliffe et al., 2001). With regard to adenovirus prevalence, these results were comparable to other studies where the prevalence of enteric adenoviruses was reported as; 1.55% in Brazil (Soares et al., 2002), 3.1% in Australia (Grimwood et al., 1995), 3% in South Africa (Kidd et al., 1996), and 3% in France (Bon et al., 1999).

NoVs GII were detected in 1.7% (6/340) of the samples from outpatient gastroenteritis cases. This is comparable to that in France, Ireland, Switzerland and Italy, where NoV prevalence was found to be 1.7% (Bon et al., 1999). Published studies report an incidence varying between 1.5% and 47.3% (Iturriza et al., 2008).
Similarly, the findings on astrovirus prevalence are similar to other studies showing an incidence of astrovirus infection in children with gastroenteritis of 1.6% in India (Shetty et al., 1995), 2% in Brazil (Stewien et al., 1993), and 3.2% in Australia (Palombo et al., 1996). However, in some studies, higher rates of astrovirus infection have also been reported. These include 6.3% in France (Bon et al., 1999), 6.8% in USA (Dennehy et al., 2001), and 7% in South Africa (Steele et al., 1998). This could be attributed to the environmental conditions during the study period, the timing of sample collection, methods of detection used and the emergence of new astrovirus variant strains at the time the studies were conducted.

The medical importance of astroviruses results from the prevalence of this pathogen in both rural and urban population, and the fact that astrovirus gastroenteritis can result in complications and lead to hospitalization among individuals suffering from poor nutritional status, immunodeficiency, severe mixed infection, or underlying gastrointestinal disease (Koopmans et al., 1998). Several previous reports have noted a fairly high prevalence of astrovirus infection among immuno-compromised patients including adult AIDS patients, adult bone marrow transplant patients, and pediatric transplant and oncology patients (Grohmann et al., 1993).

With the licensing and routine use of the rotavirus vaccine, the impact of astrovirus infection on the morbidity of infants and children may become increasingly important (Koopmans et al., 1998). The development of an effective astrovirus vaccine would be a worthwhile goal, especially for the protection of young children, the institutionalized elderly, and those at risk for the development of complicated disease.
In this study, the most common age group identified with acute rotavirus diarrhoea was 7–12 months age group. The study revealed that the under 1 year age group contributed to over 73% of positive cases and over 88% of the children had rotavirus diarrhoea by the age of 2 years, emphasizing the fact that rotavirus infection occurs early in life. The prevalence of rotavirus disease is similar in children in both developed and developing countries, however, children in developing countries die more frequently possibly due to poorer access to hydration therapy and prevalence of malnutrition (Parashar et al., 2003). Published data indicate that the highest rotavirus prevalence occurred in children aged 6 to 18 months with severe infections occurring more frequently in younger children aged 6 to 12 months in developing countries (Bishop et al., 2004). Also in Central Africa, rotaviruses were found most frequently among children less than 1 year-old (Georges et al., 2006).

A Venezuelan study by Perez-Schael et al. (2003) reported the generation of rotavirus-specific IgA antibodies following rotavirus infection, and by the age of 11 months, about 85% of the infants had acquired rotavirus IgA antibodies. Neutralizing IgG antibodies also increased markedly as the children reached one year of age, reflecting exposure to natural infection and as expected, infection in this age group is generally mild with few deaths reported worldwide. In this study, a low prevalence to rotavirus was noted in older children between 2 to 5 years of age (12%), and this could be explained by the acquisition of neutralizing antibodies to rotavirus early in life as a result of multiple exposures to rotavirus infection (Soares et al., 2002).
In adenoviruses, the majority of infections were detected in children between 7 to 12 months old and none above two years. This finding is in agreement with a study conducted in Saudi Arabia, which showed that all infections were detected in children below two years of age (Akhter et al., 1999). Seroprevalence studies have shown that 50% of children less than four years of age have antibodies to enteric adenoviruses (Soares et al., 2002), and this indicates that there is an initial exposure to adenovirus infection in infants and in toddlers.

Unlike in rotavirus and enteric adenovirus infections, norovirus and astrovirus infections were detected in all ages under study, with most of the infections observed among children between the ages of 7 to 24 months. This was attributed to the fact that these two viruses are known to cause epidemic outbreaks involving subjects of any age in favoring conditions, and therefore children take long before developing neutralizing antibodies due to absence of frequent exposure (Sebunya et al. 2000). In fact, noroviruses are estimated to be responsible for 60–90% of all epidemic outbreaks involving subjects of any age (Kirkwood, 2001). In a study by Kurtz and colleagues (1992), astrovirus antibodies were detected in 7% of infants 6 to 12 months of age, 70% in children aged between 1 to 3 years, and 85% of children 5 years of age and above. This suggested that in most cases, antibodies to astroviruses are not developed in early age (Sebunya et al. 2000).

This 15-month survey of enteric virus infection in and around Mukuru slums indicated that rotaviruses and enteric adenovirus infections were present in all seasons, while norovirus and astrovirus infections were sporadic. However, all the viral infections peaked in the cold and wet months of July and August. The seasonal distribution of enteric viral infection remains largely
unexplained even though it has been well documented that most infections are noted during the winter months in temperate climates and during the rainy season in tropical climates (Labaron et al. 1990). While the high incidence noted during the winter months is extremely puzzling, it is likely that in tropical climates, the rainy season creates conditions which increase the opportunities for transmission such as the breakdown of sanitation in developing nations (Koopmans et al., 1998).

Precisely, 65% of rotavirus gastroenteritis incidence occurred during the cold months, while only 7% of these infections were found among diarrhoeic patients in worm months of January to March. These results are in agreement with other African studies conducted in Botswana by Sebunya et al. (2000) and Kasule (2001), and in Zambia Zimbabwe and South Africa by Cunliffe et al. (2001). This pattern is usually observed in tropical climatic regions and it is not applicable to all climatic conditions as shown by Labaron et al. (1990). His findings showed that the seasonal nature of viral gastroenteritis is not universal and in countries within 10° of the equator, infection occurred all the year-round.

No significant difference was observed in the infection rate between male and female patients in the current study. This confirmed other studies done in China and Bahrain, where viral diarrheoa infections had no gender difference (Qiao et al., 1999). The major clinical symptoms associated with viral gastroenteritis in this study were fever, watery stool, vomiting and abdominal cramps. However, symptoms due to rotavirus infection appeared more severe than in the other three enteric diarrhoea viruses. These findings were similar to studies conducted in Indonesia (Roman et al., 2003), Thailand (Gomwalk et al., 2000) and Egypt (Naficy et al., 1999).
Dual infections involving two viruses were present in 8 (2.4%) of the tested samples, corresponding to approximately 7.8% of the 102 viral positive stool samples. Adenoviruses, noroviruses and astroviruses were the most frequently detected viruses implicated in mixed infections in association with rotavirus. Previously published studies identified mixed infection in percentages ranging from 4.4 to 29% of the virus-positive stool samples in different countries using classic or molecular techniques (Roman et al., 2003). A recent French study reported the presence of 11% of mixed infections among the positive stool samples during 1997 and 1998 winter periods (Marie et al., 2002).

No specific clinical severity of gastroenteritis was observed in cases of mixed infection diseases. The observation in this study that adenoviruses were found at similar frequencies in both rotavirus-positive (dual infection) and rotavirus-negative diarrhoeic stools suggests that there was no synergy between infections by these two viruses. However, the presence of mixed viral infections has previously been associated with the development of necrotizing enterocolitis in some premature infants (Hunter et al., 2008).

The results of this study describe the first genotyping results for rotavirus strains associated with acute diarrhoea in infants and young children in Mukuru slums Kenya. Genotyping is a well established and recognized epidemiological tool for examining strain diversity, and the correlation between genotypes is well understood. The significance of determining serotypes of circulating rotaviruses has become increasingly recognized (Taniguchi et al., 1994) and RT-PCR has been shown to be the most sensitive assay for determining genotypes (Ushijima et al., 1994).
Two major RNA patterns designated as short and long profiles were detected by PAGE, the long strain being dominant (32/40; 80%). The short strains were detected in 8 samples (20%). This corresponds to a study carried out in Ahwaz Iran on children below five years of age which showed that the long electropherotypes dominated at 83% (Samarbafzadeh et al., 2005). This incidence appears to be the norm globally (Coluchi et al., 2002). This study showed that the long strains existed throughout the year whereas the short strains were found in existence only between April and December. These results confirm the findings of other studies carried out in South Africa and Hungary (Banyai et al., 2002).

The monthly distribution of the long electropherotypes appeared to be random and no apparent seasonal variations could be detected. The short profiles were more prevalent during cold and wet months and were not observed in dry months of the study period. This data shows similar epidemiologic pattern to what have been found in the African developing countries (da Silva-Vaz et al., 1999). The distribution of long RNA electropherotypes did not appear to vary with age and it occurred in all age ranges (one month to five years old), while the short RNA profiles were only identified among children aged up to two years old (Table 4.0).

The majority of the genotypes identified in this study fall within the range of globally common strains. As expected from similar surveys carried out worldwide, G1 was the most predominant type detected in each of the 13 months in Mukuru; identified in 40.5% of the G types. This has been the dominant genotype in most parts of the world during the past decade (Gentsch et al., 2005), and studies carried out in India from 1996 to 2001 (Husain et al., 2002), and in South Korea during 2002–2003 (Kang et al., 2005) showed that G1 was the most common prevailing
genotype. In other studies however, G9 was the predominant genotype in Thailand in 2002 (Jiraphongsa et al., 2005) and emerged as a significant type with a detection rate of 32.4%. This seems to indicate a notable relative shift in the prevalence of circulating viruses, which should be monitored over the coming years.

After the P-typing assays, it was discovered that the Mukuru results matched those observed in studies from seven other countries in that the P[8] type prevailed (Gentsch et al., 2005). It was also observed that its relative incidence over the 15 months varied, with a detection peak in the month of August. Subsequently, P[6] was the second in detection after P[8] and appeared in 22.9%, followed by P[4] which was detected in 11.4% of the P types. The incidence of P[6] in recent American (Ramachandran et al., 1998), Brazilian (Timetesky et al., 1994), and Indian (Ramachandran et al., 1998) surveys seems to highlight this strain as a significant emerging genotype. The slightly lower efficiency relative to P typing can be partially attributed to the RNA degradation from some of the earlier isolates, since the G-typing assays were completed first.

Only five samples (6.7%) could not be assigned a G or a P type, which suggests that the incidence of unusual genotypes among Mukuru isolates is rare. This figure is low when compared to the data from other studies in which, approximately 30% of rotavirus positive stool specimens could not be P or G typed (Cunliffe et al., 2001), (Yeager et al., 2005). The reason for the occurrence of untypeable strains is unclear. It is possible that inhibitors present in extracted RNA specimens prevent enzyme function in RT and PCR steps of the VP7/VP4 typing assays, hence no amplification. In a similar fashion, subtle changes in the primer binding regions could prevent typing. The use of more recently developed VP4/VP7 first round primers may improve P
typing rates (Simmonds et al., 2008). The third possibility is that these strains may present truly novel G and P types, and only sequence analysis of their PCR products would confirm this. Previous studies have shown that characterization of non-typeable strains have occasionally led to the identification of novel G and P genotypes (Gentsch et al., 2005). The untypeable strains in this study may be due to the emergence of new genotypes and thus further research by characterization and sequence analysis should be considered in the near future.

Regarding G–P combinations, the major ones identified during the study were G1 P[8] and G3 P[8] which are both globally common combinations (Moe, 2006)]. In addition, G9 P[6] was also identified at a higher rate but it is not commonly encountered worldwide (Cunliffe et al., 2001). This would seem to indicate a significant genotypic shift, which will be of major importance for future studies carried out in Mukuru slums. The VP4 P[6] protein was traditionally thought to be associated with asymptomatic neonatal disease (Min et al., 2004). However, in this study it was identified in young children with acute disease, similar to that reported in other recent studies conducted in Africa and Asia (Steele et al., 1998). The accessibility of molecular typing methodology has enhanced the knowledge about G and P genotype diversity and in this study, the rarely seen G9 P[4] and G3/G9P[8] combinations were also identified, further highlighting the ability of rotaviruses to undergo re-assortment (Gentsch et al., 2005).

The detection of rotaviral genotypes for the first time in children from Mukuru slums, and the indication that the prevalence of certain genotypes may change over a rotavirus season is significant and mirrors observations from studies in other developing countries (Haffejee et al., 1991).
5.2 Conclusion

- Gastroenteritis played a major part in the overall morbidity for acute infectious diseases in Mukuru slums Nairobi County.

- Rotaviruses, enteric adenoviruses, astroviruses and noroviruses GII were confirmed important etiologic agents of acute gastroenteritis in this region, and some untypable strains observed remain a challenge to diagnosis and disease epidemiology.

- Rotavirus infection is mainly observed in children below two years of age with the rate of disease declining thereafter, while adenovirus infection is not found in children above two years. Astrovirus and norovirus infections are found in all age groups aged five years and below.

- Both the short and long strains of rotavirus are in circulation among children in Mukuru slums, with the long strain leading in both the rate of infection and age distribution with serotypes G1, G3 and G9 in combination with P[4], P[6] and P[8] being the main genotypes circulating in children below five years in Mukuru slums.

5.3 Recommendations

- Further studies on viral pathogens in diarrhoea diseases for both outbreaks and sporadic cases should be carried out in order to understand the burden of viral gastroenteritis.

- The surveillance of enteric viruses should be carried out among children aged five and below country wide.

- Simple and rapid tests be introduced for the diagnosis of viruses causing gastroenteritis in local medical and health centers to improve patient care.
The untypeable strains in this study may be due to the emergence of new genotypes hence, further research by characterization and sequence analysis should be considered in the near future to determine whether they are novel isolates or otherwise.

In order to reduce the prevalence of rotavirus infections in the slum areas, the government needs to provide vaccines for free or at affordable rates.


REFERENCES


APPENDIX

1.1 Extraction of adenovirus DNA using QIAamp MiniElute™ Virus Kit (QIAGEN)

Protocol: Using Stool Tubes for Isolation of DNA from Stool for pathogen Detection

For easier measurement of stool samples, stool tubes with integrated measuring spoons in their lids are available. The spoons in these tubes are designed to collect 200 mg stool. This protocol is optimized for usage with such stool tubes. Lysis conditions in this protocol are optimized to increase the ratio of nonhuman DNA to human DNA. Human DNA is not excluded by this procedure.

Important points before starting

- For detection of cells that are difficult to lyse, such as those of some bacteria and parasites, the lysis temperature in step 4 can be increased to 95°C, if necessary.

- All centrifugation steps should be carried out at room temperature (15–25°C) at 20,000 x g approximately 14,000 rpm).

- Increase the centrifugation time proportionately if your centrifuge cannot provide 20,000 x g (e.g., instead of centrifuging for 5 min at 20,000 x g, centrifuge for 10 min at 10,000 x g).

- The 2 ml tubes used in step 6 should be wide enough to accommodate an InhibitEX Tablet.
Things to do before starting

- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on the labels.
- Mix all buffers before use.
- If a precipitate has formed in Buffer ASL or Buffer AL, dissolve by incubating at 70°C.
- Prepare a 70°C water bath for use in steps 4 and 13.

Procedure

1. Use the spoon integrated into the cap of a stool tube (not provided) to measure 180–220 mg of the stool sample. A level spoonful will correspond to approximately 200 mg stool. Close the tube and place it on ice. A spatula should be used to remove excess stool from the spoon.

2. Add 2 ml Buffer ASL to each stool tube. Use the pipet to wash the stool sample from the spoon while transferring the buffer. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.

Note: It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final eluate. After Buffer ASL has been added, all the following steps can be carried out at room temperature (15–25°C).

3. Pipet 1.6 ml of the stool lysate into a labeled 2 ml microcentrifuge tube. Cut the ends off the pipet tips to make pipetting viscous samples easier.
4. Heat the suspension for 5 min at 70°C. This heating step increases total DNA yield 3- to 5-fold and helps to lyse bacteria and parasites. The lysis temperature can be increased to 95°C for cells that are difficult to lyse (such as Gram-positive bacteria).

5. Vortex for 15 seconds and centrifuge sample at full speed for 1 min to pellet stool particles.

6. Pipet 1.2 ml of the supernatant into a new 2 ml microcentrifuge tube (not provided) and discard the pellet.

Note: The 2 ml tubes used should be wide enough to accommodate an InhibitEX Tablet. Transfer of small quantities of pelleted material will not affect the procedure.

7. Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

8. Centrifuge sample at full speed for 3 min to pellet stool particles and inhibitors bound to InhibitEX matrix.

9. Pipet all the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min. Transfer of small quantities of pelleted material from step 8 will not affect the procedure.

10. Pipet 15 µl proteinase K into a new 1.5 ml microcentrifuge tube (not provided).
11. Pipet 200 µl supernatant from step 9 into the 1.5 ml microcentrifuge tube containing proteinase K.

12. Add 200 µl Buffer AL and vortex for 15 seconds.

Note: Do not add proteinase K directly to Buffer AL. It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.

13. Incubate at 70°C for 10 min. Centrifuge briefly to remove drops from the inside of the tube lid (optional).

14. Add 200 µl of ethanol (96–100%) to the lysate, and mix by vortexing. Centrifuge briefly to remove drops from the inside of the tube lid (optional).

15. Label the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully apply the complete lysate from step 14 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. Close each spin column in order to avoid aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.
16. Carefully open the QIAamp spin column and add 500 μl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.

17. Carefully open the QIAamp spin column and add 500 μl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

18. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

19. Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided). Carefully open the QIAamp spin column and pipet 200 μl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA. For long-term storage, keep the eluate at -20°C.
Note: When using eluates in PCR, for maximum PCR robustness we highly recommend adding BSA to a final concentration of 0.1 µg/µl to the PCR mixture. For maximum PCR specificity we recommend using QIAGEN HotStarTaq Plus DNA Polymerase. For best results in downstream PCR, use the minimum amount of eluate possible in PCR; the volume of eluate used as template should not exceed 10% of the final volume of the PCR mixture. Also, note that high amounts of template DNA may inhibit the PCR. DNA yield is typically 15–60 µg but, depending on the individual stool sample and the way it was stored, may range from 5 to 100 µg. DNA concentration is typically 75–300 ng/µl.
FROM: Dean, Graduate School

TO: Gikonyo Joshua Ndung’u
C/o Department of Biochemistry and Biotechnology

REF: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that the Graduate School Board at its meeting of 28th March, 2011 approved your research proposal for M.Sc degree Subject to editing the title to read “Identification of Diarrhoea causing Viral Agents and Molecular Characterization of Group A Rotaviruses from Children in Mukuru Slums, Nairobi, Kenya”.

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