PREVALENCE OF ADENOVIRUS, ASTROVIRUS AND ROTAVIRUS DIARRHOEA IN CHILDREN INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS IN NAIROBI, KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted for a degree in any University.

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DEDICATION

This thesis is dedicated to my mother Sarah Achieng', and my father Dr. Frederick Ogodo Oluoch for their unwavering faith in my capabilities and their unconditional support in all my endeavours. It is also dedicated to my sisters and brothers who have been a source of inspiration.

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ABSTRACT

Diarrhoea is one of the commonest symptoms of disease in children. In some communities 25% of children younger than one year may suffer from diarrhoea episodes in any two-week period. Worldwide, each child under five years of age may suffer an average of 3 episodes of diarrhoea per year. Majority of these diarrhoeal cases are due to acute self-limiting intestinal viral infection and can be successfully managed without recourse to health services. However HIV predisposes infected persons to intestinal infection with various pathogens leading to persisting severe diarrhoea. The present study evaluated the viral causes of diarrhoea in children with HIV and compared them with children with unknown HIV status. Between February 1999 and June 2000 stool samples were collected from 113 HIV positive children and 94 samples from children with unknown HIV status suffering from diarrhoea. The samples were assayed for astrovirus, adenovirus and rotavirus, three common causes of severe diarrhoea in young children. Antiviral antibody ELISA was used to screen the three viruses, but further characterisation of rotavirus was done using SDS-PAGE, VP6 subgroup antibody ELISA, VP7 monoclonal antibody ELISA and RT-PCR for genotyping of VP7 and VP4 genes. The results showed that the incidences of adenovirus, astrovirus and rotavirus did not vary significantly in HIV infected and uninfected children. Astrovirus was also shown to be an important cause of diarrhoea in children in Kenya (5.8%). The main rotavirus genotypes in children, (G3P[6] and G8P[6]) differed from the predominant types known (G1P[8]). This shows that the important genotype pairs that are responsible for diarrhoea in Kenya differ from those that are important in causing diarrhoea in most parts of the world. This has significant implications in vaccine development since this requires knowledge of the molecular characteristics of circulating strains within a regions population.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Infants and young children with HIV infection commonly suffer from gastrointestinal manifestations (Ramos-Soriano *et al.*, 1996). Many HIV infected children have evidence of persistent diarrhoea, malabsorption, malnutrition or growth failure (Ramos-Soriano *et al.*, 1996). In Kenya, diarrhoeal diseases rank second after pneumonia as the major cause of childhood morbidity (Gatheru *et al.*, 1991).

The aetiology and pathogenesis of gastrointestinal dysfunction in HIV infected children have not been well defined (Ramos-Soriano *et al.*, 1996). Diarrhoea occurs frequently among persons with AIDS, but the cause often remains unknown (Grohmann *et al.*, 1993). It has been estimated that by the year 2000, five to ten million children will have been infected with the human immunodeficiency virus (HIV) (Scarlatti, 1996). Acute and chronic cases of diarrhoea are both major sources of morbidity and mortality in these infected children, particularly in developing countries (Kotloff *et al.*, 1994; Pavia *et al.*, 1992; Thea *et al.*, 1993a), but the etiology and pathogenesis of these gastrointestinal problems are not well understood. Functional and structural intestinal abnormalities, infection with HIV itself, and multiple opportunistic infections have all been implicated as causes of diarrhea (Sharpstone and Gazzard, 1996).

Infectious gastroentritis results from a viral agent in 70% of the cases in young children (Buzby, 1992). Several viruses cause diarrhoea in children including rotaviruses, adenoviruses, caliciviruses, coronaviruses, and even astroviruses (Krugman *et al.*, 1985). The

vast majority of diarrhoea cases in children are due to acute self-limited intestinal infection and are successfully managed at home without recourse to health services.

Immunosuppression as a result of malnutrition and/or HIV infection predisposes the children to intestinal infection. It is however not yet clear whether paediatric HIV infection is associated with severe viral diarrhoea.

Gastrointestinal viruses, predominantly rotaviruses and adenoviruses, can be detected by enzyme-linked immunosorbent assay, electron microscopy or cell culture in greater than 50% of homosexual men with symptomatic HIV infection who do or do not have diarrhoea (Cunningham *et al.*, 1988).

1.2 JUSTIFICATION

Paediatric HIV infection is a common scourge in Sub-Saharan Africa. It has therefore become a major cause of debility and death in children. Most of the morbidity and mortality is due to opportunistic infections. Diarrhoea is a common problem in HIV infected children. The causes are many and include viral organisms. It is important to determine the causes particularly with reference to enteric virus types, strains and serotypes. In this study, special emphasis was made on rotavirus diarrhoea, a major cause of diarrhoea even in children without HIV. This study also focused on adenoviruses and astrovirus, both of which are increasingly implicated, in HIV- related viral diarrhoeas in children.

1.3 OBJECTIVES

1.3.1 Overall Objective

The main objective of this study was to determine viral causes of diarrhoea in HIV infected children.

1.3.2 Specific Objectives

1. To determine the prevalence of rotavirus, adenovirus and astrovirus among HIV-infected children.
2. To determine whether the prevalence of enteric viruses in HIV-positive children differs from that of children without HIV.
3. To characterise the rotavirus strains isolated from the children.

1.3.3 Expected Outcome of Study

In this study we hope to come up with the comparative prevalence of astrovirus, adenovirus and rotavirus among children with HIV and as a control study the prevalence in children without HIV.

CHAPTER 2

INTRODUCTION AND LITERATURE REVIEW

2.0 DIARRHOEA AS A SYMPTOM OF DISEASE

Diarrhoea continues to be a major cause of morbidity and mortality worldwide resulting in an estimated 1000 deaths among children each day, the highest incidence being in developing countries of the world (Fontaine and Newton, 2001).

Diarrhoea is defined by the World Health Organisation as the passage of loose or watery stools at least three times in a 24-hour period (WHO, 1995). There may be abdominal pain (colic) which is less after a stool is passed. Diarrhoeal diseases are usually classified as acute, persistent or chronic depending on the duration of the diarrhoeal episode. Acute diarrhoea comes on suddenly and lasts a short time. Chronic diarrhoea affects someone over a long period of time. Most diarrhoeal attacks are acute, lasting for 5 to 7 days (WHO, 1998). When the stool contains visible red blood the diarrhea is termed as dysentery (WHO, 1994). In some cases the episode can last up to 14 days leading to persistent diarrhea.

Diarrhoea is caused when excess fluid is passed (secreted) from the blood-stream into the bowel, for example in gastroenteritis such as happens in when one takes laxatives. It can also be caused when the bowel moves its contents through too quickly and too little fluid is passed back into the bloodstream. This is one way in which anxiety for example produces diarrhoea.

Acute diarrhea can be caused by food poisoning, gastroenteritis, anxiety, antibiotic treatment, or even excess alcohol. Chronic diarrhoea occurs when diarrhoea goes on for a long time. The most likely cause is the irritable bowel syndrome. It is called a 'functional' condition. Because the bowel produces stools which are looser or more frequent than normal,

although the bowel is not diseased. Less commonly, there are also several conditions which cause chronic diarrhoea:

- inflammation of the bowel - eg. ulcerative colitis or Crohn's disease. The diarrhoea in ulcerative colitis often contains blood.
- poor absorption of food - eg. coeliac disease or chronic disease of the pancreas, when the stools may be very pale, offensive smelling and difficult to flush away.
- hormonal changes - eg. diabetes or an overactive thyroid gland.
- some bowel cancers.
- chronic bowel infections.
- some stomach operations.
- some drugs including antibiotics, magnesium-containing antacids, pills for blood pressure or arthritis and laxatives.
- foods including milk in some people who digest milk sugar (lactose) poorly and wheat products in patients with coeliac disease and consumption of unusually large amounts of alcohol.
- True food allergy, which is very rare.

2.1 HUMAN IMMUNODEFICIENCY VIRUSES

History: Human Immunodeficiency Virus (HIV) is the aetiological agent associated with Acquired Immunodeficiency Syndrome (AIDS) and is an RNA virus that belongs to the lentivirus family of non- oncogenic, cytopathic retroviruses (Chiu *et al.,* 1985). It includes at least two known types. The first, HIV-1, was isolated in 1984 from a patient with AIDS and previously designated lymphadenopathy associated virus (LAV), human T cell lymphotropic virus (HTLV-III), or AIDS-related virus (ARV) (Barre-Sinoussi *et al.,* 1983; Gallo, 1984; Popovic *et al.,* 1984; Levy *et al.,* 1984). These viruses now referred to as HIV-1 are

responsible for the current AIDS epidemic all over the world (Coffin *et al.*, 1986). The second type, HIV-2, previously known as LAV-2, was isolated from AIDS patients in West Africa (Clavel *et al.*, 1986).

Structure: The HIV-1 genes that code for core proteins are ***gag*** and ***env*** for viral envelope glycoproteins and ***pol*** for the protease, reverse transcriptase and the endonuclease. The outer shell of the virus is known as the *viral envelope*. Embedded in the viral envelope is a complex protein known as *env*, which consists of an outer protruding cap *glycoprotein* (gp) *120*, and a stem *gp41*. (Figure 1and 2). Within the viral envelope is an HIV protein called *p17* (matrix), and within this is the viral core or *capsid*, which is made of another viral protein *p24* (core antigen). The major elements contained within the viral core are two single strands of HIV RNA, a protein *p7* (nucleocapsid), and three enzyme proteins, *p51* (reverse transcriptase), *p11* (protease) and *p32* (integrase) (figure 1 and 2).

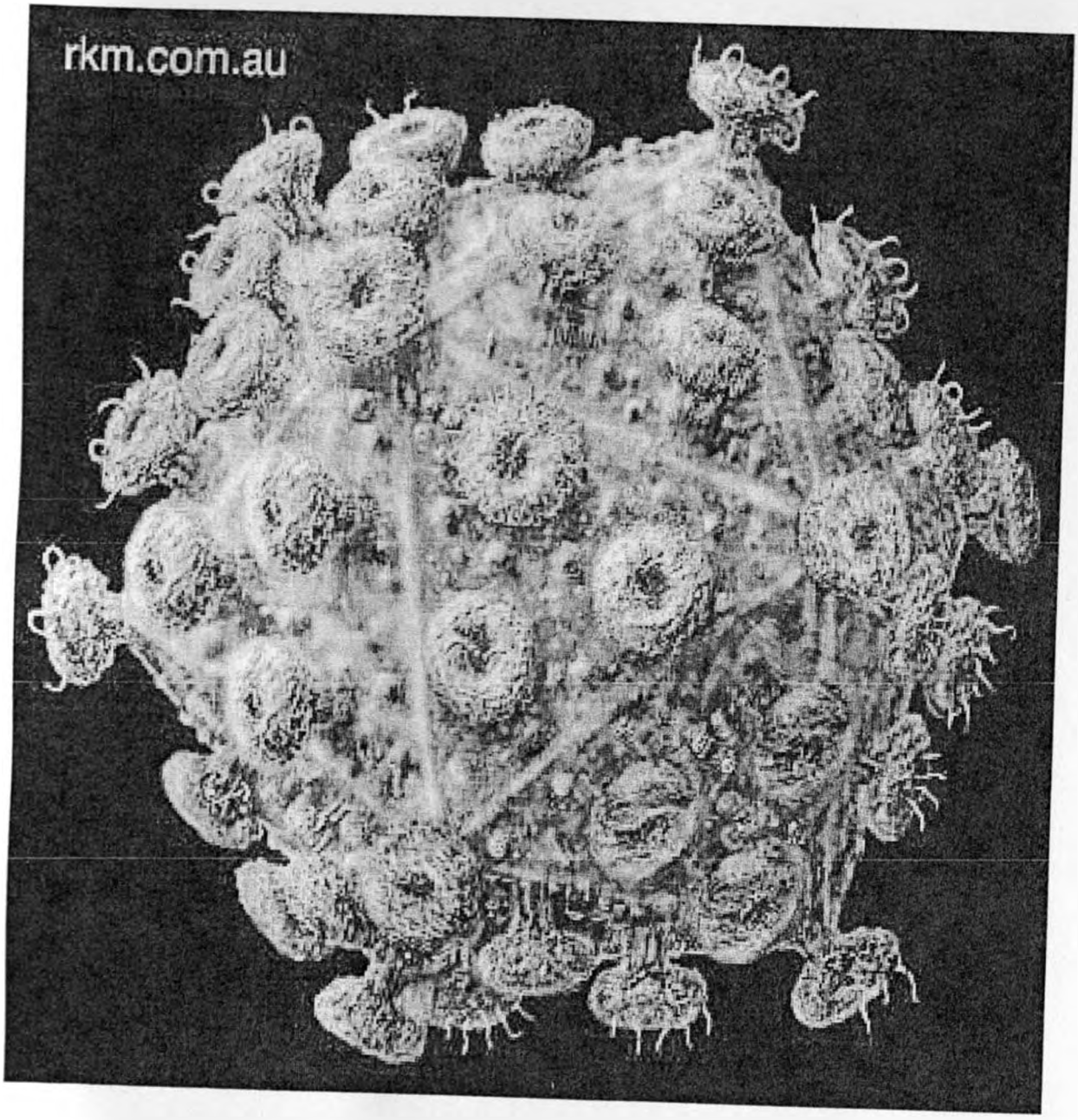

Figure 1 **Illustration of the HIV Virus**

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www.rkm.com.au/biograph.html

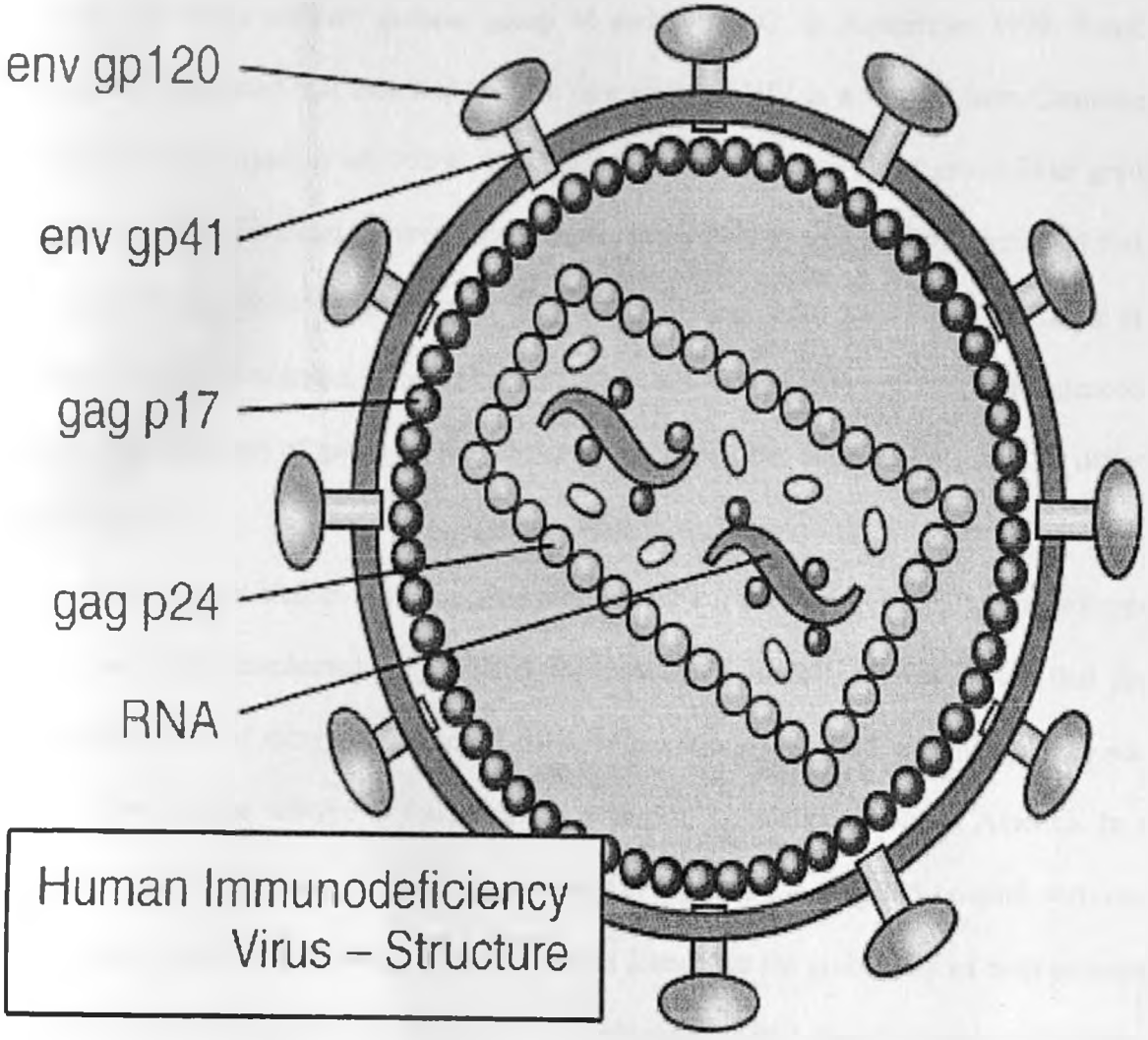


Figure 2 **Human Immunodeficiency virus structure**

(Diagram Courtesy of AVERT, http://www.avert.org/virus.htm)

HIV Subtypes: HIV-1 is a highly variable virus, which mutates very readily. So there are many different strains of HIV-1. These strains can be classified according to groups and subtypes and there are two groups, group M and group O. In September 1998, French researchers announced that they had found a new strain of HIV in a woman from Cameroon in West Africa (Roques *et al.,* 1999). The strain does not belong to either group M or group O, and has only been found in three other people, all in the Cameroon. Within group M there are currently known to be at least 10 genetically distinct subtypes of HIV-1. These are subtypes A to J. In addition, Group O contains another distinct group of very heterogeneous viruses. The subtypes of group M may differ as much between subtypes as group M differs from group O.

Some recent studies have suggested that subtype E spreads more easily than subtype B. In one study conducted in Thailand (Mastro *et al.*, 1994), it was found that the transmission rate of subtype E among female commercial sex workers and their clients was higher than that for subtype B found among a general population in North America. In a second study conducted in Thailand (Kunanusont *et al*, 1995), among 185 couples with one partner infected with HIV subtypes E or B, it was found that the probability of both partners in a couple becoming infected was higher for subtype E (69%) than for subtype B (48%). This suggests that subtype E may be more easily transmissible.

Today, five major (A to E) and several minor subtypes of HIV-1 are recognized throughout the world (Blakeslee, 1996). However, because of the highly variable nature of the viruses and the ever-changing dynamics of the AIDS pandemic, it is unlikely that the subtypes seen today will be the same ones that will exist years from now (Blakeslee, 1996). In Kenya subtypes A C and D have been identified from Mombasa and Nairobi truck drivers and commercial sex workers (Robbins *et al*, 1999).

Current Statistics in the HIV Pandemic: Currently it is estimated that at least 36.1 million people are living with HIV or AIDS (UNAIDS, 2000). Africa is home to 70% of the adults and 80% of the children living with HIV and AIDS in the world (UNAIDS, 2000).

2.2 PAEDIATRIC HIV AND AIDS

The first cases of paediatric AIDS were described in 1982, a year after AIDS had been described in adults (CDC, 1982). Since then there has been a rapid increase of AIDS cases among children (Oleske *et al.*, 1983; Rubinstein *et al.*, 1983; European Collaborative Study, 1991; CDC 1987; CDC 1992). Paediatric AIDS comprises about 3% of all reported AIDS cases in developed countries and as many as 20% of all AIDS cases in developing countries (WHO Global Programme on AIDS, 1993; Gwinn *et al.*, 1991; European Collaborative Study, 1991). The rate of vertical transmission of HIV in developing countries is as high as 50%, while in developed countries it is approximately 10-39% (Lepage *et al.*, 2000). However, rates of transmission as low as +/- 1% have been observed with the combination of antiretroviral prophylaxis and elective caesarean delivery (Lepage *et al.*, 2000).

Vertically acquired paediatric HIV infection accounts for over 70% of reported paediatric HIV cases world-wide (WHO Global Programme on AIDS, 1993). Paediatric AIDS most often results from transmission of HIV from an infected mother to her newborn infant (Anderson and Zevallos, 1993). Transmission of HIV from mothers to children may occur through the transplacental, intrapartum, or breastfeeding routes (Fawzi, 2000). Approximately 30% of children born to mothers who are seropositive for HIV will seroconvert (Grattan-Smith *et al.*, 1992).

Infants infected with HIV often develop immunodeficiency and die sooner after infection than do HIV-infected adults. About 33% of HIV-infected infants have a rapidly fatal disease course and die within one year, while the majority develop clinical disease more slowly and survive for more than 5 years (Oxtoby, 1994; Wilfert *et al.*, 1994).

It has been estimated that approximately 20% of HIV infected infants will develop AIDS during the first year of life (Ryder *et al.,* 1989). It has also been shown that greater than 90% of infected infants can be expected to develop HIV-related symptoms by one year of age (European Collaborative Study, 1991; Blanche *et al.,* 1989; Tovo *et al.,* 1992). In the past decade an increase in paediatric HIV infection has had a substantial impact on childhood morbidity and mortality world-wide (Domachowske, 1996).

The common feature of HIV infections in infants and adults is a profound immunodeficiency that renders the host susceptible to neoplasm and opportunistic infections (Goudsmit *et al.,* 1986). The virus exerts direct and indirect effects on the host's immune system that may be particularly serious in infants and children because of the ongoing maturation of the immune system (Otsyula, 1995).

The pathogenesis of HIV in children infected *in utero*, during the perinatal period, or in early childhood, includes a short latency followed by the development of symptomatic disease (Connor and Ho, 1994). In children infected with HIV-1, there is a short latency period before the development of clinical illness (Otsyula, 1995). Lower levels of HIV burden are found in the plasma and peripheral blood mononuclear cells (PBMC) of asymptomatic or mildly symptomatic children, whereas severely symptomatic children may have viraemia comparable with that found in adults (Srugo *et al.,* 1991; Alimenti *et al.,* 1992). Some perinatally infected infants may fail to produce significant levels of anti-HIV antibodies, and they develop a profound deficiency of cell-mediated immunity (Andiman, 1989), suggesting that they may be at a higher risk of developing AIDS.

Studies have shown that AIDS can take either of two forms in children (European Collaborative Study, 1991; Blanche *et al.,* 1989; Tovo *et al.,* 1992). Namely, that one third of HIV-infected infants develop AIDS within the first years of life, while the remaining two thirds of HIV infected children, the risk of developing AIDS resembles that of HIV-infected

adults (European Collaborative Study, 1991; Blanche *et al.*, 1989; Tovo *et al.*, 1992). The latter is characterised by a primary infection and seroconversion. This is followed by an asymptomatic phase that can last up to 11 years and is characterised by low levels of viral replication, a continuous decline in CD4+ T cell counts and relatively few clinical manifestations. Finally a clinical disease marked by rapidly decreasing CD4+ T cell counts and a high virus load in both plasma and PBMC (European Collaborative Study, 1991; Blanche *et al.*, 1989; Tovo *et al.*, 1992; Lemp *et al.*, 1990).

Data on the immunological status of neonates are limited and not sufficient to account for the bimodal course of the disease seen in infected neonates. HIV infection of the foetus or neonate can result in one of several immunological outcomes, including development of an effective immune response, immunopathology due to the cell mediated immune response or establishment of immunologic tolerance (Otsyula, 1995). The immune system in HIV positive children involves dysfunction in both the cellular and humoral immunity, which increases susceptibility of the children to a variety of infections, particularly opportunistic organisms. These most often result in complications of the lungs, cardiovascular system, gastrointestinal tract, genito-urinary system and neurological system.

There is urgent need to strengthen the area of paediatric HIV/AIDS care in developing countries (Lepage *et al.*, 1998). This study would like to investigate gastro-intestinal tract involvement, which is usually due to opportunistic organisms that produce oesophagitis, gastritis, enteritis and colitis.

2.3 VIRUSES THAT CAUSE DIARRHOEA IN CHILDREN

2.3.1 Rotaviruses

Rotaviruses represent 80% of recognised viral aetiologies amounting to 140 million cases of diarrhoea per year (Bajolet and Chippaux, 1998). They strike young children with similar frequency throughout the world, but the mortality rate is high in developing countries, with an estimated 870,000 to one million deaths per year (Cook *et al.*, 1990; WHO, 1997). This accounts for an estimated 20-25% of all deaths due to diarrhoea and 6% of all deaths among children less than five years old (Cook *et al.*, 1990).

Severe rotavirus disease in young children may be followed by extended excretion of rotavirus (Richardson *et al.*, 1998). Extended excretion could also explain some cases of the post-gastro-enteritis syndrome (Richardson *et al.*, 1998). Rotavirus has been found to be the most common enteric pathogen in HIV-infected children (Italian Paediatric Intestinal/HIV study group, 1993) and is often associated with severe protracted diarrhoea in immunodeficient children (Saulsbury *et al.*, 1980). In Zambia, 25% of consecutively studied children under 5 years of age hospitalised for diarrhoea were positive for both rotavirus and HIV and had a higher mortality rate than those children with only rotavirus infection alone (Oshitani *et al.*, 1996). However this study also showed that there did not appear to be a more frequent association of rotavirus infection in children infected with HIV than in HIV-negative children. Other studies have also shown this finding (Pavia *et al.*, 1992; Thea *et al.*, 1993a).

Structure: Rotaviruses belong to the family Reoviridae and are segmented bicatenary (double stranded) ribonucleic acid (dsRNA) viruses (Fields, 1996) which explains their genetic variability, the presence of mixed infections, and the establishment of molecular epidemiology by electrophore types. The viruses are "naked" and thus resistant to the outside environment. The virus morphology comprises of particles that are 70 nm round, double shelled, enclosing a genome of 11 segments of double stranded ribonucleic acid (Figure 3).

The virus is hardy and may even survive in sewage, despite stringent treatment. Human rotavirus has proved difficult to culture *in vitro*, but the serologically related monkey and calf rotaviruses grow easily in cell culture. Rotaviruses possess a double stranded ribonucleic acid (dsRNA) genome comprising of 11 discrete segments contained within the virus core capsid (Fields, 1996). The segments fall into four size classes based on contour length measurements by electron microscopy and confirmed by nucleotide sequence analysis of the genus (Estes and Graham, 1985). The distribution of the eleven segments into these four size classes is evident using PAGE of RNA (Figure 4).

Structural Proteins, Genotype and Serotype Specificity: The structural proteins of the virus particle are organized into three layers (figure 5): the outer capsid shell is formed by VP7 and the viral hemagglutinin, VP4; an inner capsid shell is formed by VP6; and the virus core is formed by proteins VP1, VP2 and VP3 which encapsidate the dsRNA segmented genome (Estes and Cohen, 1989, Shaw *et al.*, 1993; Yeager *et al.*, 1994).

VP6 plays several important roles in the replication cycle of rotavirus and it is the subgroup-specific antigen for rotavirus (Matsui *et al.*, 1989). It is known that IgA neutralizing antibodies directed against VP6 can protect against rotavirus infection (Burns *et al.*, 1996).

The two outer capsid layer proteins of the virus, VP7 (encoded by gene segment 9) and VP4 (encoded by gene segment 4), are capable of inducing the production of neutralising antibodies (Estes, 1996), which have been shown to play an important role in inducing resistance to disease (Hoshino *et al*, 1985; Offit *et al*, 1986). The major neutralising antigen, VP7, is a glycoprotein and carries the G-serotype specificity while the minor neutralising antigen, VP4, carries the P-serotype specificity. The serotypic specificity is defined and characterised by serological methods, therefore the terms G type and P type (genotype) are used for typing of rotavirus by molecular biological methods. . The nomenclature for G genotypes and serotypes are identical (followed by an open number e.g., G1) whereas a P

genotype is denoted by closed brackets (e.g., P[8]) and serotype is indicated by an open number (e.g., P8) (O'Mahony *et al.*, 1999).

To date at least 14 G types and 18 P types have been identified in humans and animals (Frühwirth *et al.*, 2000). G serotypes 1 to 4 are the most prevalent in humans, with between 71 and 97% of the strains characterised (Gentsch *et al*, 1996). Eight P genotypes have been found in humans, although the majority of strains belong to only two P genotypes (P[4] and P[8]) (Gentsch *et al*, 1996).

The G and P typing of rotavirus strains is important in vaccine development. Most rotavirus vaccines in development include live, oral, attenuated strains,(Kapikian and Chanock, 1996) and concern exists regarding their use in infants who might be immunocompromised. Specifically, the US Centres for Disease Control and Prevention's Advisory Committee on Immunization Practices recommended that the (now suspended),(CDC, 1999a) tetravalent rhesus-human reassortant rotavirus vaccine should not be given to infants born to HIV-infected mothers (unless HIV infection in the infant has been excluded), and suggested further research in this area (CDC, 1999b).

Grouping: Rotaviruses are divided into seven serogroups A to G based on their distinct antigenic and genetic properties. Each group contains closely related viruses with a unique genome profile, in addition to a unique group antigen, and a unique terminal fingerprint sequence of the genome segments (Bohl *et al.*, 1982; Bridger and Brown, 1985). Viruses belonging to serogroups A, B, and C are known to cause infections in humans, with group A being the most important pathogens (Sen *et al.*, 2000).

Group A rotaviruses (especially subtypes 1,2,3,4) are the major cause of severe gastro-enteritis in children under 4 years of age world–wide (Koopmans and Brown, 1999). Group A rotaviruses have also been identified in many animal and bird species, they are antigenically complex, and multiple serotypes infect humans. Some of the serotypes in

humans include rotavirus serotypes G1-4 and in Europe these comprise 90% of group A rotaviruses that have been typed.

Group B rotaviruses mainly infect pigs and rats and were found to be the cause of extensive annual epidemics of severe diarrhoea affecting both adults and children in China in the last decade (Nakata *et al*, 1987).

Group C rotaviruses were first recognised as animal pathogens infecting piglets and later identified as the aetiological agents causing diarrhoeal illness in children and adults (Caul *et* al., 1990). They are fastidious in their in vitro cell culture requirements. Recent surveys indicate that antibody to group C rotavirus is present in 3-45% of the human population in certain geographic locations (Teixeira *et al.*, 1998), suggesting that rotavirus group C infections is more prevalent than previously believed and that the low rate of detection of these agents is probably due to the lack of sensitive diagnostic assays. Group C rotaviruses also infect pigs. Group D rotaviruses have been reported in birds while group E in pigs.

Clinical Disease: Clinically, rotavirus infection causes a spectrum of disease, from asymptomatic infection in the newborn babies to severe dehydrating diarrhoea in infants and young children. In older children and adults the infection is usually sub-clinical and it may be that these individuals act as a reservoir for the virus. The massive elimination of rotavirus, 10^8 to 10^{10} viral particles per gram of faeces begin with the first day of diarrhoea. Rotavirus is spread by the faecal-oral route and by direct contact. Oral-faecal transmission is facilitated by deficient sanitary conditions. Clinical studies have indicated that the incubation period of rotavirus illness is less than 48 hours but can last from 5 to 7 days. Vomiting is a prominent early symptom of infection, followed by profuse and watery diarrhoea. Dehydration is more strongly associated with rotavirus diarrhoea than bacterial gastro-enteritis and has been indicated as potentially fatal.

The shedding of rotaviruses by neonates is well documented world-wide and has been observed in Africa. Studies in Kenya have shown that rotavirus gastroenteritis occurs all year round (Urasawa *et al.*, 1987). Excretion was found to begin as early as the first day after birth and was generally found not to be associated with any clinical symptoms in neonates shedding the virus. The sub-clinical infection in the neonatal stage has been reported to offer protection against subsequent severe rotavirus infection up to 3 years of age (Bishop *et al.*, 1983).

Rotavirus species in Animals: Numerous animal species are infected with rotavirus species, distinct from the human ones (Bajolet and Chipaux-Hyppolite, 1998). The pathology in animals is of economic importance and interest as an experimental and vaccinal model (Bajolet and Chipaux-Hyppolite, 1998). Between human and animal rotaviruses there can be genetic re-matching and the VP6 protein is an antigen common to the group A rotaviruses (Bajolet and Chipaux-Hyppolite, 1998).

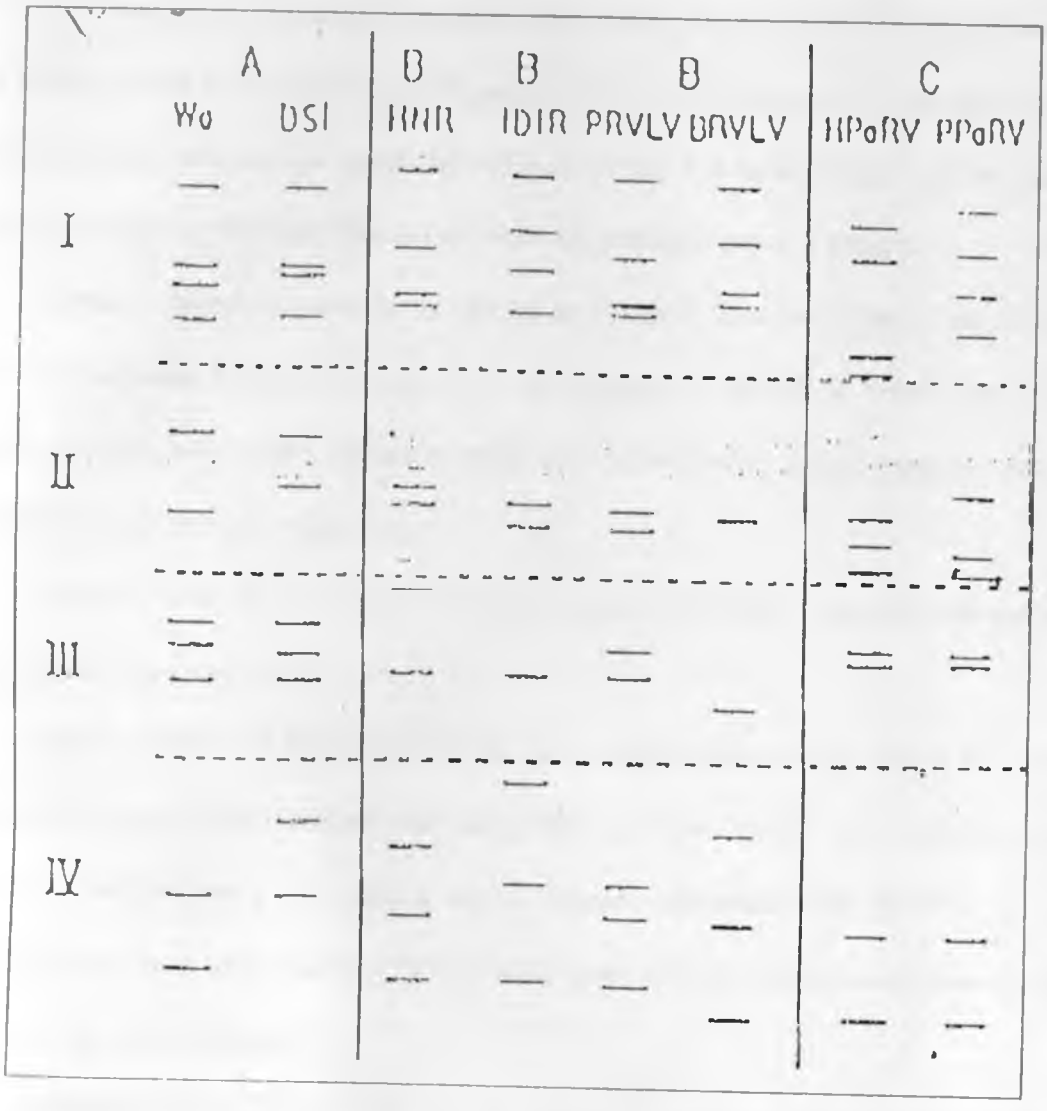


Figure 3 RNA Electrophoresis of Group A human (Wa and DS-1) rotaviruses showing the distribution of the 11 segments into four size classes. There are four large segments (Class I), two medium segments (Class II), three smaller segments (Class III) and two smallest segments (Class IV), Tao, 1988).

Diagnosis of Rotavirus: The diagnosis of rotavirus infection is not sufficient by clinical symptoms only and requires the detection of rotavirus in stools. Direct visualisation of the stool material by electron microscope after staining the stool with 2% phosphotungstic acid (PTA), is one of the methods of diagnosis. This method has a very high specificity due to the distinctive morphologic appearance of rotaviruses, it is rapid and permits the detection of the non-group A rotaviruses that do not share the common group A antigen.

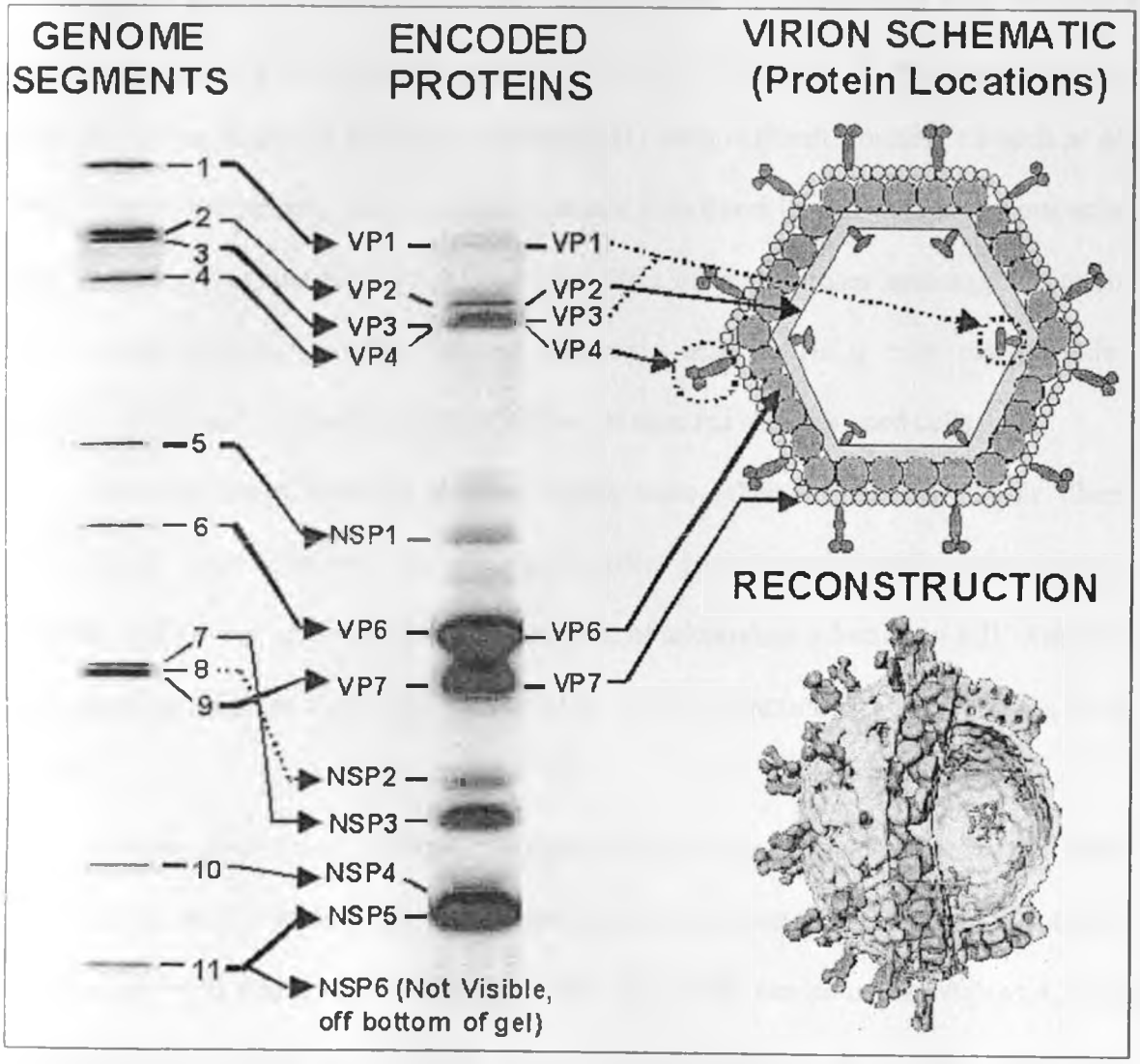
Another diagnostic method is the enzyme-linked immunosorbent assay (ELISA), where an indicator antibody conjugated to an enzyme is utilised to detect the rotavirus antigen. This method requires the use of group specific antibodies so that group A rotaviruses are detected with group A antibodies.

Reverse transcription polymerase chain reaction (RT-PCR), is used for the molecular characterisation of rotaviruses.

Other methods of detection include latex agglutination assay, where the virus is detected by a slide agglutination test using latex particles coated with antibody against rotavirus. Electrophoresis of rotavirus double-stranded ribonucleic acid (dsRNA) in a gel, immunoblot enzyme assay, and dot hybridisation assay and cell culture techniques can all be used for diagnostic purposes.

Treatment: For persons with healthy immune systems, rotavirus gastroenteritis is a self-limited illness, lasting for only a few days. Treatment is nonspecific and consists of oral rehydration therapy to prevent dehydration. About one in 40 children with rotavirus gastroenteritis will require hospitalization for intravenous fluids (Glass RI, 2000).

Figure 4 **Coding assignments and virion locations of rotavirus proteins and 3D structure of the rotavirus particle.**



Genome Segments: Shows SDS-PAGE separation of the 11 segments of the rotavirus genome.
Encoded proteins: Shows the PAGE separation of the intracellular proteins synthesized by SA11 and the genome segment in which they are encoded. Six structural (VP) and six nonstructural (NSP) proteins are synthesized in the infected cell. Note that NSP6 runs far below NSP5 and is not shown on the gel presented here.
Virion Schematic: Shows the locations of the various structural proteins within the rotavirus virion. Note the arrangement of the proteins into 3 concentric capsid layers, and the location of the VP1/VP3 complexes at the 5-fold verticies of the icosahedral structure.
Reconstruction: A 3D reconstruction of the rotavirus virion (23Å resolution) with the genome computationally removed. The color scheme is the same as the schematic, to indicate locations of the various proteins. Courtesy of J. Lawton and B.V.V. Prasad).

Figure supplied by Eric Mossel , Mary Estes and Frank Ramig

(From: dsRNA virus, RNA/protein tables: edited by P. P. C. Mertens and D. H. Bamford http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/rotavirus%20figure.htm)

2.3.2 Adenoviruses

These are icosahedral-shaped double-stranded DNA viruses measuring 60 to 70nm and having 41 serological types which react independently in neutralization tests but share a common group complement fixing antigen (Timbury, 1991). The 41 serotypes of human adenoviruses are classified into six subgenera (A-F) with different tropisms (Wadell *et al.*, 1987). These viral particles have a cubic symmetry with fibres topped with knobs projecting from their vertices (Timbury, 1991) (Figure 6). Most of these viruses haemagglutinate and grow slowly in tissue cultures (human embryonic cells or HeLa cells recommended, Timbury, 1991) with cytopathic effect of clusters of rounded and ballooned cells.

The adenovirus infection in man causes acute respiratory illness, febrile illness accompanied with exanthema, keratoconjunctivitis, gastro-enteritis and acute nephritis (Hayashi, 1983). The most common manifestation of adenovirus infection in HIV disease is diarrhoea (Cunningham *et al.*, 1988; Kaljot *et al.*, 1989; Grohmann *et al.*, 1993; Thea *et al.*, 1993b).

Enteric adenoviruses are important agents of paediatric gastro-enteritis (Brown *et al.*, 1996) and in several studies they were found to be only second in prevalence to rotavirus infection but with a much lower prevalence rate. In a study carried out in Malawi 4.2% of 186 children with gastro-enteritis were reported with adenovirus (Pavone *et al.,* 1990); 9.8% of 234 paediatric diarrhoeal stool samples from children with gastro-enteritis in Gauteng province, South Africa (Moore *et al.,* 1998) and 2.3% of 87 children below the age of 36 months in Dar-es-Salaam, Tanzania (Mhalu *et al.,* 1988) had adenovirus infection. Faecal adenoviruses are representatives of adenovirus serotype 40 and 41(Wadell *et al.*, 1987) and maybe serotype 38 (Krugman *et al.,* 1985) with typical cytopathic effect produced on cell culture (Krugman *et al.,* 1985). Faecal adenoviruses are found in endemic infections in the

community and also in outbreaks. Infection is often associated with prolonged excretion (Timbury, 1991).

Clinical features of enteric adenovirus diarrhoea in children include watery stools, vomiting and moderately elevated temperature; respiratory symptoms are infrequent. The diarrhoea is protracted with a mean duration of 8.6 and 12.2 days for adenovirus 40 and 41 respectively (Wadell *et al.*, 1987). In spite of the difficulty of isolating adenovirus 40 and 41, they can be directly identified in stool by enzyme-linked immunoassay (ELISA) and solid-phase immuno-electron microscopy (Wadell *et al.*, 1987) and the amount of viral DNA in stool preparations is sufficient for identification by DNA restriction or dot blot analysis.

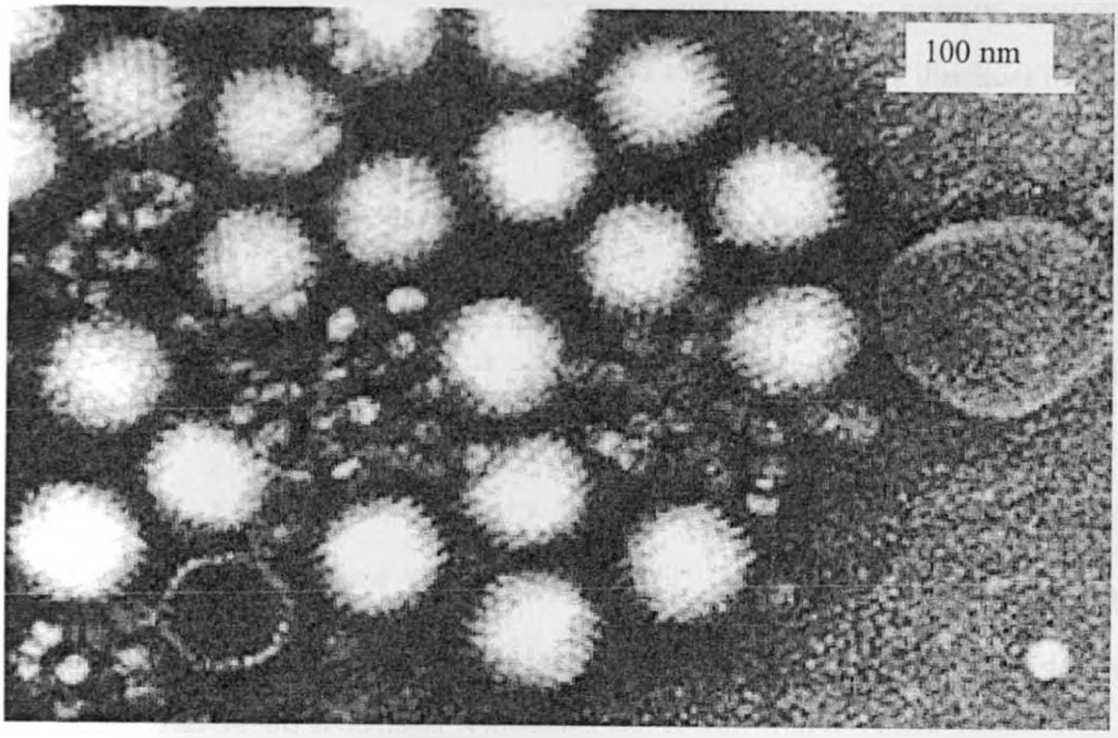


Figure 5 Negative-stain Transmission Electron Microscopy of Adenovirus from a stool sample from an individual with gastroenteritis

The adenovirus virion is made up of 252 identical protein building blocks or subunits, which are wrapped around a DNA molecule. Fibre projections from the molecule help the virus attach to the cells lining the gastrointestinal tract. Note the two virus types present in the photograph. The larger virus particles with prominent capsomeric detail on their surface are adenovirus particles. The small featureless particles seen mostly clumped between the adenovirus particles are parvovirus particles. Photo by FP Williams, EPA, USA.

2.3.3 Astroviruses

Human astroviruses are recognised as a common cause of infantile gastroenteritis (Matsui and Greenberg, 1996). Initially associated with an outbreak of diarrhoea in infants in a maternity unit, these viruses were given the name astrovirus because of the characteristic five- or six-point star shape they display when viewed by electron microscopy after negative staining of faecal extracts (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975).

Novel enteric viruses such as astrovirus and picorbinavirus are important etiologic agents of diarrhoea in HIV-infected patients than previously recognised and may be more common than either bacterial or parasitic enteropathogens (Grohmann *et al.*, 1993). Astroviruses are star-shaped single-stranded RNA viruses(figure 7) of 28nm particles (Krugman *et al.*, 1985) with a virion composed of a single non-enveloped capsid layer of between 27 and 34 nm in diameter (Greenberg and Matsui, 1992). They belong to the family Astroviridae and 8 serotypes have so far been reported (Sakamoto *et al*, 2000) and are described according to the reactivities of the capsid proteins with polyclonal sera and monoclonal antibodies (Matsui and Greenberg, 1996).

Astroviruses are also classified into genotypes on the basis of the nucleotide sequence of a 348bp region of the open reading frame region 2 (ORF) (Noel *et al*, 1995). Three ORFs designated ORF1a, ORF1b and ORF2 have been identified so far (Jiang *et al*, 1993). There is a good correlation between genotype and serotype (Noel *et al*, 1995) and there are currently 8 established serotypes of human astrovirus that correlate with 8 genotypes.

The medical importance of human astrovirus infection has been established by reports which have shown that in some settings astrovirus is the second most common cause of diarrhoea in children (Glass *et al*, 1996). They are seen significantly more often in stools of children with diarrhoea than in those from healthy controls (Timbury, 1991). Infection with astroviruses has been associated with gastro-enteritis in children, and serologic surveys

indicate that this infection may be frequent (Hermman *et al.*, 1991). Most children are infected during the first two years of life (Glass *et al.*, 1996). The elderly and immuno-compromised are also at a high risk of infection wit astrovirus (Glass *et al.*, 1996).

In early surveys based on electron microscopy (EM), astroviruses appeared to be a rare cause of gastro-enteritis, being found in less than 1% of children with diarrhoea (Glass *et al.*, 1996). The development and use of monoclonal antibodies and enzyme immunoassays led to a report of a higher prevalence (2.5-9%) of astrovirus infection among patients hospitalised with diarrhoea (Glass *et al.*, 1996). The cloning and sequencing of astroviruses has also led to more sensitive assays to detect the viruses by reverse transcription, polymerase chain reaction (RT-PCR). As in rotavirus infection, immunity to infection with astrovirus develops early in life. The main symptom of infection is watery diarrhoea, which is often associated with vomiting, fever and abdominal pain (Matsui and Greenberg, 1996).

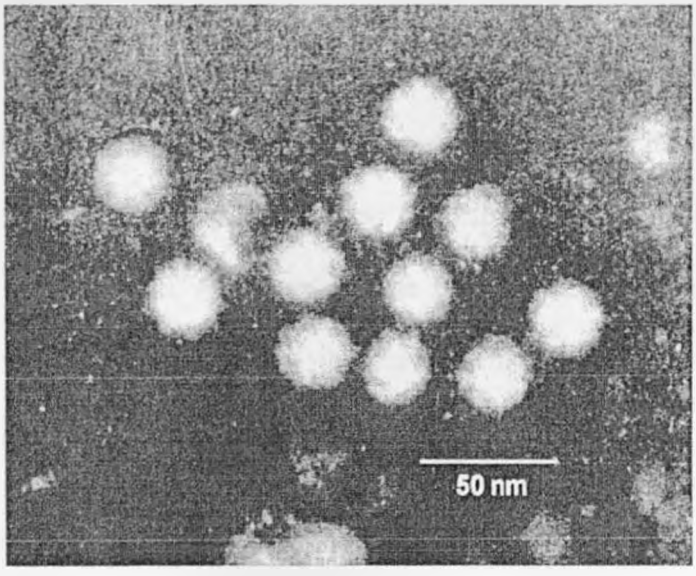


Figure 6 Negative Stain Transmission Electron Micrograph of Astroviruses

Small round viruses with a distinctive five- or six-pointed star-like surface structure. Note the star-like images exhibited by individual virus particles. These are distinct from the 'Star of David' image exhibited by typical calicivirus particles. Bar = 50 nanometers. This is from a stool sample from an individual with gastroenteritis. Photo by FP Williams, EPA, USA.

CHAPTER THREE

MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

A total of 207 children were enrolled in the study. The experimental group consisted of 43 children who were HIV positive with diarrhoea. The principal control group consisted of 94 children without HIV with diarrhoea. A second control group studied were 70 children with HIV without diarrhoea. Samples were first collected from the children in the period of February 1999 to June 2000. All samples were stored at -20°C. Once all the samples were collected, they were then analysed for the presence of rotavirus, astrovirus and adenovirus using various kits and methods as described below.

3.2 MATERIALS

3.2.1 Human Subjects

The ethical approval for this study was obtained from three institutions:

(i) The Institutional Scientific and Ethical Review Committee (ISERC) of the Institute of Primate Research

(ii) The Kenyatta National Hospital Ethical Review Committee (KNH-ERC)

(iii) The Nyumbani external Ethical and Scientific Review Committee (NESB)

The study involved 113 HIV infected children at Nyumbani Hospice and Nyumbani's foster care programme (Lea Toto) and 94 children (whose HIV status was unknown) seen at city council clinics and hospitals in Nairobi and its environs. Stool samples were collected from all the children at Nyumbani with or without diarrhoea. This group of children were housed at Nyumbani and so it was possible to collect samples even when no diarrhoea was reported. Samples from the city council clinics and hospitals were collected only from

children with diarrhoea. This is because only those suffering from diarrhoea visited the clinics for treatment.

The clinical data of the Nyumbani children was obtained from the Nyumbani clinic. The Nyumbani Diagnostic Laboratory provided data on the HIV status, CD4/CD8 counts and haematology. CD4+/CD8+ counts were determined by T-lymphocyte phenotyping using methods described by Becton Dickinson® FACSCOUNT system (1994). HIV status in this laboratory was determined by the detection of anti HIV-specific IgG antibodies.

3.2.2 Faecal Samples

Faecal samples were collected from all children at Nyumbani Children's Hospice, children with diarrhoea in Lea Toto program and from clinics around Nairobi over the period covering February 1999 to June 2000. All faecal samples collected were stored in -20°C and used for detection of viral pathogens.

3.3 LABORATORY METHODS

3.3.1 Diagnostic Methods

3.3.1.1 Diagnostic ELISA (Antigen ELISA) for detection of rotavirus in faeces

The detection of rotavirus in human faecal specimens was done using the IDEIA™ Rotavirus kit (DAKO Diagnostics Ltd, Cambridgeshire, UK) which is an immunoassay for the detection of group A rotaviruses in faecal specimens utilises a polyclonal antibody to detect group specific proteins, including the major inner capsid protein (VP6), present in group A rotaviruses. This kit was used to screen all faecal samples following the procedures described in the kit.

3.3.1.2 Detection of Rotavirus in Faeces by Transmission Electron Micrography

The technique used was negative stain electron microscopy. A 10% suspension of stool prepared in distilled water was adhered to a formvar carbon-coated grid and stained with 3% phosphotungstic acid (PTA). The technique enabled the visualization of the virus (an essentially trqansparent object) by surrounding it with a coloured solution (the PTA stain).

3.3.1.3 Detection of Adenovirus by ELISA in Faeces

This was done using the IDEIA™ Adenovirus test kit (DAKO Diagnostics Ltd, Cambridgeshire, UK), which is a qualitative enzyme immunoassay for the detection of adenovirus in human faeces or in infected cell culture monolayers. The IDEIA™ test utilises a monoclonal antibody in a solid-phase sandwich enzyme immunoassay to detect a genus–specific hexon epitope of adenovirus.

3.3.1.4 Detection of Astrovirus by ELISA in Faeces

The detection of astrovirus in the faecal samples was done using the IDEIA™ Astrovirus kit (DAKO Diagnostics Ltd, Cambridgeshire, UK). This is a qualitative enzyme immunoassay for the detection of astrovirus in human faeces. It utilises a monoclonal

antibody and polyclonal antibody in a solid-phase enzyme immunoassay to detect astrovirus antigen.

3.3.1.5 Rotavirus dsRNA Extraction from Faeces

The dsRNA genome was extracted from all positive specimens according to methods described previously by Steele and Alexander (1987), with slight modifications (See Appendix II).

3.3.2 Further Characterisation of Rotavirus Positive Samples

3.3.2.1 Polyacrylamide Gel electrophoresis (SDS-PAGE)

Electrophoretic analysis was performed using discontinuous buffer system with 3% stacking gel and 10% resolving gels. A 10% polyacrylamide vertical slab gel was loaded with 30µl of extracted RNA and electrophoresced overnight at 100 volts using the discontinuous buffer system (Appendix III, Adapted from the WHO Rotavirus Workshop Manual, 1998). A 3% stacking gel was employed to enhance the resolution of the gel. The gels were stained using the silver staining technique (Appendix IV) of Herring *et al.*, (1982). The gels were then dried using a gel dryer (Easy Breeze Gel Dryer, Hoefer, San Franscisco, USA).

3.3.2.2 Rotavirus VP6 Subgroup ELISA

All rotavirus positive specimens were subjected to the VP6 subgroup specificity assay according to methods described previously (Steele and Alexander, 1988). The coating antibody for this assay was rabbit anti-human rotavirus #0903 (from DAKO, Cambridgeshire, UK). The monoclonal antibody for group antigen detection, A3M4 (Beards *et al.*, 1984) and the subgroup specific antibodies (Greenberg *et al.*, 1983) were all obtained from the laboratory of A. D. Steele (Refer to Appendix V for detailed protocol).

3.3.2.3 Rotavirus VP7 Monoclonal antibody (Mab) ELISA

The VP7 protein is the major neutralising antigen of rotavirus detected by hyperimmune serum and has been used as the basis for the identification of rotavirus serotypes. The VP7 protein forms the smooth external surface of the triple layered particle and contributes to 30% of the virion protein VP7 elicits the production of neutralising antibodies.

VP7 G typing was carried out initially by ELISA (typing also done using RT-PCR) with little variation as described by Taniguchi *et al.,* (1987). This was done using Mab 60 to detect the presence of the intact outer shell of the virus (Shaw *et al.*, 1985) and rabbit anti-rotavirus hyperimmune serum (# 702, Beards, 1982) as the capture antibody. The following monoclonal antibodies were used; Serotype G1 (KU-4 [1:5000] & 5E8 [1:10,000]); Serotype G2 (S2-2G10 [1:5000] & IC10 [1:5000]); Serotype G3 (YO-1E2 [1:5000] & 159 [1:5000]); Serotype G4 (ST-2G7 [1:5000]). The test was carried out in duplicate and each plate had appropriate controls. The reaction was read at 450nm wavelength on the spectrophotometer (see Appendix Vi for detailed protocol).

3.3.3 Molecular Characterisation of Rotavirus

3.3.3.1 Purification of Rotavirus dsRNA for PCR Amplification

Two methods of extraction were used, namely,

(i) RNaid Extraction

In this method, 50µl 1MnaAc (pH 5.0) containing SDS was added to 500µl of 10% faecal suspension which was then vortexed for 10 seconds and then incubated at 37°C for 15 minutes. This was followed by the addition of 500µl phenol/chloroform mixture, vortexed for 1 minute and then incubated at 56°C for 15 minutes. The upper phase was removed into a new eppendorf tube and the phenol/chloroform extraction repeated with ½ volume (i.e.

250µl). The second upper phase was then removed into a new eppendorf tube and 500µl of 6M guanidine thiocyanate (GTC, from RNaid kit Bio 101, Carlsbad, California, USA) was added to 400µl of the recovered suspension. This was vortexed to mix followed by spinning at 12000rpm for 5 minutes. The solution was poured into a new eppendorf. Following this, 10µl of well vortexed RNaid matrix (RNaid kit Bio 101, Carlsbad, California, USA) was added and vortexed for 30 seconds before incubation on a rocker for 15 minutes. The solution was then spun for 10 seconds at 5000rpm and the supernatant aspirated into a new tube (this was stored for re-extraction in case required). The pellet was washed with 400µl RNaid wash (RNaid kit Bio 101, Carlsbad, California, USA) and then gently re-suspended in the same. Before spinning for 30 seconds at 12000rpm, the supernatant was aspirated off and the wash was repeated on the pellet once more.

A final wash with 100µl RNaid wash was done followed by spinning at 12000rpm for 1 minute. The pellet was then dried in a vacuum pump spinning at medium temperature for 10 minutes to remove excess alcohol. The pellet was then re-suspended (using a pipette) in 40µl of DEPC water (RNaid kit Bio 101, Carlsbad, California, USA) and incubated at 65°C for 10 minutes. This was followed by spinning at 10000rpm for 2-3 minutes. The supernatant was then removed into a new eppendorf tube and stored at -20°C until ready for PCR.

(ii) Extraction of Viral RNA by TRIzol Method

A 10% faecal suspension was centrifuged at 5000rpm for 5 minutes in 4°C in a microfuge. 200µl of the supernatant were combined with 500µl TRIzol (TRIzol kit from LS ultrapure, GibcoBRL, Life technologies, Karlsruhe, Germany) and vortexed for 30 seconds. The sample was then incubated for 5 minutes at room temperature. 120µl of chloroform was then added to each sample, which in turn was vortexed for 30 seconds and then incubated at room temperature for 3 minutes. The aqueous phase of the resulting mixture was transferred into a clean tube and an equal volume of isopropyl alcohol was added and the mixed gently

by hand. The samples were then incubated at room temperature for 20 minutes. Following this the samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was poured off and the samples spun at 12000rpm for 1 minute. The remaining supernatant was then removed using a pipette. The tubes were then allowed to dry uncapped at room temperature for 5 minutes. The viral RNA pellet was then re-suspended in 16µl of DEPC treated distilled water. 5-7µl of sample would then be used for each RT-PCR reaction.

3.3.3.2 Rotavirus VP4 and VP7 Reverse Transcriptase PCR (RT-PCR)

Reverse transcription-PCR (RT-PCR) was used for G and P genotyping of the VP4 and VP7 gene. Rotavirus ds-RNA was isolated from stool as described in 2.2.8.

(i) RT-PCR amplification of Rotavirus dsRNA

The ds-RNA was reverse transcribed and amplified by PCR using two oligonucleotide primers for VP4 (Con3 and Con2, see Appendix IX) that correspond to regions that are highly conserved among all known human rotavirus gene 4 types. The 876bp ds-RNA products were obtained by this method. For VP7 the reverse transcription and amplification for the full-length gene 9 was done using two primers (sBeg9/Beg9 and End9, see appendix VIII) that resulted in the 1062bp gene products.

Protocol:

Denaturing ds RNA: The dsRNA extracted either by TRIzol method or by RNaid extraction was initially denatured by first adding the oligonucleotide primers EndA/sBeg (for VP7 PCR typing) and Con2/3 (for VP4 PCR typing) and boiling the mixture for 5 minutes followed by immediate chilling.

Reverse transcription: The four nucleotide bases (dATP, dCTP, dGTP and dTTP), reverse transcriptase enzyme (AMV reverse transcriptase, Promega Madison Wisconsin USA) and buffer (AMV reverse transcriptase, Promega Madison Wisconsin USA) were

added and the mixture incubated for 26-30 minutes in a 42°C water bath (The resulting mixture is called the master mix). This enabled the production of DNA from RNA.

Amplification of cDNA: Finally the 4 bases (dNTP's from Gibco Life Technologies, Karlsruhe, Germany), *Taq* polymerase, *Taq* buffer, Magnesium chloride ($MgCl_2$) (all from Gibco Life Technologies, Karlsruhe, Germany) and distilled water (dH_2O) were added to the master mix after incubation. The amplification of copy DNA was then done using the DNA Thermal Cycler (Perkin-Elmer® Cetus, Norwalk, USA) running at 30 cycles at 94°C for 1 minute, 42°C for 2 minutes and 72°C for 3 minutes.

Gel Electrophoresis: The products were run on 1.5% agarose gel stained with 2µl ethidium bromide (Sigma®) for band visualization against UV-light background. A molecular weight marker of 100-1500bps (Boehringer Mannheim®) and a negative control were included in the experiment. The gels were electrophoresced at 80-100V and visualised using a gel reader (UVP White/UV Transilluminator, California, USA). The gels were then printed on thermal paper. The above process resulted in the 876bp (VP4) and 1062bp (VP7) gene products.

(ii) Re-amplification of cDNA (second amplification)

The 876bp ds RNA gene product (for VP4) were re-amplified by PCR in the presence of a cocktail containing one conserved plus sense primer and 4 type-specific minus sense primers (con3and 1T-I, 2T-I, 3T-I, 4T-I, 5T-I) selected from the hypervariable region of gene ¤ (Appendix IX; Gentsch *et al.*, 1992).

For VP7 the 1062bp ds RNA product was re-amplified using six serotype –specific primers aBT1, aCt2, aET3, aDT4, aAT8 and aFT9 and the common primer VG (Appendix VIII; Gouvea *et al.*, 1990).

Protocol

PCR: Primers for VP7 and VP4 (see above), the four bases (dATP, dCTP, dGTP, dTTP), *Taq* polymerase, *Taq* buffer, $MgCl_2$ and dH_2O were added to the master mix. The PCR was then carried out in a DNA Thermal Cycler and the reaction was subjected to 35 cycles of PCR. Each cycle consisted of 1-minute at 94°C, 1-minute at 50°C and 1-minute at 72°C.

Agarose gels: 10ul of PCR products were loaded onto 1.5% agarose gel containing 2µl ethidium bromide (Sigma®) for band visualization against UV-light background. A molecular weight marker of 100-1500bps (Boehringer Mannheim®) and a negative control were included in the experiment. The gels were electrophoresced at 80-100V and visualised using a gel reader (UVP White/UV Transilluminator, California, USA). The gels were then printed on thermal paper.

3.3.4 Statistical Analysis

The mean ages of the HIV-seropositive and HIV-seronegative populations were compared by using the two-tailed, two-sample t-test and generate a confidence interval. The rates of virus detection in the specimens from both groups of patients were tested for significance with the approximation of the binomial distribution to the normal distribution test.

CHAPTER FOUR

RESULTS

4.1 HIV AND GASTROINTESTINAL DISEASE STATUS

A Total of 207 stool samples were analysed from children reported with diarrhoea and those not having diarrhoea. Out of these samples 94 were from children with diarrhoea whose HIV status was unknown, while 113 were from children infected with HIV (Table 1). A total of 137 children had diarrhoea out of which 47 were found to be positive for a viral agent causing diarrhoea (34%).

4.2 ROTAVIRUS ELISA

Of 207 samples evaluated by ELISA (DAKO® kit) 29 (14%) were positive by this method. Of these 17 out of 94 (18.1%) were from children with diarrhoea whose HIV status was unknown. Two out of 70 (2.9%) were from children infected with HIV without diarrhoea and 10 out of 43 (23.3%) were from children infected with HIV with diarrhoea. These figures are illustrated in Table 2 below. Samples from children with unknown HIV status were all from clinical cases of diarrhoea.

4.3 ADENOVIRUS ELISA

Six out of the 207 (2.9%) samples tested for adenoviruses were positive. Of these 4 out of 94 (4.3%) were from children with diarrhoea whose HIV status was unknown, 1 out of 70 (1.4%) were from children without diarrhoea who were HIV positive, while 1 out of 43 (2.3%) were from children with diarrhoea who are HIV positive (See Table 2).

4.4 ASTROVIRUS ELISA

Twelve of the 206 (5.8%) samples were positive for astrovirus. Of these, 9 out of 94 (9.6%) were from children with diarrhoea with unknown HIV status; 2 out of 69 (2.9%) were

from children without diarrhoea who were HIV positive; while 1 out of 43 (2.3%) where from children with diarrhoea who were HIV positive (See Table 2).

Table 1: Sample Distribution

HIV status	Samples with Diarrhoea	Samples without Diarrhoea	TOTAL
HIV POSITIVE	43	70	113
UNKNOWN STATUS	94	0	94
TOTAL	137	70	207

Table 2: Percentage infection with Group A rotavirus, adenovirus and astrovirus

	HIV POSITIVE		UNKNOWN STATUS	
	DIARRHOEA N = 43	NO DIARRHOEA N = 70	DIARRHOEA N = 94	NO DIARRHOEA N = 0
ROTAVIRUS	10 (23.3%)	2 (2.9%)	17 (18.1%)	0
ADENOVIRUS	1 (2.3%)	1 (1.4%)	4 (4%)	0
ASTROVIRUS	1 (2.3%)	2 (2.9%)	9 (9.6%)	0

4.5 Electron Micrograph of Faecal Rotavirus

One sample identity number 1631/99 that was initially positive for rotavirus by ELISA was taken for electron micrograph resulting in positive demonstration of rotavirus particles in stool as shown in figure 7.

4.6 SDS-PAGE

An electrophyte profile (4-2-3-2) characteristic of group A (see figure 8 a and b) was demonstrated by PAGE in samples positive by ELISA. Samples positive for PAGE were noted to be strong (+++) ELISA positives. Two ELISA negative samples were assayed using PAGE. Fourteen (53.8%) of 26 samples positive by rotavirus ELISA were positive for PAGE. These comprised of 5 short types and 9 long types (length of the 11 segments seen). Most of the rotavirus strains detected by PAGE showed the "long" electrophoretic pattern (64.3%) while the remaining exhibited the "short" electropherotypes (Figure 8 a and b).

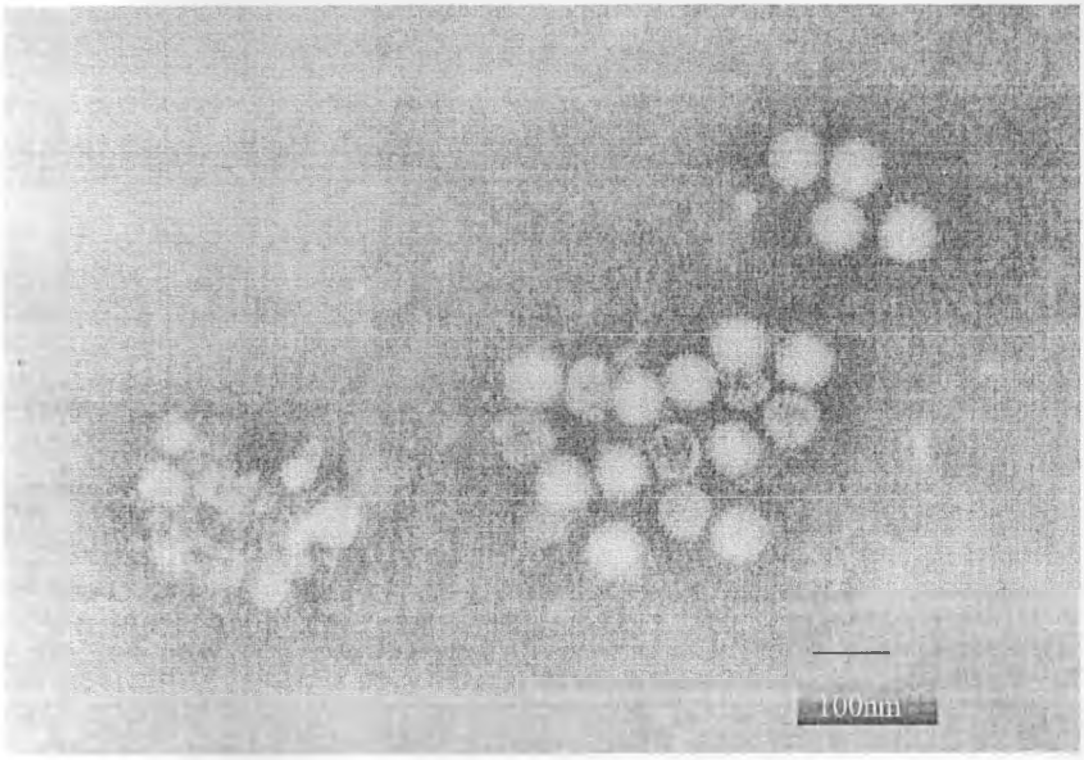
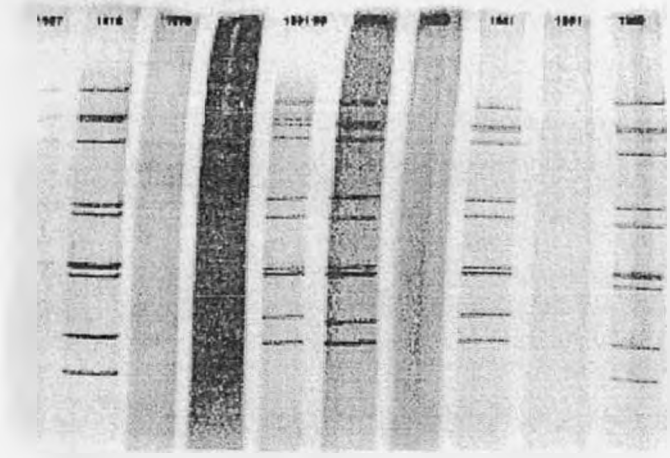


Figure 7 A Negative stain Electron Micrograph of Rotavirus from one of the children (ID# 1631/99) who tested positive by ELISA. Note the wheel-like appearance of some of the rotavirus particles.



(a) 1 2 3 4 5 6 7 8 9 10

Figure 8 **Analysis of human rotavirus dsRNA**

(a) PAGE analysis of the dsRNA genome extracted from stool suspensions taken from children with diarrhoea is shown. All samples exhibited typical 4-2-3-2 pattern of group A rotavirus. In a, lanes 2 and 10 show "long" elctrophoretic pattern while lanes 5,6 and 8 show the "short" electrophoretic pattern. Lane 1 also shows the long pattern although the results are faint and not too clear.

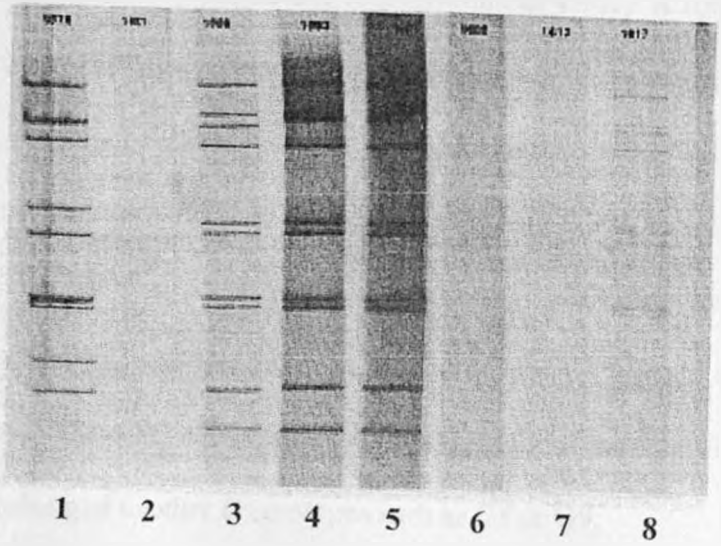


Figure 8 **Analysis of human rotavirus dsRNA**

(b) PAGE analysis of the dsRNA genome extracted from stool suspensions taken from children with diarrhoea is shown. All samples exhibited typical 4-2-3-2 pattern of group A rotavirus.. In **b**, lane 1 shows the short pattern while lane 3,4 and 5 show the long pattern. Lane 8 shows the long pattern but this is very faint.

4.7 ROTAVIRUS VP6 SUBGROUP DETERMINATION

This assay was used for rotavirus positive specimens to determine the subgroup specificity of the rotavirus present. Thirteen out of 28 (46.4%) of the rotavirus positive samples run were positive for the VP6 ELISA. Six were positive for subgroup I (46%) while 7 for subgroup II (54%) antigens.

4.8 ROTAVIRUS VP7 MONOCLONAL ANTIBODY ELISA

This assay was run to enable the identification of Group A rotavirus G serotypes in the stool specimens. Previously there have been 14 G types identified in humans with G1 to 4 being the most prevalent (Frühwirth *et al.*, 2000; Gentsch *et al*, 1996). The assay was only able to test for the presence of G1 to G4 . G1 (3 samples), G2 (1 sample) and G3 (1 sample) serotypes were identified in the rotavirus positive by ELISA samples. Two samples also showed mixed infections with G1/2/3 types. Rotavirus type G4 was not detected in any of the 24 samples tested. The other samples did not test for any of the four G types but this could mean that they belonged to other G serotypes such as G8 or G9.

4.9 RT-PCR AND PCR GENOTYPING

Twenty three samples were selected for PCR and subjected to G and P genotyping by reverse transcription PCR (RT-PCR), with primers specific for human rotavirus genotypes (See Appendix VIII for VP7 primers and Appendix IX for VP 4 primers). The samples were a mixture of strong ELISA positive samples, SDS-PAGE positive samples and a few negative samples (see Table 3). Only a few samples were selected due to the high cost of running this assay. The gene product of the RT-PCR of gene 9 (VP7) using sBeg9 and end9 was a 1062bp (figure 9a) while that for gene 4 (VP4) was 876bp (see figure 9b).

Table 3: Combinations of VP7 and VP4 identified in single Rotavirus Strains

	MEDUNSA #	IPR #	EIA	SDS-PAGE	VP7 type	VP4 type
1.	-	02	Not Done	+	G 1	P [8]
2.	-	57	Not Done	+	G 8	P [4]
3.	1561/00	60	+++	+S	G 3	P[6]
4.	-	62	Not Done	+	G 9	P [4]
5.	1563/00	63	+++	+L	G 1	P [6]
6.	1576/00	79	+++	+S	G 8	P [6]
7.	1589/00	93	+++	+L	G 1, 8	P [6]
8.	1593/00	97	+++	+L	G 3, 8	P [6]
9.	1601/00	105	+++	+L	G 3	P [6] [8]
10.	1602/00	107	+	-	G NT	P [6]
11.	1617/00	122	+++	+L	G 4	P [6]
12.	1618/00	123	+++	+L	G 4	P [8]
13.	1619/00	124	+	+L	G 3, 8	P [6]
14.	1631/00	136	-	+	G NT	P [8]**
15.	1632/00	137	-	+	G NT	P [8]**
16.	1631/99	138	+++	+S	G 8	P [6]
17.	1634/00	140	+++	+S	G 3	P [6]
18.	1635/00	141	+	-	G 3	P [6]
19.	1641/00	147	+++	+S	G 3	P [6]
20.	1709/00	217	-	Not Done	G 1*	P [8]*
21.	1963/00	234	+++	+L	G 1	P [6]
22.	1964/00	235	+	-	G 3	P [6]
23.	1967/00	238	+	-	G 3	P [6]

Key: * Indicates use of alternative primers, that is to say, a divergent type

** Indicates use of degenerate primers divergent from prototype

NT Non-typable with any of the available G or P primers

(+) Positive result; (-) Negative result

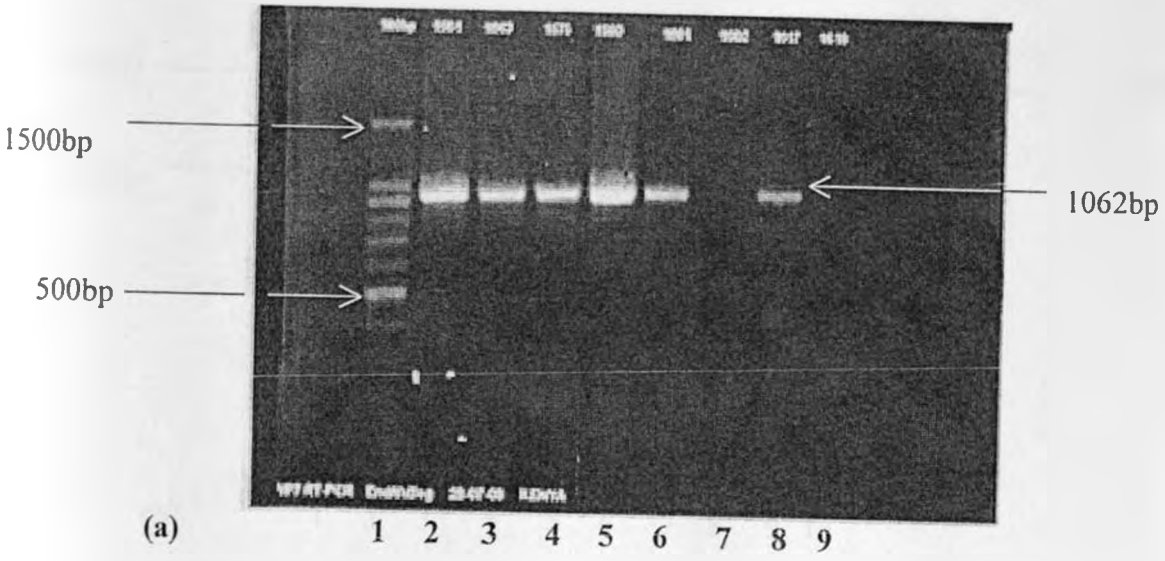


Figure 9 **Amplification of the VP7 genes by RT-PCR**

(a) Amplification products corresponding to the VP7 in 9 different patients. Lane 1 has the molecular weight marker (100bp ladder). The sizes of amplified bands are indicated.

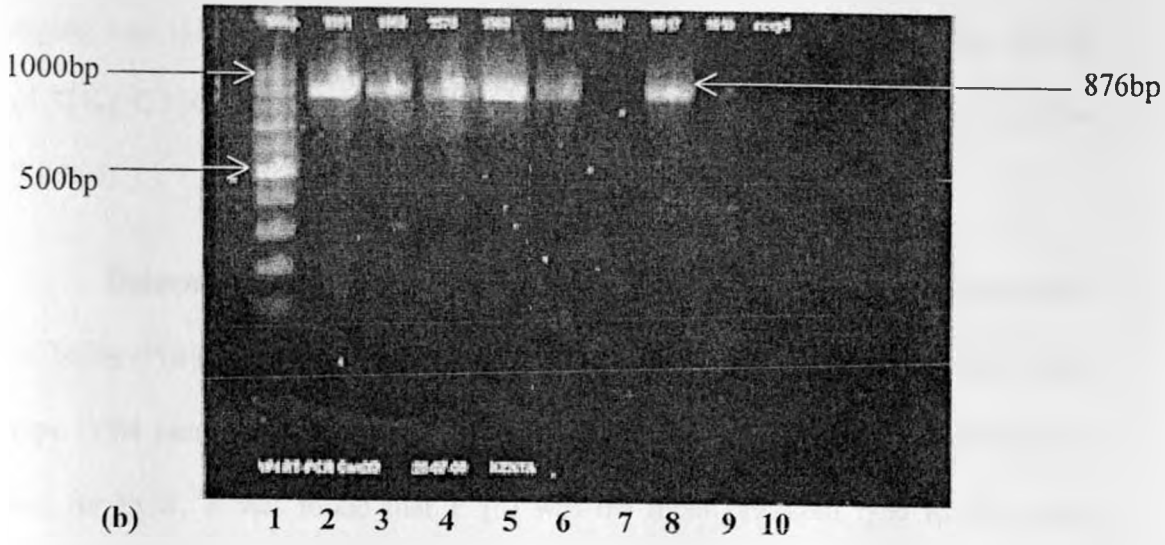


Figure 9 **Amplification of the VP7 and VP4 genes by RT-PCR**

(b) Amplification products corresponding to the VP4 genes in 9 different patients. Lane 1 has the molecular weight marker (100bp ladder) while a negative control is in lane 10. The sizes of amplified bands are indicated.

4.9.1 **Determination of G type:** The six sets of primers used for typing yielded bands of distinct lengths – 630, 250, 460, 765, and 186bp – belonging to serotypes 1, 3, 4 8 and 9 respectively (see figure 10a). The G type (VP7 associated) was successfully determined in 20 (87%) of the 23 samples run for PCR. This shows a slightly lower efficiency relative to P typing (100%). The overall incidence for G typing was G1 (17.4%), G3 (34.8%), G4 (8.7%), G8 (8.7%), G9 (4.35%), G1+G8 (4.35%), G3+G8 (8.7%) and 3 samples (13%) could not be typed for the G type (See Table 3).

4.9.2 **Determination of P type:** The products of PCR of gene 4 resulted in products of 345bp (P[8]), 483bp (P84]), 267bp (P[6]), and 594bp (P[10] (see figure 10b). The P type (VP4 associated) was successfully determined in all 23 (100%) of the samples run for PCR. It was found that P [6] was the most prevalent type in this group (65.2%). Others were P [4] (8.7%) and P [8] (26.1%) (see table 3).

4.9.3 **Correlation of G and P types:** Table 4 shows the correlation of VP4 and VP7 genotypes identified in the same specimen. The serotype G and genotype P was assigned to 20 (87%) of the 23 samples by using the procedures outlined. G3P[6] (7 out of 23) was recorded most common (30.4% of all type viruses). The others were G8P[6] (1 out of 23; 4.3%), G1P[6] (2 out of 23; 8.6%), G1P[8](2 out of 23 ; 8.6%), G8P[4](1 out of 23; 4.3%),G3P[8], G9[4], G4P[6] and G4P[4] all (1 out of 23 for each; 4.3%) . The mixed infections were G1+8 P[6] (1 out of 23; 4.3%) and G3+8 P[6] (2 out of 23; 8.6%).

4.10 COMPARISON OF ELISA, PAGE AND RT-PCR

Electropherotypes: In the study, G1 and G4 rotaviruses were associated with "long" electropherotypes whereas G3 electropherotypes were "short". Mixed infections of G3/8 showed the short electrophoretic pattern, while G1/8 showed "long" patterns. Only 53.8% of the samples positive by ELISA were positive by PAGE.

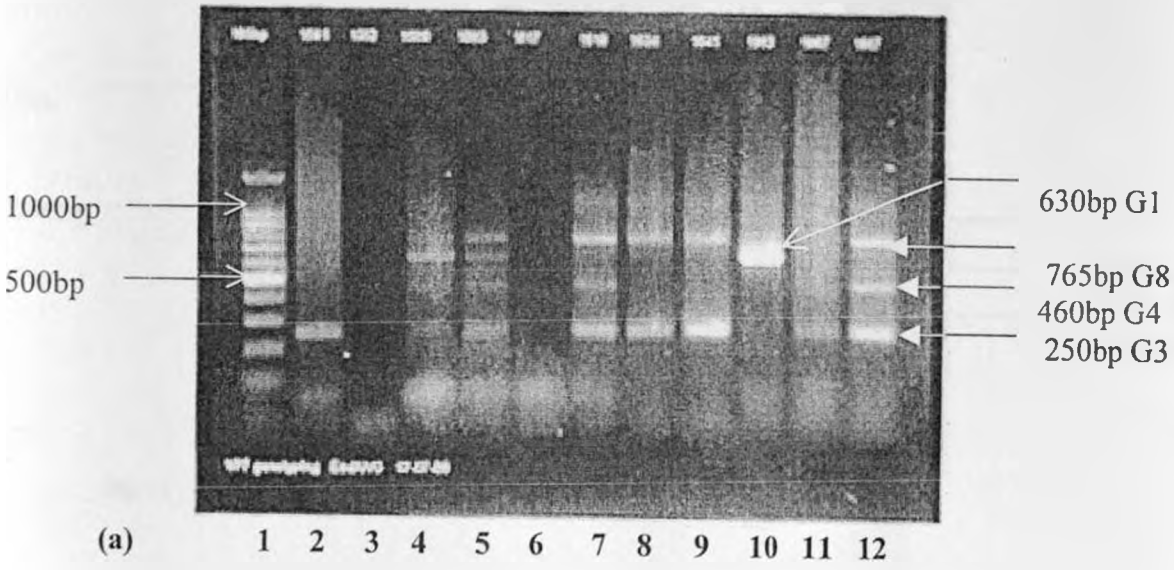


Figure 10 **Typing of human group A rotavirus VP7 and VP4 gene by PCR**

(a) Amplification products of the VP7 genes in stool samples from 11 different patients with diarrhoea. Lane 1 has the molecular weight marker (100bp ladder). Lane 2 shows type G3; lanes 8 and 9 mixed infection of G3 and G8; lane 10 shows G1; lanes 7 and 12 show mixed infections of G3, G4 and G8.

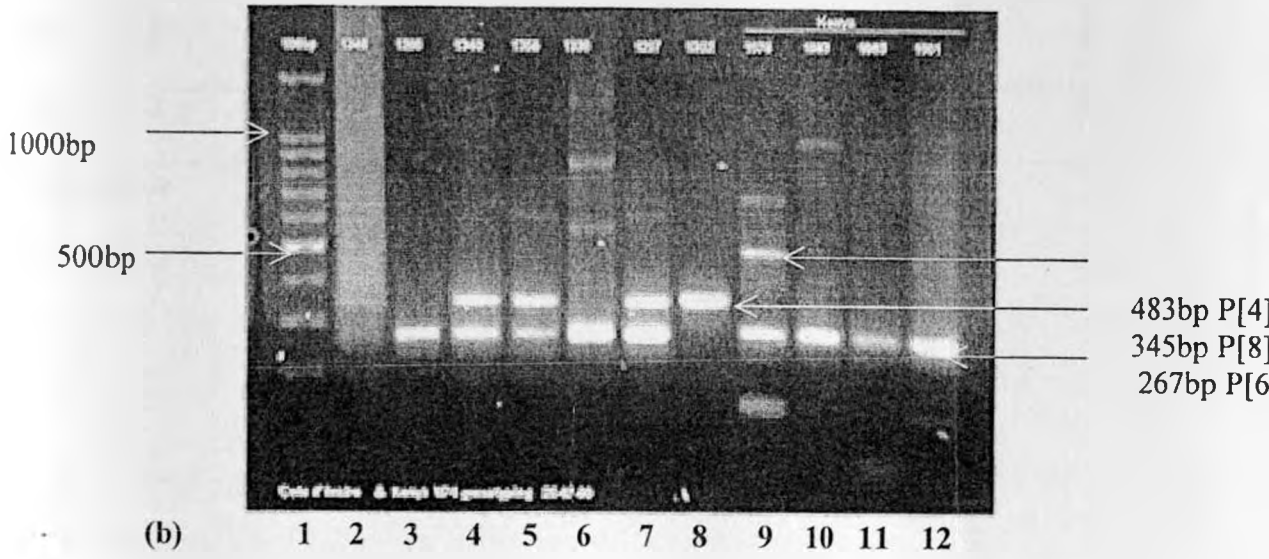


Figure 10 Typing of human group A rotavirus VP7 and VP4 gene by PCR

(b) Amplification products of the VP4 genes in stool samples taken from 12 different patients with diarrhoea. Lane 1 has the molecular weight marker (100bp ladder); Lanes 3,6,10,11 and 12 show type P[6]; lanes 4,5 and 7 show co-infection with P[6] and P[9]; lane 8 shows P[9] and lane 9 shows mixed infection with P[4] and P[6].

Table 4 Circulating P and G types in Kenya

	Serotype VP7 →								
Genotype VP4 ▼	NT	G1	G3	G4	G8	G9	G1+8	G3+8	Total
P[4]		0	0	1	1	1	0	0	3
P[6]	1	2	7	1	1	0	1	2	15
P[8]	2	2	1	0	0	0	0	0	5
Total	3	4	8	2	2	1	1	2	23

NT: Non-typable

4.11 STATISTICAL ANALYSIS

The samples from the HIV infected children were from those with diarrhoea (43 samples) and those without diarrhoea (70 samples). These samples were collected during the period February 1999 to June 2000. The group of children with HIV had a mean age of 6.3 years (75.48 months). The youngest child was 5 months old while the eldest was 14 years (168 months) old. The median age was 6 years (72 months), while the most commonly occurring age (mode) was 8 years (96 months).

The samples from the children whose HIV status was unknown were all collected from those with reported cases of diarrhoea (n=94). These samples were collected during the period February 1999 to June 2000. The mean age in this group was 1.8 years (20.9 months) making this group on the average younger than the HIV infected children. The youngest child in this group was 1 month old while the eldest was 9 years (108 months). The median age was 1-½ years (18 months) while the most commonly occurring age was 2 years (24 months).

4.11.1 Comparison of Mean Age for HIV Infected (μ_1) and Children of unknown status (μ_2)

Two-Sample T-Test was used to perform an independent two-sample t-test and generate a confidence interval. Use of 2-Sample t-test to perform a hypothesis test and compute a confidence interval of the difference between two population means when the population standard deviations, are unknown.

Assume that the data constitutes two independent random samples, one from a population of apparently normal subjects and another from a population of subjects with disease (in this case HIV).

Hypotheses:

H_0: $\mu_1 - \mu_2 = 0$ versus H_A: $\mu_1 - \mu_2 \neq 0$

Basic Statistics (N = Total Number of Samples; StDev = Standard Deviation;

SE Mean = Standard Error of Mean):

	N	Mean	StDev	SE Mean
HIV	98	75,5	36,0	3,6
NHIV	91	20,9	17,9	1,9

Difference = μHIV - μ NHIV

Estimate for difference: 54.56

95% Confidence Interval for difference: (46.47; 62.64)

t-Value = 13.34

p-Value < 0.0001 & Degrees of Freedom = 144

Conclusions: The population means are different.

4.11.2 Statistical Analysis of Prevalence Rates

Rotavirus

(i) The prevalence of rotavirus is 10 (x_1) out of 43 (n_1) in HIV positive children with diarrhoea and 17(x_2) out of 94(n_2) in children with unknown HIV status with diarrhoea.

Test: Is there a significant difference in the occurrence of rotavirus in the two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of diarrhoeic children with and without HIV.

Hypotheses: The null hypothesis is that the prevalence rate of rotavirus in HIV infected children (p_1) is not different from that of children with unknown HIV status (p_2). Hence

H_0: $p_1 = p_2$ or $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of rotavirus in the two groups. Hence **H_A: $p_1 \neq p_2$ or $p_1 - p_2 \neq 0$**

Test Statistic: $z = \dfrac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\dfrac{p(1-p)}{n_1} + \dfrac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H_0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 10/43 = 0.23$, $\hat{p}_2 = 17/94 = 0.18$

And $p = (10 + 17)/(43 + 94) = 0.197$

$$z = \frac{(0.23 - 0.18)}{\sqrt{\dfrac{(0.197)(0.803)}{43} + \dfrac{(0.197)(0.803)}{94}}} = 0.683$$

Statistical Decision: Do not reject H_0 since $0.683 < 1.645$ **Statistical Conclusion:** The prevalence of rotavirus does not differ significantly between HIV-infected children with diarrhoea and children with unknown HIV status with diarrhoea (p value = 0.2483).

(ii) The prevalence of rotavirus is 10(x_1) out of 43(n_1) in HIV positive children with diarrhoea and 2 (x_2) out of 70 (n_2) in HIV positive children without diarrhoea

Test: Is there a significant difference in the occurrence of rotavirus in these two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of HIV-infected children with and without diarrhoea HIV.

Hypotheses: The null hypothesis is that the prevalence rate of rotavirus in HIV infected children with diarrhoea (p_1) is not different from that of HIV infected children with diarrhoea (p_2). Hence $\mathbf{H_0}$: $p_1 = p_2$ or $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of rotavirus in the two groups. Hence $\mathbf{H_A}$: $p_1 \neq p_2$ or $p_1 - p_2 \neq 0$

Test Statistic: $$z = \frac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\frac{p(1-p)}{n_1} + \frac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 10/43 = 0.23$, $\hat{p}_2 = 2/70 = 0.03$

$p = (10 + 2)/(43 + 70) = 0.106$

$$z = \frac{(0.23 - 0.03)}{\sqrt{\frac{(0.106)(0.894)}{43} + \frac{(0.106)(0.894)}{70}}} = 3.353$$

Statistical Decision: Reject H_0 since $3.353 > 1.645$

Statistical Conclusion: The prevalence of rotavirus differs significantly between HIV-infected children with diarrhoea and HIV-infected children without diarrhoea (p value = 0.0004).

Adenovirus

(i) The prevalence of adenovirus is 1(x_1) out of 43 (n_1) in HIV positive children with diarrhoea and 4 (x_2) out of 94 (n_2) in children of unknown HIV status with diarrhoea.

Test: Is there a significant difference in the occurrence of adenovirus in the two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of diarrhoeic children with and without HIV.

Hypotheses: The null hypothesis is that the prevalence rate of adenovirus in HIV infected children (p_1) is not different from that of children of unknown HIV status (p_2). Hence

H_0: $p_1 = p_2$ or $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of adenovirus in the two groups. Hence **H_A: $p_1 \neq p_2$ or $p_1 - p_2 \neq 0$**

Test Statistic: $$z = \frac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\frac{p(1-p)}{n_1} + \frac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H_0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 1/43 = 0.023$, $\hat{p}_2 = 4/94 = 0.042$

$p = (1 + 4)/(43 + 94) = 0.036$

$$z = \frac{(0.023 - 0.042)}{\sqrt{\frac{(0.036)(0.964)}{43} + \frac{(0.036)(0.964)}{94}}} = 0.547$$

Statistical Decision: Do not reject H_0 since $0.547 < 1.645$

Statistical Conclusion: The prevalence of adenovirus does not differ significantly between HIV-infected children with diarrhoea and children of unknown HIV status with diarrhoea (*p* value = 0.2929).

(ii) The prevalence of adenovirus is 1(x_1) out of 43 (n_1) in HIV positive children with diarrhoea and 1 (x_2) out of 70 (n_2) in HIV positive children without diarrhoea

Test: Is there a significant difference in the occurrence of adenovirus in these two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of HIV-infected children with and without diarrhoea HIV.

Hypotheses: The null hypothesis is that the prevalence rate of adenovirus in HIV infected children with diarrhoea (p_1) is not different from that of HIV infected children with diarrhoea (p_2). Hence $\mathbf{H_0}$: $p_1 = p_2$ **or** $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of adenovirus in the two groups. Hence $\mathbf{H_A}$: $p_1 \neq p_2$ **or** $p_1 - p_2 \neq 0$

Test Statistic: $z = \dfrac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\dfrac{p(1-p)}{n_1} + \dfrac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H_0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 1/43 = 0.023$, $\hat{p}_2 = 1/70 = 0.0143$

$p = (1+1)/(43 + 70) = 0.0177$

$$z = \frac{(0.023 - 0.0143)}{\sqrt{\dfrac{(0.0177)(0.9823)}{43} + \dfrac{(0.0177)(0.9823)}{70}}} = 0.340$$

Statistical Decision: Do not reject H_0 since 0.340 <1.645

Statistical Conclusion: The prevalence of adenovirus does not differ significantly between HIV-infected children with diarrhoea and HIV-infected children without diarrhoea (*p* value = 0.3669).

Astrovirus

(i) The prevalence of astrovirus is $1(x_1)$ out of $43(n_1)$ in HIV positive children with diarrhoea and 9 (x_2) out of 94 (n_2) in children of unknown HIV status with diarrhoea.

Test: Is there a significant difference in the occurrence of astrovirus in the two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of diarrhoeic children with and without HIV.

Hypotheses: The null hypothesis is that the prevalence rate of astrovirus in HIV infected children (p_1) is not different from that of children of unknown HIV status (p_2). Hence

H_0: $p_1 = p_2$ or $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of astrovirus in the two groups. Hence **H_A: $p_1 \neq p_2$ or $p_1 - p_2 \neq 0$**

Test Statistic: $$z = \frac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\frac{p(1-p)}{n_1} + \frac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H_0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 1/43 = 0.023$, $\hat{p}_2 = 9/94 = 0.096$

$p = (1 + 9)/(43 + 94) = 0.073$

$$z = \frac{(0.023 - 0.096) = 0.073}{\sqrt{\frac{(0.073)(0.927)}{43} + \frac{(0.073)(0.927)}{94}}} = 1.524$$

Statistical Decision: Do not reject H_0 since $1.524 < 1.645$

Statistical Conclusion: The prevalence of astrovirus does not differ significantly between HIV-infected children with diarrhoea and children of unknown HIV status with diarrhoea (*p* value = 0.06365).

(ii) The prevalence of astrovirus is 1(x_1) out of 43 (n_1) in HIV positive children with diarrhoea and 2 (x_2) out of 70 (n_2) in HIV positive children without diarrhoea

Test: Is there a significant difference in the occurrence of astrovirus in these two groups?

Assumptions: Assume that the patients in the study constitute independent simple random samples from populations of HIV-infected children with and without diarrhoea HIV.

Hypotheses: The null hypothesis is that the prevalence rate of astrovirus in HIV infected children with diarrhoea (p_1) is not different from that of HIV infected children with diarrhoea (p_2). Hence $\mathbf{H_0}$: $p_1 = p_2$ or $p_1 - p_2 = 0$

The alternate hypothesis is that there is a difference in the prevalence rates of astrovirus in the two groups. Hence $\mathbf{H_A}$: $p_1 \neq p_2$ or $p_1 - p_2 \neq 0$

Test Statistic:

$$z = \frac{(\hat{p}_1 - \hat{p}_2) - (p_1 - p_2)}{\hat{\sigma}_{\hat{p}1 - \hat{p}2}}$$

Where $\hat{\sigma}_{\hat{p}1 - \hat{p}2} = \sqrt{\frac{p(1-p)}{n_1} + \frac{p(1-p)}{n_2}}$ and $p = (x_1 + x_2)/(n_1 + n_2)$

Decision Rule: Let $\alpha = .05$. That is to say 95% confidence interval. Hence the critical value will be 1.645. H_0 is rejected if computed z is greater than 1.645.

Calculation of test statistic: From the sample data:

$\hat{p}_1 = 1/43 = 0.023$, $\hat{p}_2 = 2/70 = 0.03$

$p = (1 + 2)/(43 + 70) = 0.027$

$$z = \frac{(0.023 - 0.03)}{\sqrt{\frac{(0.027)(0.973)}{43} + \frac{(0.027)(0.973)}{70}}} = 0.087$$

Statistical Decision: Do not reject H_0 since $0.087 < 1.645$

Statistical Conclusion: The prevalence of astrovirus does not differ significantly between HIV-infected children with diarrhoea and HIV-infected children without diarrhoea (*p* value = 0.4701).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.0 GENERAL

The causes of diarrhoea in HIV-infected children are not well understood. In an attempt to better understand the aetiology of this syndrome, we studied the prevalence of three enteric viruses in faecal samples collected from HIV-positive and HIV-negative children. The objective of this study was to determine the prevalence of rotavirus, adenovirus and astrovirus among children with and without HIV. In addition, however we are able to report some of the P and G genotypes of circulating rotaviruses isolated from children with diarrhoea in Nairobi.

In this study, stool specimens were obtained from 207 young children. There were 137 children with diarrhoea and 70 children without diarrhoea. The latter group were all HIV positive. The group of 137 children with diarrhoea consisted of 43 children who were HIV infected and 94 children with unknown HIV status. The general outcome of this study showed that rotaviruses were detected in 14% of all the samples compared to, 5.8% infected with astrovirus and 2.9% infection with adenovirus. A comparative study carried out in South Africa (Steele *et al.*, 1998a), showed that rotaviruses were detected in 20% of 225 samples from young children under the age of five years with gastroenteritis, astroviruses were detected in 7% while adenovirus in 3% of these children. This shows similar results to our study. It is possible that the prevalence rates of these viruses could have been higher in our studies if the average age of the children was lower for the HIV positive children (75 months or 6 and half years in the HIV infected children and 21 months or 1year and nine months in the children with unknown HIV status). Also, some of the samples from the children with HIV were not from cases of diarrhoea. Therefore this lowered the possible rates of infection expected in such a group.

5.1 ROTAVIRUS

It has been shown in this study that overall 14% (29 out of 207) of the children tested were positive for rotavirus infection. Of these 18.1% (17 out of 94) were children with unknown HIV status (all these samples were from children with diarrhoea) and 23.3% (10 out of 43) were from HIV infected children with diarrhoea. These results further support the fact that group A rotavirus is a very important aetiological agent of gastro-enteritis in children.

The prevalence of rotavirus did not differ significantly between the HIV-infected children with diarrhoea and the HIV-uninfected children with diarrhoca ($p > 0.05$). The latter group were children routinely visiting local city council clinics to be treated for paediatric diarrhoea and not coming specifically for HIV testing. Rotavirus is the most important viral agent associated with severe gastroenteritis in children (Kapikian and Chanock, 1996). In comparison, the results obtained in this and previous studies (Fontana *et al*, 1996; Italian Paediatric Intestinal/HIV Study Group, 1993) indicate that, in children, the epidemiology of rotavirus and its association with diarrhoea do not vary significantly during HIV infection.

These results were similar to a study in Zambia, which demonstrated that while rotavirus and HIV infections commonly co-existed, rotavirus was not more prevalent nor the illness more severe in HIV infected children (Oshitani *et al*, 1994). Liste *et al.*, (2000) also showed that specimens from HIV-positive children were not more likely than those of HIV-negative children to have rotavirus (5 versus 8%; $P > 0.5$).

However, there was a difference within the group of children with HIV so that rotavirus was more prevalent in those with diarrhoea than in those without ($p = 0.0004$). This demonstrates that rotavirus is associated with diarrhoea symptoms and rarely occurs in asymptomatic children.

The VP4 and VP7 rotavirus genes encode for the outer capsid neutralisation proteins of the virus. In order to determine the prevalent serotypes and hence the genotypes of rotavirus in Kenya 23 samples were genotyped by using RT-PCR.

Of these samples tested 20 (87%) of 23 were assigned a G type. The overall incidence for G typing was G1 (17.4%), G3 (34.8%), G4 (8.7%), G8 (8.7%), G9 (4.35%), G1+G8 (4.35%), G3+G8 (8.7%) and 3 samples (13%) could not be typed for the G type. This study has shown that the predominant G type was G3. This genotype appeared in mixed infections and also individually. In contrast, a previous epidemiological survey in Kenya on the G serotype distribution of group A rotavirus showed G1 (Gatheru *et al.*, 1993) and G4 (Nakata *et al.*, 1999) to be the most prevalent. It is important to note that Gatheru and Nakata were screening for G1 upto G4 at the time of their stuidies. As result even if other G types were present, they could either have not been identified or known at the time. However, studies carried out worldwide show that G1 is the most predominant type (O' Mahony *et al.*, 1999). As a result, G1 to G4 are the most common aetiologic agents of childhood diarrhoea worldwide for which vaccines have been developed (Kapikian *et al.*, 1996; Kapikian *et al.*, 1991). In this study, G8 and G9 serotypes were also found in these children, but these are not included in current vaccine strategies.

The most prevalent of P types (65.2%) in this study was P[6]. The incidence of P[6] in recent American (Ramachandran *et al.*, 1998), Brazilian (Timenetsky *et al.*, 1994, Indian (Ramachandran *et al.*, 1996) and African (Steele *et al.*, 1995) studies seems to highlight this strain as a significant emerging genotype. Genotype P[6] strains are usually associated with asymptomatic infection in neonates (Gorziglia *et al.*, 1988) and are considered uncommon pathogens in older infants with diarrhoea(Steele *et al*, 1992). However in this study, P[6] was the most common of the VP4 genotypes identified from diarrhoeic cases. The common P

types that prevail according to literature are P[4] and P[8] (Gentsch *et al.*, 1996; O'Mahony *et al.*, 1999). It is therefore possible that P[6] is a common P type in Nairobi, Kenya, just as it is an unusual type in the other reported studies. There were no P[9] types reported in this study but P[4] (8.7%) and P[8] (26.1%) were reported.

The results also show that the prevalence of G3P[6] (30.4% of all type viruses), as the most common combination. The others were G8P[6] (1 out of 23; 4.3%), G1P[6] (2 out of 23; 8.6%), G1P[8](2 out of 23 ; 8.6%), G8P[4](1 out of 23; 4.3%),G3P[8], G9[4], G4P[6] and G4P[4] all (1 out of 23 for each; 4.3%) . The mixed infections were G1+8 P[6] (1 out of 23; 4.3%) and G3+8 P[6] (2 out of 23; 8.6%). Studies done in Malawi have reported the isolation of serotype G8 possessing P[6] and P[4] genotype specificity(Cunliffe *et al.*,1999; 2000, 2001) as a novel P/G strain. However G1P[8] and G8P[6]were found to be the most prevalent strain types in the same area (Cunliffe *et al.*, 2001). A study in Zambia demonstrated the prevalence of G1P[8] but also G4P[8] (Steele *et al.*, 1998b) These results are of interest because it has been repeatedly reported that G1P[8] and G2P[4] are the G-P type combinations frequently found among tested samples (Das *et al.*, 1994; Gentsch *et al.*, 1996; Gouvea *et al.*, 1990; Santos *et al.*, 1994 and Timenetsky *et al.*, 1994). It has also been reported that G1P[8] has been found in children in the United States (Gentsch *et al.*, 1996; Gouvea *et al.*, 1990; Ramachandran *et al.*, 1998) and New Delhi (Matson *et al.*, 1993; Ramachandran *et al.*, 1996).

The diversity of rotavirus VP7 serotypes and VP4 genotypes in a specific geographical area indicates the need for continual monitoring (Steele *et al.*, 1995). This is because it is possible that the virus is highly variable and therefore mutates constantly.

The results in this study present a very interesting aspect in vaccine strategies because it means that the circulating strains in Nairobi, Kenya may be very different from those that cause infection in other parts of the world. The molecular characterisation of isolated strains

is very important in the development of effective vaccines for various geographical regions. The significance of determining serotypes of circulating rotaviruses is currently of major importance (Wu *et a.l,* 1994) with RT-PCR being the most sensitive assay for the determination of genotypes (Masendycz *et al.*, 1997).

The typing of P and G types presents a very important finding, since current trial vaccines may not be effective against rotavirus strains in Kenya. However, this is still not a clear-cut area because it is not known whether cross-immunity can be imparted using other strains. Currently, vaccine studies are at a halt following the discovery that some children developed intussusceptions after vaccination, in the United States of America. In August 1998, a live attenuated rotavirus vaccine (Rotashield, Wyeth Laboratories, Marietta, PA, USA) was licensed in the USA for the prevention of severe diarrhoea in infants. A programme of immunisation with a three-dose schedule (at 2, 4, and 6 months of age) began in the autumn of 1998. Between Oct 1, 1998, and July 16, 1999, an estimated 1·5 million doses of Rotashield were administered to US infants (personal communication, Peter Paradiso, Wyeth Laboratories). On July 16, 1999, the US Centers for Disease Control and Prevention (CDC) reported 15 cases of intussusception in infants, which had been temporally associated with Rotashield vaccination. CDC recommended suspension of vaccination pending further information (Centers for Disease Control and Prevention, 1999c) and later provided additional data indicating that such temporally associated intussusceptions were clustered predominantly within a week of the first Rotashield dose, with an estimated relative risk of more than 20 during days 3-7 after vaccination. (Advisory Committee on Immunization Practices, 1999). Overall, the odds ratio for intussusception was estimated to be 1·6-1·8 for Rotashield recipients compared with never-vaccinated infants (Advisory Committee on Immunization Practices, 1999). This estimate of relative risk was converted into estimates of population attributable risk, which ranged from one excess intussusception

event in 2500 to one in 5000 Rotashield-vaccinated infants(Advisory Committee on Immunization Practices, 1999) Another projection was that if a national programme of vaccination with Rotashield had been fully implemented, up to 1600 excess infant intussusception events per year would have occurred ((Advisory Committee on Immunization Practices, 1999).

On the basis of this information, the Advisory Committee on Immunization Practices (ACIP) withdrew its recommendation for routine use of Rotashield, effectively ending the vaccine's availability after 9·5 months of use. Hence the phase one clinical trials of rotavirus vaccines never got far and have never even been tested in many parts of the developing world where rotavirus diarrhoea is a major debilitating disease.

As of March 1, 2001, Rotashield remains licensed but unavailable, despite benefit/risk ratios presumed to weigh heavily in favour of rotavirus vaccination in developing countries with a large burden of rotavirus diarrhoea mortality (Gay *et al.*, 1999; WHO, 2000; Emmanuel *et al.*, 2000; Melton, 2000; Weijer, 2000; Levin, 2000). Safety concerns in the USA have created a climate in which the sole manufacturer is currently not producing Rotashield (Simonsen *et al.*, 2001). In assessing the benefit/risk ratio of Rotashield, policy-makers should, in addition to the diarrhoeal disease burden prevented, consider the fact that some studies found that the association with intussusception in the immediate post-vaccination weeks7-9 have not led to an increase in intussusception admissions during the Rotashield use period in the USA (Simonsen *et al.*, 2001). Ethical considerations about Rotashield use should include the different perceptions of intussusception events directly linked in time to vaccine administration, versus intussusception events that may have been precipitated by the vaccine and thus prevented from occurring later in infancy (Simonsen *et al.*, 2001). Although the former cases are easily identified as adverse Rotashield events, the latter are not appreciated as a benefit of vaccination (Simonsen *et al.*, 2001).

5.2 ADENOVIRUS

In this study 2.9% (only 6 out of 207 samples tested) of the samples tested positive for adenoviruses. The incidence in HIV infected children with diarrhoea was 2.3% while those without diarrhoea was 1.4%. These incidence rates are low compared to other similar studies. Brandt *et al.*, 1985, reported adenovirus in 8.6% of paediatric inpatients (900) with diarrhoea as visualised by electron microscopy of throat and anal swabs. At rural health centres in India 3.8% of children with diarrhoea and 2.5% of children without diarrhoea tested positive for adenovirus (Bhan *et al.*, 1988).

Adenoviruses, known to be etiological agents of diarrhoea in children, were not detected in a study by Liste *et al.*, (2000) and were not associated with disease in another study by Fontana *et al.,* (1996). In contrast, in HIV-infected adults, rotavirus and adenovirus infections have been associated with diarrhoea in certain epidemiological settings (Albretch *et al.*, 1993; Laughon *et al.*, 1989).

There was no significant difference between the prevalence of adenovirus in HIV infected and HIV uninfected children with diarrhoea ($p = 0.3669$). Also the children with HIV and diarrhoea and those without HIV but with diarrhoea showed no significant difference in prevalence ($p = 0.2929$). The prevalence of adenovirus, like rotavirus in this case is not determined by the HIV status of the children.

It is necessary to carry out further studies using several other ELISA kits since the particular kit used in this study may not be efficient. Monoclonal antibody ELISA would also assist in confirming the results obtained here. To be even more specific, PCR could be done. However it is not cost effective to run PCR on 207 samples. It is more pertinent to run a screening test such as an antigen ELISA.

5.3 ASTROVIRUS

Surveys of the incidence of hospitalisation due to astrovirus-induced severe gastro-enteritis in developed countries have reported rates of 2 to 3% (Carter and Wilcocks, 1996, Mustafa *et al.*, 1999). Astroviruses have been described previously as the most common viral agent associated with diarrhoea in immunosuppressed adults (Cox *et al.*, 1994; Grohmann *et al.*, 1993), but the prevalence of this group of viruses in HIV-infected children has not previously been determined. Astroviruses were detected in HIV-infected children (3) and in uninfected children (9). Two of the HIV infected children with astrovirus had no diarrhoea. Frequent asymptomatic astrovirus infections have been previously observed in healthy children (Glass *et al.*, 1996).

Our results show that the overall prevalence is 5.8%. This is similar to prevalence and incidences reported in other studies involving developing countries. In Mexico, human astroviruses were detected in 6.3% of stool samples (23 out of 365) from children with diarrhoea less than 18 months old (Walter *et al.*, 2001). In a study in Japan 5.9% (82 out of 1,382 samples) were detected with astrovirus (Sakamoto *et al.*, 2000). A study by López *et al.*, 2000 found astrovirus in 15 (5.5%) patients out of 270 tested. Studies in Chile show prevalence of up to 20% (Gaggero *et al.*, 1998). This suggests that the burden of astrovirus disease may be greater in developing countries.

A comparison between HIV infected children with diarrhoea (2.3%) and those without diarrhoea (2.9%) showed no significant difference in prevalence. However in the group of children with diarrhoea whose HIV status was unknown, the prevalence was 9.6%, which is relatively high. However, statistically, this difference was still not significant (p = 0.0636). One of the reasons that could explain this is that most of the children seen in the city council clinics are from communities that have very low incomes and hence they live in an environment where hygiene and exposure may be higher. The population density in these

areas is much higher. In contrast some of the children at Nyumbani children's hospice live in a clean environment where exposure would be less. However a group included from the extended care programme were also from high-density areas. These results require further investigation to determine the HIV status of these children. This was not possible at the time of the study because consent was not granted for this purpose.

This study has established that astrovirus is an important cause of diarrhoea in children in Nairobi. However, further studies are required to determine the circulating serotypes and also to establish whether there is a seasonal variability in infection rates. Currently there is no clearly defined seasonality.

5.4 CONCLUSIONS

This study has attempted to evaluate the common viral causes of diarrhoea and to further characterise rotavirus strains found in Kenya. Astroviruses were found in association with gastro-enteritis more prevalently than adenovirus and with almost one-third the incidence of rotavirus. It seems then that in this study rotavirus is the most prevalent cause of diarrhoea (14%) followed by astrovirus (5.8%) and finally adenovirus (2.9%). The study has been able to show that rotavirus, astrovirus and adenovirus are important causes of diarrhoea in children.

In comparing the two groups of children with diarrhoea, namely the HIV infected (43) and those with unknown HIV status (94) we see that the prevalence of rotavirus, adenovirus and astrovirus in these groups as follows:

23.2%, 2.3% and 2.3% (HIV infected children with diarrhoea) and 18.1%, 4.3% and 9.6% (children with unknown status) respectively. It has been shown that statistically these prevalence rates are not different from each other. This shows that the prevalence of viral infection is not modified by HIV infection. It has also shown that adenovirus and astrovirus

are not significantly associated with diarrhoea in HIV infected children. Rotavirus on the other hand is significantly associated with diarrhoea in HIV infected children.

It is also evident that analysis of VP7 and VP4 rotavirus structural proteins is important for evaluating candidate vaccines, which may not be similar for different geographic regions.

Looking at our initial objectives we have therefore been able to determine the prevalence of the three enteric viruses in HIV infected children and found no significant difference in relation to HIV negative children. Understanding the causes of diarrhoea in HIV-infected children may permit the development of interventions to improve their quality of life.

5.5 FUTURE DIRECTION

It has emerged from these studies that constant monitoring and accurate diagnosis of viral diarrhoea in children is of utmost importance. Furthermore, characterisation and typing of adenovirus and astrovirus serotypes will be necessary in order to have more accurate information on these two viruses. In the case of rotavirus, nucleotide sequencing will be required of the isolated strains in order to compare these with strains representing other G serotypes and P genotypes.

Future studies should also involve the clear cut difference between HIV positive and HIV negative groups of children. The latter group is not always easily accessible because it requires the active testing of children visiting clinics for treatment for diarrhoea. Consent to test for HIV and enrol children in research studies are not easily given because such studies are usually treated with suspicion. Also random testing for HIV in a large number of children is costly.

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APPENDIX I

ABBREVIATIONS

Ad	Adenovirus
AIDS	Acquired Immunodeficiency Virus
APS	Ammonium persulphate
ARV	AIDS-related virus
As	Astrovirus
ATP	Adenosine Triphosphate
Bp	Base pairs
CBC	Complete blood count
CD	Cluster Differentiation antigen
CDC	Centers for Disease Control
cDNA	copy DNA
CTP	Cytosine triphosphate
DH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
GTC	Guanidine thiocyanate
GTP	Guanine triphosphate
H_2O	Water
H2SO4	Sulphuric acid

HIV	Human Immunodeficiency Virus
HRP	Horse-radish peroxidase
IgG	Immunoglobulin G
LAV	Lymphadenopathy-associated virus
Mab	Monoclonal Antibody
$MgCl_2$	Magnesium Chloride
Ml	Millilitres
NaAc	Sodium acetate
NaOH	Sodium Hydroxide
Nm	Nanometres
OD	Optical density
ORF	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS/T	PBS/ Tween
PCR	Polymerase chain reaction
PTA	Phosphotungstic Acid
RV	Rotavirus
RNA	Ribonucleic acid
RNAid	Ribonucleic acid buffer
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SM	Skimmed milk
T Cell	T Lymphocyte

TTP	Thiamine triphosphate
µl	Microlitres
+Ve/ -Ve	Positive/Negative
VP4/6/7/8	Viral Protein 4/6/7/9
WHO	World Health Organisation

APPENDIX II

RNA EXTRACTION FROM FAECES

500 µl of a 10-20% faecal suspension was aliquoted into an 1.5 ml eppendorf tube and subsequently 50 µl IM NaAc (pH 5.0) containing 1% SDS (before use, incubated NaAc at 37°C to prevent SDS precipitating out) was added. This was incubated at 37°C for 15 minutes in a water bath and then 500µl phenol/chloroform mixture added into the eppendorf and vortexed for 1 minute. This was followed by incubation at 56°C for 15 minutes in a water bath. After another one minute of vortexing the mixture, it was centrifuged for 2-3 minutes at 12,000rpm.

Once centrifuged, the upper aqueous phase (containing the dsRNA) was removed into a new tube. The phenol extraction was repeated if necessary using half the volume of phenol/chloroform mixture (250 µl). Carefully the upper aqueous phase was removed taking care to avoid any interface material. Into this was added 1/10 volume of 3M NaAc (40 µl) and 2 volumes of cold absolute ethanol (1ml). Double stranded RNA was precipitated at -70°C for 20-30 minutes or at -20°C overnight. The samples were centrifuged (microfuge) at 4°C for 10 minutes at 12,000rpm. The supernatant was poured off and the pellet dried under vacuum. The dry pellet contained the dsRNA. This was re-suspended in 1xTE or de-ionised water (10 µl) and stored at 4°Cor -20°C.

APPENDIX III

POLYACRYLAMIDE GEL ELECTROPHORESIS

A. Reagents

1. 30% Acrylamide Stock

30g Acrylamide + 0.8g NN' methylene bis-acrylamide. Dissolve in 50ml dH_2O and make up to 100ml. Filter before use. **Caution!! Acrylamide is a potent neurotoxin and is absorbed through the skin. Always wear gloves.**

2. Resolving gel Buffer (pH 8.9)

1N HCL	48ml
Tris	36.3g

Dissolve and make up to 100ml with dH_2O. pH 8.9

3. Spacer Gel Buffer (pH 6.7)

Dissolve 5.98g Tris and adjust pH to 6.7 with 1N HCl. Make up to 100ml with dH_2O.

4. 1N HCl

Add 86ml conc. HCL to 910ml dH_2O.

5. 10% (w/v) Ammonium persulphate

0.1g APS in 1ml dH_2O. Store at 4C for up to 3 days.

NB!! Prepare ONLY the amount you need.

6. 5 x Tris-Glycine Running Buffer

25mM Tris Base	15.1g
250mM Glycine	94g

Dissolve and make up to 1000ml with dH_2O.

NB: 1 x running buffer is used for running PAGEs. To make it, dilute 200ml of the 5 x running buffer in 800ml dH_2O.

B. **Gels**

10% Resolving Gel

	1.5 Gel		0.75 gel	
	1x	2x	1x	2x
dH_2O	15.8ml	31.6ml	9.9ml	19.8ml
30% Acrylamide Stock	10.0ml	20.0ml	6.3ml	12.5ml
Resolving Buffer (pH 8.9)	3.75ml	7.5ml	2.4ml	4.8ml
TEMED	15µl	30µl	10µl	19µl
10% Ammonium Persulphate	450µl	900µl	282µl	564µl

3% Spacer Gel

	1.5 Gel		0.75 gel	
	1x	2x	1x	2x
dH_2O	6.8ml	13.6ml	5.1ml	10.2ml
30% Acrylamide Stock	1.6ml	3.2ml	1.2ml	2.4ml
Spacer Buffer (pH 6.7)	1.25ml	2.5ml	0.9ml	1.9ml
TEMED	5µl	10µl	4µl	8µl
10% Ammonium Persulphate	150µl	300µl	112µl	225µl

Note: **Thick spacers use the recipe for 1.5 gels**

Thin spacers use the recipe for 0.75 gels

APPENDIX IV

SILVER STAINING (For SDS-PAGE Gels)

1. Remove gel from glass plates (**NB!!!** Cut off corner for orientation). Soak gel in 200ml of 40% EtOH, 5% acetic acid in dH_2O. Shake gently for 30 minutes on an orbital shaker.
2. Drain off fixing solution. Replace with 200ml of 10% EtOH, 0.5% acetic acid in dH_2O. Shake for 30 minutes.
3. Drain off 2nd fixing solution and add 11mM silver nitrate ($AgNO_3$), i.e. 0.37g $AgNO_3$ in 200ml dH_2O). Only make up $AgNO_3$ just before use and be careful when weighing it out since it stains hands and surfaces. Shake for 30 minutes.
4. Rinse gel twice for 2 minutes in dH_2O. **NB!! Rinsing time is very important.**
5. Prepare developing solution (7.5g NaOH dissolved in 250ml dH_2O, add 2ml of 36% formaldehyde solution).
6. Add ± 50ml of developing solution and shake for 30 seconds (the solution turns dark brown). Drain off the 50ml and add the remaining 200ml of developer. After 1-2 minutes the gel background turns yellow. After approximately 5 minutes the bands have turned from pale brown to dark brown or black.
7. Drain off the developer and add 5% acetic acid for 1 to 2 minutes to stop the reaction. Drain off the stopping solution and replace with a storage solution of 0.5% acetic acid and 5-10% EtOH. Shake in storage solution for 5 minutes.
8. Drain of storage solution and replace with 200ml dH_2O then shake for 5 minutes.
9. To keep the gel, dry on the gel dryer.
10. De-staining of gels: Use 5% methanol, and 0.5% acetic acid. Leave shaking in solution for 60 minutes.

Silver Staining (Continued)

Solutions:

1 Gel	**2 Gels**
1. 80ml EtOH	160ml EtOH
110ml dH_2O	220ml dH_2O
10ml Acetic Acid	20ml Acetic Acid
2. 20ml EtOH	40ml EtOH
180ml dH_2O	360ml dH_2O
1ml Acetic Acid	2ml Acetic Acid
3. 0.37g $AgNO_3$	0.74g $AgNO_3$
200ml dH_2O	400ml dH_2O
4. Rinse 2X2 minutes in dH_2O	Rinse 2X2 minutes in dH_2O
5. 7.5g NaOH	15g NaOH
250ml dH_2O	500ml dH_2O
2ml 36% Formaldehyde	4ml 36% Formaldehyde
6. 10ml Acetic Acid	20ml Acetic Acid
200ml dH_2O	400ml dH_2O
Stop Solution!	**Stop Solution!**
7. 1ml Acetic Acid	2ml Acetic Acid
20ml EtOH	40ml EtOH
180ml dH_2O	360ml dH_2O

APPENDIX V

ROTAVIRUS VP6 SUBGROUP ELISA

Microtitre (96-well Nunclon® flat bottom) plates were coated with 100µl of a 1:5000 dilution of anti-rotavirus rabbit serum (#720) in a carbonate/bicarbonate buffer (pH 9.6). These were incubated overnight at 4°C. The plates were then washed 4-6 times in Phosphate buffered saline/Tween 20 (PBS/T), drained of fluid and 100µl of PBS/T/EDTA is dispensed into the wells. Fifty microlitres (50µl) of 10% faecal extract and the control antigens were added into the appropriate wells and the plates were incubated overnight at 4°C. The plates were once again washed in PBS/T at least 4-6 times and drained before 100µl of a 1:5000 dilution of the monoclonal antibodies (to the group antigen and the group I and II antigens) was added into the appropriate wells. The plates were then incubated for 3 hours at 37°C. Once the incubation was over 100µl of a 1:1000 dilution of anti-mouse IgG alkaline phosphatase/HRP conjugate was dispensed into each well and the plates were incubated for 2 hours at 37°C. The plates were then washed in PBS/T 4 times before 100µl of TMB substrate was added into each well and the plates were again incubated for a further 10-15 minutes at 37°C. The reaction was then stopped using stop solution from the IDEIA™ rotavirus kit from DAKO. The optical density (OD) was determined spectrophotometrically at 450nm wavelength.

Interpretation of the Rotavirus VP6 Subgroup ELISA absorbances

Rotavirus Group A Antigen	OD > 1.0
Subgroup I	OD SGI:SGII > 1.7
Subgroup II	OD SGII:SGI > 2.0

Reagents for VP6 Subgroup ELISA

1. **Carbonate/bicarbonate Buffer (0.05M)**

Na_2CO_3 0.3975g
NaH CO_3 0.7325g
Dissolve in dH_2O and adjust pH to 9.6. Make up the solution to 250ml with dH_2O.
Store at 4°C. STable for 14 days.

2. **Phosphate Buffered Saline/Tween (pH 7.2) (PBS/T)**

NaCl 20.20g
KH_2PO4 0.20g
$Na_2HPO_4.2H_2O$ 1.15ml
Tween 20 0.5ml
Dissolve in dH_2O and adjust the pH to 7.2. Make up the solution to 1 litre.
Store at 4°C. STable for two months.

3. **Phosphate Buffered Saline /Tween/ EDTA (pH 7.2) (PBS/T/EDTA)**

EDTA 1.86g
Tween 20 0.5ml
PBS 500ml
Adjust the pH to 7.2 with 3M NaOH. Make up the solution to 1 litre.
Store at 4°C. STable for two months.

4. **Phosphate Buffered Saline/Tween/ Bovine Serum Albumen (pH 7.2) (PBS/T/BSA)**

BSA 5g
PBS/T 1litre
Adjust pH to 7.2
Store at –20°C

5. **3M NaOH**

12g NaOH pellets in 100ml dH_2O.

ROTAVIRUS VP6 SUBGROUP ELISA SAMPLE TEMPLATE

Date: _____

Assay: **VP6 SUBGROUP ELISA**

Samples: _____

GrpA SGI SGII GrpA SGI SGII GrpA SGI SGII GrpA SGI SGII

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Sample Dilution: _____

Monoclonal Antibody Dilution: _____

Conjugate Dilution: _____

	Incubation time Temperature	Time in	Time out	
1				
2				
3				
4				

Notes:

APPENDIX VI

ROTAVIRUS VP7 MONOCLONAL ANTIBODY ELISA

The monoclonal antibodies all as ascites fluids were used at 1:5000 dilution in PBS/T/S milk buffer. The conjugate was horseradish peroxidase (HRP) conjugated goat anti-mouse IgG used at a 1:5000 dilution in PBS/T/S milk buffer.

60µl of each monoclonal antibody (Mab) diluted appropriately in PBS (pH 7.2) was added into each well of a 96-well microtitre plate. This was then incubated overnight at 4°C. The plates were then washed six times with PBS/T. Following this 200µl of 2.5% SM/PBS was added into each well and the plates again incubated at 37°C for 2 hours. Following the incubation the SM/PBS was decanted out and 100µl of a 20% stool suspension and 100µl of 2% SM/PBS were added into the assigned well and the plates incubated at 4°C overnight. Thereafter the plates were washed six times in PBS/T and 50µl of anti-hyperimmune serum diluted at 1:10,000 with 1% SM/PBS was added into each well and the plates incubated at 37°C for 1 hour. After washing 6 times in PBS/T, 50µl of HRP-conjugated goat anti-rabbit IgG diluted at 1:2000 with 1% SM/PBS was added into each well and the plates incubated at 37°C for 1 hour. After the incubation the plates were washed 6 times in PBS/T and 50µl of TMB substrate was added into each well and the plates incubated for 10 minutes. The reaction was then stopped by the addition of 25µl of 2N H_2SO_4. The results were then read spectrophotometrically at 450nm wavelength (See Appendix VI).

Antiserum Used:

Capture antibody:	Rabbit hyperimmune sera (1:5000 dilution)	
Monoclonal antibodies:	Serotype 1 (G1)	KU-4
	Serotype 2 (G2)	S2-2G10
	Serotype 3(G3)	YO-1E2
	Serotype 4 (G4)	ST-2G7

The monoclonal antibodies all as ascites fluids used at 1:5000 dilutions in PBS/T/S milk buffer.

Conjugate: Horseradish peroxidase (HRP) conjugated goat anti mouse IgG used at 1:5000 dilutions in PBS/T/S milk buffer.

Reagents

1. **Carbonate/bicarbonate coating Buffer (0.05M)**

Na_2CO_3 0.3975g
$NaHCO_3$ 0.7325g
Dissolve in dH_2O and adjust the pH to 9.6. Make up the solution to 250ml with dH_2O.
Store at 4°C. STable for 14 days.

2. **PBS/Tween Wash Buffer**

Phosphate buffered saline (PBS) (pH 7.4)
0.05% Tween 20 (v/v)

3. **Stool Extraction Buffer** (0.1M Tris, 0.1% Tween, 1% BSA)

0.1M Tris-HCl (pH 7.4)
Dissolve 12.1g Tris in 100ml water (10 X stock)
pH using HCl Dilute 1:10 to use
0.1% Tween 20 (v/v)
1% Bovine serum albumen (fraction V) (w/v)

4. **Antibody Buffer** (PBS/T/S milk)

Phosphate Buffered Saline (pH 7.4)
0.1% Tween 20(v/v)
2.5% Skim milk powder (w/v)

5. **Substrate Buffer** (Acetate/citrate buffer, pH 5.5)

0.1M Sodium Acetate (NaAc):
Dissolve 6.8g NaAc in 500ml water. Adjust pH using a citric acid solution

6. **Citric Acid Solution**

2.1g citric acid dissolved in 10ml dH_2O

7. **Substrate Solution** (TMB)

50 X Stock solution:
Dissolve 5mg of TMB in 1 ml of DMSO
Keep in the dark and store in aliquots at 2-8°C or at –18 to –20°C

Working strength:
Dilute 0.5ml of 50 X TMB in 25ml of acetate/citrate buffer
Add 8υl of H_2O_2. **Prepared immediately before use.**

Volume of TMB (ml)	Volume Acetate/citrate Buffer (ml)	Volume H_2O_2 (υl)
0.5	25	8
0.6	30	9.5
0.7	35	11
0.8	40	13
0.9	45	14.5

ROTAVIRUS VP7 MONOCLONAL ANTIBODY ELISA SAMPLE TEMPLATE
Date: _____

Assay: **VP7 MONOCLONAL ANTIBODY ELISA**

Samples: _____

S1 S2 S3 S4 S5 S6 S7 S8 S9 S10 S11

		1	2	3	4	5	6	7	8	9	10	11	12
G1 KU4	A												B
G1 5E8	B												L
G2 S2-SG10	C												A
G2 IC10	D												N
G3 YO-1E2	E												K
G3 159	F												R
G4 ST-2G7	G												O
Mab 60	H												W

S Sample
G1 etc, Serotype

Sample Dilution: _____

Monoclonal Antibody Dilution: _____

Conjugate Dilution: _____

	Incubation time Temperature	Time in	Time out	
1				
2				
3				
4				

Notes:

APPENDIX VII

POLYMERASE CHAIN REACTION

i) **RT-PCR Amplification of Rotavirus dsRNA** **Date:**

Starting from dsRNA: _____

DsRNA Samples used: _____

Extraction Method used: _____

A. Denature dsRNA

_____ µl dsRNA

_____ µl Primer a _____

_____ µl Primer b _____

_____ µl dH_2O

6-10 µl TOTAL

Boil for 5 minutes. Chill immediately in an ice bath.

B. **Reverse Transcribe** – add 3.2 µl of master mix (MM)/tube

1 x Reaction _____ x Reactions

0.2µl 10mM dATP_____ µl dATP

0.2µl 10mM dCTP _____ µl dCTP

0.2µl 10mM dGTP _____ µl dGTP

0.2µl 10mM dTTP _____ µl dTTP

0.4µl Rtase (AMV) _____ µl Rtase _____

2.0µl 5 x AMV Buffer_____ µl 5 x Buffer _____

Note: Check manufacturers' concentration of AMV.e.g. Promega 0.4µl AMV is required whereas for Boehringer Mannheim only 0.2µl AMV is needed. Incubate for 26-30 minutes in a 42°C waterbath

C. PCR Amplification of cDNA after the addition of 40µl MM

1 x Reaction _____ x Reactions

1µl 10mM dATP _____ µl dATP

1µl 10mM dCTP _____ µl dCTP

1µl 10mM dGTP _____ µl dGTP

1µl 10mM dTTP _____ µl dTTP

0.3µl Taq Polymerase _____ µl Taq Polymerase

4µl 10 x Taq Buffer _____ µl 10 x Taq Buffer

2.4µl 25mM $MgCl_2$ _____ µl 1.5mM $MgCl_2$

30µl dH_2O _____ µl dH_2O

40µl TOTAL _____ µl TOTAL

D. Amplification

File number: _____

Cycles: _____

Temperatures: _____

ii) Re-amplification of cDNA

CDNA used: _____

Primers: _____

		1 x Reaction	_____ x Reaction
40µl	or	90µl Total	_____ Total
1µl		2µl cDNA	_____ cDNAµl
1µl		2µl Primer a	_____ µl Primer a
1µl		2µl 10mM dATP	_____ µl dATP
1µl		2µl 10mM dCTP	_____ µl dCTP
1µl		2µl 10mM dGTP	_____ µl dGTP
1µl		2µl 10mM dTTP	_____ µl dTTP
0.3µl		0.5µl Taq Polymerase	_____ µl Taq Polymerase
4µl		10µl 10 x Taq Buffer	_____ µl 10 x Taq Buffer
2.4µl		5.4µl 25mM $MgCl_2$	_____ µl 25mM $MgCl_2$
30µl		70µl dH_2O	_____ µl dH_2O

File: _____

Cycles: _____

Temperatures: _____

_____

Note: Standard Rotavirus PCR File

Cycles are 30-35.

Temperatures are 94°C for 1 minute, 42°C for 2minutes, 72°C for 3 minutes.

REAGENTS (PCR Continued)

1. 1.5% Agarose gel (20ml)

0.3g Agarose in 20ml 1 x TAE buffer (pH 7.9). Heat to dissolve then check volume and make up to 20ml with dH_2O if less. After solution has cooled, add 2µl ethidium bromide (Stock concentration – 5mg/ml).

2. Ethidium Bromide

Caution! Ethidium bromide (EtBr) is a powerful mutagen and is moderately toxic. Wear gloves when working with solutions containing this dye. To prepare, add 10mg of EtBr to 1ml dH_2O. OR dissolve 1 x 100mg EtBr Tablet in 10ml dH_2O. Store solution in a dark bottle because it is light sensitive.

3. a) 20 x TAE (pH 7.9)

	500ml	1000ml
0.4M Tris	24.22g	48.44g
0.05M NaCl	3.40g	6.81g
0.01M EDTA	1.861g	3.72g

Dissolve in dH_2O and adjust pH to 7.9 with glacial acetic acid. Make up the solution to the final volume.

b) 1 x TAE (pH 7.9)

To make up 1 litre: 50ml of 20 x TAE is added to 950ml dH_2O

4. Bromophenol Blue tracking Dye

Make a 0.1% bromophenol blue (BFB) stock solution in dH_2O. If it does not dissolve in water add 0.1% NaCl. Add 1ml BFB stock solution to 9ml of 40% sucrose made up in 1 x TE Filter the final solution.

5. Molecular Weight Markers (Boehringer Mannheim)

VIII 19-1114bp Mixture of pUCBM21 DNA, cleaved with Hpa II and pUCBM21 cleaved with Dra I and Hind III.
IX 72-1353bp X174 DNA cleaved with Hae III.
XIV 100-1500bp Prepared by restriction digests of a specifically constructed plasmid.

1.2% Agarose gels are electrophoresed at 80-100V.

APPENDIX VIII

VP7 GENOTYPING OF ROTAVIRUS dsRNA BY RT-PCR

PCR Protocol:

Step 1: RT-PCR of gene 7, 8, or 9 using two outer VP7 primers: sBeg9/Beg9 and End9. (30 cycles: 94C 1 minute, 42C 2 minutes, 72C 3 minutes).

Step 2: Typing of human rotavirus (Gouvea *et al.*, 1990). Re-amplification of gene 9 cDNA using cocktail of primers: RVG9, aAT8, aBT1,aCT2, aDT4, aET3 and aFT9. (30 cycles: 94C 1 minute, 42C 2 minutes, 72C 3 minutes)

PCR Typing Results:

Beg9/sBeg9-End9 product:	1062bp	
Human Rotavirus VP7 genotypes:	RVG + VG	EndA + VG
VP7 genotype G1:	749bp	630bp
VP7 genotype G2:	652bp	530bp
VP7 genotype G3:	374bp	250bp
VP7 genotype G4:	583bp	460bp
VP7 genotype G8:	885bp	765bp
VP7 genotype G9:	306bp	186

Oligonucleotide primers for PCR amplification

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
Beg 9	GGCTTTAAAAGAGAATTTCCGTCTGG	1-28	Wa (1)
SBeg9	GGCTTTAAAAGAGAATTTC	1-21	Wa (1)
End9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	SA11 (3)
RVG9	GGTCACATCATACAATTCT	1062-1044	SA11 (3)
Human Rotavirus Typing (Gouvea *et al.*, 1990)			
aAT8	GTCACACCATTTGTAAATTCG	178-198	69M (8)
aBT1	CAAGTACTCAAATCAATGATGG	314-335	Wa (1)
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	DS1 (2)
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	ST3 (4)
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	P (3)
aFT9	CTAGATGTAACTACAACTAC	757-776	WI61 (9)

APPENDIX IX

VP4 GENOTYPING OF ROTAVIRUS NUCLEIC ACID BY RT-PCR

PCR Protocol:

Step 1: RT-PCR of gene 4 using the two VP4 primers; con3 and con2.

(30 cycles: 94C 1 minute, 42C 2 minutes, 72C 3 minutes).

Step 2: VP4 typing of human rotavirus (Gentsch *et al.,* 1992). Re-amplification of gene 4 cDNA using a cocktail of primers: con3, 1T-1, 2T-1, 3T-1, 4T-1, and 5T-1. (30 cycles: 94C 1 minute, 42C 2 minutes, 72C 3 minutes).

PCR Typing Results:

Con3-con2 product: 876bp

Human Rotavirus VP4 genotypes:

P4 genotype P8: 345bp

VP4 genotype P4: 483bp

VP4 genotype P6: 267bp

VP genotype P9: 391bp

VP4 genotype P10: 594bp

Oligonucleotide primers for VP4 PCR typing

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
Con3	TGGCTTCGCCATTTTATAGACA	11-32	
Con2	ATTTCGGACCATTTATAACC	868-887	
Human Rotavirus Typing (Gentsch *et al.*,1992)			
1T-1	ACTTGGATAACGTGC	339-356	KU (P8)
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5 (P4)
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076 (P6)
4T-1	TGAGACATGCAATTGGAC	385-402	K8 (P9)
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M (P10)