DECLARATION

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

JAMES WYCLIFFE OLUOMO NYANGAO

SIGNATURE. ... DATE 3/9/2004

This thesis is submitted for examination with our approval as the university supervisors

Dr. F. W. MULI,
Department of Biochemistry
Kenyatta University.

SIGNATURE. DATE 3/9/2004

Dr. E. U. KENYA,
Department of Biochemistry
Kenyatta University.

SIGNATURE. DATE 3/09/04

PROF. A.D. STEELE,
Diarrheal Pathogens Research Unit,
Medical University of Southern Africa (MEDUNSA)
Pretoria, South Africa.

SIGNATURE. DATE 03/09/2004
DEDICATION

This thesis is dedicated to my father Absalom Nyangao and mother Janet Amwayi, whose tenderly love coupled with discipline made me what I am today.

I would like to honour my graduate supervisors for their untiring support and guidance in shaping this thesis as well as for their advice while I was working on my Biochemistry B.Com degree at Kenyatta University. Under the guidance of the Department, Kenyatta University, with kind support, have been facing us and encouraging us and made it possible.

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<tr>
<td>AIDS</td>
<td>Acquired immuno deficiency syndrome</td>
</tr>
<tr>
<td>ADRV</td>
<td>Adult diarrhea rotavirus</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CVR</td>
<td>Centre for Virus Research</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EM</td>
<td>Electron Microscope</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rotavirus</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>MEDUNSA</td>
<td>Medical University of Southern Africa</td>
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<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NT</td>
<td>Non typable</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NaAc</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBS/T</td>
<td>Phosphate buffered saline/Tween 20</td>
</tr>
<tr>
<td>PBS/T/BSA</td>
<td>Phosphate buffered saline/Tween 20/Bovine serum albumin</td>
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RT-PCR - Reverse transcriptase – Polymerase chain reaction
RNA - Ribonucleic acid
RRV-TV - Rhesus rotavirus tetravalent vaccine
SDS-PAGE - Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
T - Tween 20
UV - Ultra violet
VP - Viral protein
ABSTRACT

Rotavirus is known to be the most common cause of severe infantile diarrhea worldwide. Severe rotavirus disease may be preventable by vaccination. This study focused on serological and molecular characterization of rotavirus positive strains from infants and young children presenting with gastroenteritis at three hospitals in Kenya. Two hundred and eighty five virus strains were examined for RNA genome diversity by a classical method, polyacrylamide gel electrophoresis of the double stranded RNA and visualized by silver staining. The rotavirus strains were further characterized by monoclonal antibodies directed to the inner capsid, VP6 subgroup antigen. The VP7 serotype was characterized by the use of a nested PCR method using a cocktail of primers specific to the six human VP7 serotypes (G1,G2,G3,G4,G8,G9). Furthermore, the VP4 genotype was characterized by the use of a nested PCR method using a cocktail of primers specific for the five human VP4 genotypes (P4, P6, P8, P9 & P10). It was possible to determine the electropherotypes in 214 (75%) of the samples. 155/214 (72.4%) showed the long pattern and 56 (26.2%) the short pattern. Additionally three samples (1.4%) were showing a possibility of mixed infections, of both long and short patterns. Five long electropherotype patterns and five short patterns were observed. Subgroup II viruses occurred more commonly than subgroup I viruses (69% vs 29%). It was also found that 2% of the samples were expressing both subgroup I and II antigens. It was shown that the “long” electropherotype is associated with subgroup II and the “short” electropherotype with subgroup I. Interestingly a single strain of long electropherotype had subgroup I antigens. It was demonstrated that VP7 serotype G1 predominated (22%), followed by
G2 (9%), then G8 (8%), and then G3 and G4 (1%) in each case. No G9 serotypes were identified. Furthermore 2% of strains with dual serotype (G1/G3 & G1/G2) were identified by the RT-PCR typing method. A large number of strains (57%) could not be typed to determine the serotype. The common VP4 genotypes P8 (22%) and P4 (14%) were identified, as well as the genotype P6 (9%), which has classically been associated with asymptomatic neonatal strains. Furthermore 2% of the strains were identified to have dual genotype (P8/P4 & P8/P6). A large number of strains (53%) could not be typed to determine the genotype. The combination of VP7 serotype and VP4 genotype demonstrated that G1P[8] strains predominated (52%), followed by G2P[4] (18%), then G8P[4] (12%) and G8P[6] (12%). The least identified were G1P[6] (3%) and G3P[8] (3%), in each case. This study has characterized the human rotavirus strains from Kenya by antigenic and genomic analysis. It has also contributed much needed epidemiological data of rotavirus infection in Africa. The results of this study show an emergence of serotype G8 as an important serotype in Kenya and suggesting that future human rotavirus vaccines will need to protect against disease caused by this serotype.
CHAPTER ONE

INTRODUCTION

1.1 Background

Rotaviruses are non-enveloped, icosahedral viruses of the family Reoviridae with eleven segments of double-stranded RNA (dsRNA), each encoding at least one structural or non-structural protein (Estes, 1996).

Since the first recognition of human rotavirus in Australia in 1973 (Bishop et al., 1973), human rotaviruses have been convincingly implicated as one of the most important agents associated with diarrheal diseases among infants and young children under five years of age throughout the world. Group A rotaviruses are firmly established as the most important etiological agents of dehydrating gastroenteritis in infants and young children worldwide (Hoshino et al., 1985).

Rotavirus serotypes are determined by neutralizing antibody responses to each of the two outer capsid proteins, VP7 (termed G serotype) and VP4 (termed P genotype). Ten G serotypes and 7 P genotypes have been identified in humans (Hoshino and Kapikian, 2000). Since serotype G1, G2, G3, and G4 together account for greater than 80% of global human rotavirus strains, some vaccines aim to provide serotype-specific protection against these four serotypes (Hoshino and Kapikian, 2000). However, in certain geographical settings, other G serotypes such as G5, G8, and G9 may be epidemiologically important (Cunliffe et al., 2001). VP4 genotypes, P[8] and P[4] are
recovered from children with symptomatic rotavirus infection while genotype P[6] is recovered from neonates with asymptomatic infection in hospital nurseries (Flores et al., 1986, Gorziglia et al., 1988, Gorziglia et al., 1986, Hoshino et al., 1985, Steele et al., 1992). In a study done in South Africa VP7 serotype G1 was identified most frequently, occurring in 51% of the rotavirus strains tested. VP7 serotypes G2 and G4 occurred in similar numbers, although their distribution varied regionally. Few serotype G3 strains and no G8 or G9 strains were identified. The P8 VP4 genotype occurred most frequently overall (66%), followed by P4 genotype (21.7%). The P6 genotype was identified in 28 asymptotically infected neonates and in 8 symptomatic infants. Few P9 strains were identified (Steele et al., 1995).

In a case-control study of 36 infants and children admitted to Kenyatta National Hospital in Kenya with acute gastroenteritis and an equal number of age and sex-matched controls, rotavirus was found to be the major aetiological agent. 39% of the 36 children had evidence of rotavirus infection as opposed to 2 (6%) in controls (Mutanda, 1980). In another study of human rotavirus strains from Kenya, children with gastroenteritis in an urban area (Nairobi) and three rural areas were characterized by antigenic and genomic analysis. While in all areas strains with subgroups II and G serotype 1 antigens were most common, two unusual strains were detected, one strain (G4, subgroup II) possessed an additional RNA band on electrophoresis and the other (G1, subgroup II) had a short RNA pattern (Gatheru et al., 1993).
The expected impact of rotavirus vaccines in reducing disease and death from rotavirus infection will be most evident in developing countries, where rotavirus causes up to 500,000 childhood deaths annually (Miller and McCann, 2000) and the development of vaccines against the infection would reduce the incidences drastically. In the United States, as in other industrialized countries, rotavirus is generally mild and rarely fatal (20-40 deaths per year), although it incurs substantial morbidity and costs (Glass et al., 1996). Around 50,000 children are hospitalized per year, representing 3-4% of all hospitalizations in children under five years of age. The economic impact exceeds $300 million in medical costs and $1 billion in total costs, including indirect costs such as the loss of salary of the care-giver (Smith et al., 1995).

1.2 Justification

In developing countries, rotavirus has consistently been identified as the most frequent cause of diarrheal illness in children under 5 years of age. In addition, it has been estimated that in a single year rotavirus infection may account for up to 680,000 deaths in the world. The bulk of these deaths are in the developing world. Very few rotavirus serotyping and genotyping studies have been undertaken in Africa and therefore, little is known of the rotavirus serotypes and genotypes circulating in Africa.

Before the possible introduction of vaccines in Africa it is important to describe and document the epidemiology and diversity of rotavirus. This is important for evaluating future rotavirus vaccines. This study which focuses on Kenyan children will contribute towards our knowledge of circulating rotavirus serotypes in Africa before the future implementation of a vaccination program against rotaviruses.
1.3 Objectives

1.3.1 General objective

To determine and characterize antigenically and genetically human rotaviruses causing gastroenteritis in children aged 5 years and below in Nairobi and Kisumu cities in Kenya.

1.3.2 Specific objectives

(i) To screen stool samples by ELISA to detect Rotavirus group A

(ii) To determine the rotavirus electropherotypes using electrophoretic techniques.

(iii) To determine the rotavirus VP6 subgroup using a solid-phase enzyme immunoassay.

(iv) To determine the rotavirus VP7 serotypes and VP4 genotypes using reverse transcriptase polymerase chain reaction (RT-PCR).
CHAPTER TWO

LITERATURE REVIEW

2.1 Rotavirus infections

There is a universal agreement that rotaviruses are the single most important aetiologic agent of severe diarrhoeal illness of infants and young children worldwide. Rotaviruses are estimated to cause over 800,000 deaths annually in children aged less than 5 years in developing countries, and are responsible for over 500,000 visits to a medical practitioner annually in the U.S.A. alone (Hoshino and Kapikian, 2000). Development of an effective vaccine against rotavirus would diminish the high infant mortality associated with the disease.

In Africa, rotavirus infection occurs at an early age and virus shedding is predominantly observed in infants under 12 months of age with over 90% of the rotavirus cases occurring in children by the age of 18 months (Cunliffe et al., 1998). Some of the factors, which may play a role in this early age of infection, include an increased environmental viral load, lack of sanitary facilities and poor nutritional status of the children. The infective dose is presumed to be 10-100 infectious viral particles. Because a person with rotavirus diarrhea often excretes large numbers of virus (10^8-10^{10} infectious particles/ml of feces), infection doses can be readily acquired through contaminated hands, objects, or utensils. Asymptomatic rotavirus excretion has been well documented and may play a role in perpetuating endemic disease (Bad bug book). Although rotavirus infection is observed throughout the year in tropical climates, rotavirus gastroenteritis shows a distinct seasonal
variation in temperate climates. In studies performed at various regions in Africa with
different climatic conditions, rotavirus infection was seen to occur all year round.
However, there was a pronounced increase in rotavirus shedding during the cooler and
drier months in each region. Seasonal patterns of disease was examined by plotting the
monthly detections of rotavirus and comparing them with the local wet and dry seasons.
In all countries except Ghana, rotavirus detection occurred throughout the year. Seasonal
peaks were commoner during dry seasons than wet seasons, but this pattern was not
consistent for every country. In northern Africa (Egypt and Morocco), rotavirus had a
single peak in the autumn and winter, but not during the dry season. In southern Africa
(South Africa, Madagascar, Zambia and Zimbabwe), rotavirus had a single peak in the
autumn and winter, which overlapped with the dry seasons. In sub-Saharan Africa
(Central African Republic, Ethiopia, Ghana, Kenya, Nigeria and Somalia), rotavirus
detections were 6 times more likely to have peaks during the dry season (Cunliffe et al.,
1998).

2.1.1 Clinical symptoms associated with rotavirus infection
Rotavirus infection causes a spectrum of disease syndromes, from asymptomatic
infection in newborn babies to severe dehydrating diarrhea in infants and young children
(Steele, 1998). 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 (Steele, 1998). Rotavirus is
spread by the faecal-oral route and by direct contact. Clinical studies have indicated that
the incubation period of rotavirus illness is less than 48 hours and usually will last from 5
to 7 days (Steele, 1998). Rotavirus gastroenteritis can vary from mild watery diarrhea lasting less than 24 hours to overwhelming and occasionally fatal gastroenteritis. Gastroenteritis is more severe in malnourished children. In patients with Acquired immuno deficiency syndrome (AIDS) severe clinical symptoms are not common, although they may have an increased and prolonged viral shedding (Cunliff et al., 2001). Group C rotaviruses have also been detected from an HIV-seropositive adult male with diarrhea of sudden onset in Brazil (Texeira et al., 1998). Vomiting is a prominent early symptom of infection, followed by a profuse, watery diarrhea. Dehydration is more strongly associated with rotavirus diarrhea than bacterial gastroenteritis and has been indicated as potentially fatal. Other symptoms which are associated with rotavirus infection are fever, irritability and lethargy (Rodrigues et al., 1987). The shedding of rotavirus by neonates has been well documented worldwide and has been observed in Africa (Oelofsen et al., 1985). Furthermore, the subclinical infection in the neonatal stage has been reported to offer protection against subsequent severe rotavirus infection for up to 3 years of age (Bishop et al., 1983). The main complication arising from rotavirus infections is that of severe dehydration with electrolyte disturbances, vascular collapse and death. Other complications include intussusception, aspiration of vomitus, necrotizing enterocolitis and neurological abnormalities (Smit, 1998). Rotavirus has also been associated with aseptic meningitis, acute myositis, hepatic abscess, pneumonia, Kawasaki disease and Crohn's disease (Offit et al., 2000).

2.1.2 Pathogenesis and treatment

Maintenance of adequate levels of intravascular volume is the mainstay of treatment. While mild to moderate degrees of dehydration can be managed at home with oral
rehydration solutions, severe dehydration requires hospitalization for intravenous fluid replacement. A gradual approach to refeeding with breast milk or diluted formula is encouraged especially in malnourished children. Rotaviruses infect intestinal enterocytes, and the early events in infection are mediated by virus-epithelial cell interactions. The most important pathological mechanism causing diarrhea is the destruction of enterocytes resulting in malabsorption. Infected cells are killed and detach from villi (Estes et al., 2001). The immature cells that remain are not capable of absorbing sufficient amount of water, salt and glucose. Diarrhea and dehydration result.

2.2 Rotavirus classification, structure and antigenic composition

The mature particle consists of a triple shelled capsid consisting of the outer, intermediate, and inner layers. The outer capsid consists of two proteins (VP4 and VP7), whereas the intermediate layer is formed by VP6 and the inner by VP2 which encloses two other proteins VP1 and VP3, as well as the viral genome consisting of 11 segments of double-stranded RNA, the latter encoding six structural and five non structural proteins (Kapikian et al., 1996; Merten and Chair, 1998; Estes et al., 1996). Because of the segmented nature of the rotavirus genome, genetic reassortment occurs at high frequency during infection. Rotavirus carries three important antigenic specificities: group, subgroup, and serotype. Based on group specificity which is conferred predominantly by VP6, rotavirus are divided into 7 groups (A to G). Human rotavirus (HRV) associated infections are predominantly caused by group A, and less commonly by group B or C, and thus, the emphasis of vaccine development has been targeted at group A associated disease. Of note is the finding that group B rotavirus (adult diarrhea rotavirus [ADRV])
which caused large outbreaks in China in the 1980s was recently (1997 and 1998) detected in patients with diarrhea in India, marking the emergence of group B ADRV outside China (Krishnan et al., 1999). Subgroup specificity, which is also determined by VP6, has been used for characterizing the antigenic properties of various rotavirus strains in epidemiologic surveys. Most HRVs belong to either subgroup I or subgroup II. Outer capsid proteins VP4 and VP7 specify rotavirus serotype specificities independently (Figure1)(Kapikian et al., 1996; Estes, 1996; Hoshino and Kapikian 1994).

2.2.1 The rotavirus genome

The rotavirus genome consists of 11 segments of double-stranded RNA which range in molecular weight from $2 \times 10^5$ to $2.2 \times 10^6$, with size range of approximately 660 to 3300 base pairs (Appendix 1). The RNA segments fall into four size classes based on contour length measured by EM and RNA migration profiles after electrophoresis through polyacrylamide gels. The genetic relatedness of rotavirus strains has been studied using radio-labelled, single stranded RNA transcripts which are hybridized to the ds RNA of other rotaviruses. This technique has demonstrated that rotaviruses recovered from different animal hosts are distinct. Moreover, the technique has shown that human rotaviruses with short or long RNA electropherotypes are distinct, and this gave rise to the concept of rotavirus genogroups or “families”. There are at least three human rotavirus genogroups represented by the prototype strains Wa, DS-1 and AU-1. Analysis of the gene coding assignments and rotavirus proteins of simian SA11 established that there are six structural and five non-structural proteins (Appendix 1). Rotavirus is a
triple-layered particle which consists of a core, made up of VP1, VP2 and VP3, encapsulating the RNA genome; an inner capsid of VP6, which bears the group and subgroup antigens; and an outer capsid which consists the VP7 protein enclosing the particle and 60 VP4 spikes which protrude through this layer (Figure 1).

**Figure 1.** Gene coding assignments and three-dimensional structure of rotavirus particles. (Estes 1996)

Fig.1 above shows double-stranded RNA segments separated on polyacrylamide gel (left) which codes for individual proteins, which are localized in the schematic digital reconstruction of virus particle (center) illustrating the outer capsid, inner VP6 shell and
the core (right), by image processing of electron micrographs of particles embedded in vitreous ice (Estes, 1996).

2.2.2 The group or common antigen of rotavirus

Early investigation into antigenic relationships suggested that all rotaviruses shared a common group antigen. This was believed to be situated within the inner capsid layer of rotavirus particles. Thus it was thought that any one of the viruses from any species could be used for the preparation of antigen in humans and animals (Woode et al, 1976). As investigations proceeded, viruses with rotavirus morphology, but without the common group antigen were found in humans and in animals (Nagesha et al, 1988; Pedley et al, 1983). Serological studies have also confirmed that there are viruses which carry group antigens (group epitopes) which are different from the original rotavirus group antigens. To date, rotaviruses have been divided into seven groups A-G based on this. Furthermore, 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). The group A rotaviruses have been most extensively studied. The group epitopes of this group of rotaviruses are contained on VP6 (Ramig, 1994).

2.2.3 The subgroup antigen

Subgroup antigens have been defined serologically on VP6 and have been used for antigenic classification of both human and animal rotaviruses that shared the common
group (group A) antigen (Hoshino and Kapikian., 1994). Monoclonal antibodies have been generated (Greenberg et al., 1983) and extensively used in epidemiological studies of both humans and animal rotaviruses (Steele and Alexander., (1988). Currently, four subgroup specificities are recognized (Hoshino and Kapikian., 1994). Subgroup I, found mostly in animals and certain human rotavirus strains, Subgroup II found mostly in humans and few porcine and lapine rotavirus strains, Subgroup I & II found rarely both in humans and animals, but have been reported in murine rotavirus strains, also those which are neither Subgroup I nor II which occurs rarely in humans and animals, but have been reported in avian rotavirus strains (Appendix 2).

2.2.4 The VP7 Protein

The VP7 protein is the major neutralizing antigen of rotavirus detected by hyperimmune serum and has been used for the basis for identification of serotypes. The VP7 protein is encoded by the 7th, 8th or 9th gene depending on the strain. The resulting protein has a molecular weight of 34,000 Da. VP7 forms the smooth external surface of the triple layered particle and contributes to 30% of the virion protein. It is the second most abundant rotavirus protein. VP7 is a glycoprotein that contains only N-linked high mannose oligosaccharide residues, which are processed by trimming in the endoplasmic reticulum (ER). The group A rotaviruses are classified into serotypes on the basis of the two outer capsid proteins, VP7 and VP4. Both VP7 and VP4 elicit the production of neutralizing antibodies. These antibodies have been shown to play an important role in protective immunity. A binary system to account for the serotypic specificities of both VP7 and VP4 was adopted. The VP4 genotype system utilises the prefix P (for protease
sensitive), whereas the prefix G (for glycoprotein) is used for the VP7 serotype specificity. To date, 14 different VP7 serotypes have been described among group A rotaviruses isolated from different species (Estes, 1996). Ten rotavirus serotypes have been identified in humans. From serotyping studies conducted in human populations, VP7 serotypes G1, G2, G3 and G4 are of epidemiological importance. Serotypes G8 and G9 have been identified far less in humans. However, in a recent small study in Libya, Kenya and Cuba, G9 was found to have emerged as an important serotype (Cunliffe et al., 2001). G12 serotype has also been recovered from humans (Wakunda et al., 2003). Recently the classical bovine serotypes, G6 and G10, were also recovered from young children. Unusual serotypes have also been reported from Brazil where G5 rotaviruses were found in infant specimens recovered from five regions. It has been postulated that these G5 strains may represent reassortants between humans and animal rotaviruses. In most studies to date, the VP7 serotype G1 has most frequently been identified, although other VP7 serotypes may predominate at certain times and in certain locations.

2.2.5 The VP4 Protein

The VP4 is present on the outer capsid as a series of 60 short spikes with a knob-like structure. VP4 has been studied because it is associated with a number of biological properties which include, haemagglutination (Kalica et al., 1983), protease-cleavage enhancement of viral infectivity (Estes et al., 1981), growth restriction in tissue culture (Greenberg et al., 1983), viral virulence (Offit et al., 1986), neutralisation antigen (Hoshino et al., 1985). The proteolytic cleavage of VP4 into VP5 and VP8 is associated with enhancement of viral infectivity. The VP4 induces the production of neutralizing
antibodies. In animal models such as the mouse and the piglet, these VP4 neutralising antibodies protect against experimental rotavirus illness (Offit et al., 1986). The VP4 has been implicated as a virulence determinant in both mice and piglets. The VP4 gene determined the differences in gastrointestinal virulence in a murine model (Offit et al., 1986) and monoclonal antibodies directed against the VP4, passively protected against diarrhea caused by the virus (Offit et al., 1986). VP4 is coded for by gene segment 4 (Liu et al., 1988) and is between 2362 and 2364 nucleotides in length, depending on the strain. Molecular studies, involving nucleotide and amino acid sequence analysis, have revealed six distinct human rotavirus VP4 genotypes derived from the following prototype strains: P8 (strain Wa), P4 (strain DS-1), P6 (strain M37), P9 (strain AU228), P10 (strain 69M) and P12 (strain PA169). Although no monoclonal antibodies are available for the serological determination of this range of VP4 antigens, genetic based analyses are possible. The VP4 gene has a hyperdivergent region which is highly variable between strains with different VP4 genotypes and highly conserved between strains of the same genotype. This region has been used successfully to differentiate between the VP4 genotypes by utilizing PCR-derived probes. The P8 and P4 genotypes have been detected in children with gastroenteritis (Flores et al., 1986; Gorziglia et al., 1986). The P8 genotype has been reported among strains with VP7 G1, G3 and G4 serotype, while the P4 genotype has been detected exclusively in strains with G2 VP7 serotype (Flores et al., 1986; Steele et al., 1993). Conversely, the P6 genotype has been detected in strains exhibiting G1-4 specificity but associated with asymptomatic infection in neonates (Flores et al., 1986; Gorziglia et al., 1988). Less commonly seen in nature are the P9, P10 and P12 genotypes.
2.3 Vaccines against rotavirus

It is generally agreed that a rotavirus vaccine is required in both developing and developed countries, because of the high burden of mortality and morbidity in young children worldwide. Rotaviruses are believed to be responsible for approximately 875,000 deaths in children less than five years of age each year. It has been estimated that the administration of an efficacious rotavirus vaccine could prevent 16% of all diarrhoeal deaths in children under 5 years of age (De Zoysa and Feachem, 1986). Development of new rotavirus vaccines necessitates the need for large, prelicensure, clinical trials to determine safety. Candidate vaccines currently in clinical trials include a bovine-human reassortant pentavalent vaccine and an attenuated human rotavirus monovalent vaccine. Important issues to be addressed include the acceptable, if any, degree of risk of developing intussusception and economic issues concerning the distribution of the vaccine in developing countries (Offit, 2002).

2.3.1 Early rotavirus vaccines

The most extensively studied strategy for rotavirus vaccination has been a “Jennerian” approach, in which an antigenically-related, live, animal rotavirus (bovine or simian) is used as the vaccine to induce protection against human rotavirus. It was determined early on, that human and animal rotaviruses shared a common group A antigen and that children developed a serological response against the animal rotavirus antigens. When it was recognized that bovine or simian rotaviruses did not cause significant illness in the humans and protected against illness with the virulent human rotavirus this approach was
embraced. Both initial vaccine candidates were extensively investigated and it was soon concluded that the vaccine candidates seemed to produce a homotypic serological response which did not protect against rotavirus infection of differing serotypes. This led to a modification of the vaccines.

2.3.2 Tetravalent rhesus rotavirus vaccine

In order to achieve a broader antigenic response, Dr Albert Kapikian and his team at the National Institute of Allergy and Infectious Diseases, Bethesda, U.S.A developed a vaccine based on single gene substitutions of the VP7 gene. This modified Jennerian approach used by the NIH research team have utilized a rhesus rotavirus strain (VP7 serotype G3), which is naturally attenuated for children, and through manipulation of the virus, reassorted the VP7 serotype genes from the other human rotavirus serotypes (G1, G2 and G4) into the rhesus rotavirus background. This has produced a tetravalent vaccine which is naturally attenuated for children but carries the four important VP7 serotype genes to produce a vaccine which can protect against all four human rotavirus serotypes (Kapikian et al., 1996). The RRV-TV has been tested on almost 18,000 children and has been shown to be safe and highly effective in developed countries such as the United States and Finland (Rennels et al., 1996; Joensuu et al., 1997). In the latest study, conducted in Venezuela, more than 2,200 infants were immunized with the higher dose of the vaccine and 88% of the babies were protected from a severe rotavirus infection (Perez-Schael et al., 1997). The vaccine does not block infection entirely, but is able to prevent the severe, life-threatening disease which results in hospitalization and can lead to death in young children. In August 1998, a tetravalent, rhesus-human reassortant
rotavirus vaccine was licensed in the United States. This vaccine consists of VP7-serotype antigens (G1-G4) representing the most common human rotaviruses. After inclusion of this vaccine in the immunization schedule for infants in the USA, and immunization of almost 1 million individuals, several cases of vaccine-associated intussusception were reported. The period of greatest risk of intussusception was shown to be 3-10 days after the first of three oral doses. Although the true overall incidence of this adverse event proved difficult to assess, a group of international experts suggested a consensus rate of 1 per 10,000 vaccinated infants. The pathogenic mechanisms involved are currently unknown (Canada communicable disease report, 2003). As a consequence of this rare but potentially dangerous adverse effect, the manufacturer withdrew the vaccine from the US market 9 months after its introduction. Although still licensed, the vaccine has not been tested since then or licensed in other parts of the world.

2.3.3 Different strategies for new rotavirus vaccines

A lamb rotavirus vaccine is licensed and used in China, but no rotavirus vaccines are currently available on the international market. Of the several candidate vaccines under development, only two have reached phase III trials. A pentavalent vaccine (WC-3) based on a bovine rotavirus strain reassorted with the common VP7 and VP4 genes of human rotaviruses has been well tolerated in phase II and III studies and provided good protection both against severe rotavirus disease and against any rotavirus disease. This vaccine is currently undergoing large-scale safety trials to exclude any potential association with serious adverse events such as intussusception (Clark et al 1996). A
monovalent human rotavirus vaccine candidate is now in phase II and phase III evaluation. It represents the most common of the human rotavirus VP7 and VP4 antigens. In early trials, this candidate vaccine showed high protective efficacy both against any rotavirus diarrhoea and against very severe rotavirus disease. Large-scale safety and efficacy trials are in progress in developing countries. Other vaccine candidates under development include a human neonatal rotavirus strain and two human-bovine reassortant vaccines. Subunit rotavirus vaccines are also being investigated (Canada communicable disease report, 2003).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study site involved 2 hospitals within Nairobi city and 1 hospital in Kisumu city. The samples were collected from Gertrude’s Garden Children Hospital, The Aga Khan Hospital, Nairobi and Kisumu District Hospital, Kisumu. Gertrude’s hospital is the only children hospital in Kenya and therefore served as a good catchment site. Aga Khan Hospital is also a busy hospital that attends to a big population of children. Samples from Kisumu city served in looking at rotavirus from a different geographical setting.

3.2 Study Population

Rotavirus isolates from children attending or previously admitted at the two hospitals in Nairobi and the hospital in Kisumu over a period between 1998 and 2002 were analysed. The isolates were retrieved from freezing storage.

3.2.1 Inclusion criteria

i) Samples analysed were those previously collected from children of 5 years and below presenting with diarrhea in the Paediatric clinic.
ii) Samples collected from children of 5 years and below admitted at the Paediatric ward of the hospital due to diarrhea.

3.2.2 Exclusion criteria

Samples collected from children above 5 years of age who presented at the Paediatric clinic or were admitted in the Paediatric ward due to other reasons other than diarrhea.

3.3 Sample size

Assuming a Rotavirus detection rate of 22.2% from stool samples (Nakata et al., 1999) and a level of significance of 5%, and applying the formula by Fisher et al., 1998:

\[
N = \frac{Z^2 \cdot a \cdot P(1-P)}{d^2}
\]

Where 
- \(N\) = minimum sample size required
- \(Z\) = 1.96 (standard errors from the mean)
- \(a\) = absolute precision set at 5%
- \(P\) = detection rate for rotavirus (0.222)
- \(d\) = degree of accuracy desired set at 0.05

Hence \(N = \frac{1.96^2 \cdot 0.05 \cdot 0.222 \cdot (1-0.222)}{0.05^2 \cdot 0.05} = 252\) samples

Therefore, a minimum of 252 stool samples were required for the study.
Two hundred and eighty five samples were examined for this study.

3.4 Methodology

3.4.1 Specimen collection

Stool specimen was collected from every child attending or admitted in the study hospitals presenting with diarrhea. The stool samples were collected in a clean stool container, then transferred to the Centre for Virus Research, Kenya Medical Research Institute. They were immediately kept in freezing storage at -20°C.

3.4.2 Processing of stool samples

The stool samples were removed from cold storage and allowed to thaw. Stool suspension was made by adding a pea-sized (Approximately 0.6 gms.) amount of faecal material from each sample to approximately 5 ml distilled water (12% suspension) and mixed well and allowed to settle. The supernatant was then used in all the procedures.

3.4.3 Detection of rotavirus in stool samples by ELISA

Detection of human rotaviruses in stool was done by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DAKOPATTS A/S, Copenhagen, Denmark) as described by Grauballe et al.,(1981).

Test wells were coated by diluting 110 µl of Rabbit immunoglobulins to rotavirus (human) in 5.5 ml of coating buffer and 100 µl of the dilution was then added into all
wells in columns numbered 1, 2, 5, 6, 9, and 10 on the 96 wells microtiter plate. Negative control wells coated by diluting 110 μl of Rabbit immunoglobulins negative for rotavirus (human) in 5.5 ml of coating buffer and 100 μl of the dilution was then added into all wells in columns numbered 3, 4, 7, 8, 11, and 12 on the microtiter plate. The plate was sealed and kept at 37°C for 1 hour or 4°C overnight. The plate was then washed 5 times with washing solution. Two hundred microlitres of dilution buffer was added into all wells, sealed and kept at 37°C for 1 hour or 4°C until examined. Coated plates should be used within 2 weeks after coating. Dilution buffer was then discarded and 50 μl of dilution buffer was added into all wells. Fifty microlitres of each sample or positive control or negative control was applied into 4 wells (2 test wells and 2 negative control wells) and sealed. The plate was kept at 37°C for 1 hour or 4°C overnight. Samples were discarded and the plate was washed 5 times. Blocking of non-specific reaction was done by diluting 440 μl of normal rabbit immunoglobulin fraction in 11 ml of dilution buffer and 100 μl of the dilution added into all wells. The plate was sealed and kept at 37°C for 30 minutes. Peroxidase conjugate was prepared by adding 44 μl of Peroxidase conjugated rabbit immunoglobulin to rotavirus (human) in 11 ml of dilution buffer and then 100 μl of the dilution was then added onto the blocking reagent of the wells. The plate was sealed and kept at 37°C for 30 minutes. The plate was washed 5 times with washing solution and once with 0.1M Citrate-Phosphate buffer. Fifteen microlitres of substrate solution with 5 μl of Hydrogen Peroxide was prepared just before use and then 100 μl of substrate was added into all wells and kept at room temperature for 15 minutes. One hundred microlitres of 1M H₂SO₄ was added into all wells to stop the reaction. The amount of yellow color caused by the reaction of the substrate and enzyme bound in the
above steps was measured in a spectrophotometer which was capable of determining absorbance directly in microtitre plates at 492nm wavelength. The criteria for a positive test was that the absorbance of the test well minus the negative control well was greater than 0.1.

3.4.4 Extraction of Rotavirus ds-RNA from stool for PAGE

Samples that tested ELISA positive, rotavirus ds-RNA was extracted. Before beginning the extraction Sodium acetate(NaAc) containing 1% SDS was placed in the 37°C water bath. Four hundred and fifty microlitre of the stool suspension was placed in an eppendorf tube. Fifty microlitre of the pre-warmed 1M sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS) (pH 5.0) was added to the suspension. This was incubated in a water bath at 37 °C for 15 minutes.

Five hundred microlitre phenol / chloroform (1:1) was added to the eppendorf tube and vortexed for 1 minute. This mixture was incubated for a further 15 minutes in water bath at 56 °C. Tubes were opened and immediately resealed prior to vortexing. This reduces the air pressure within the tube and prevents the tubes popping open during mixing. These were vortexed for 1 minute and centrifuged for 2-3 minutes at 12 000 rpm. The upper aqueous phase which contained the dsRNA was carefully removed and placed in a clean eppendorf tube. Any interface material was avoided as this contains protein and DNA that will contaminate the extraction and potentially degrade the RNA. Approximately 40 μl of 3M NaAc and 1ml ice-cold absolute ethanol was added to the sample and mixed gently by turning the tube over 4-6 times. This was kept at -20°C overnight to precipitate
the ds-RNA. This was then centrifuged at 4°C for 15 minutes at 12,000 rpm to pellet the dsRNA. The supernatant was poured off and the sample allowed to air-dry. The pellet was resuspended in 30 µl sample buffer (Bromophenol blue, 45% sucrose solution) before loading on a PAGE gel.

3.4.5 Polyacrylamide gel Electrophoresis

Electrophoresis of the extracted RNA was carried out in 10% polyacrylamide slab gels with a 3% stacking gel, using the discontinuous buffer system described by Laemmli et al., (1970). Approximately 30 µl of each sample was loaded and electrophoresis carried at 100 V for 16 to 18 h at room temperature.

Glass plates were carefully cleaned with ethanol. The equipment was assembled for gel casting and the level of resolving gel marked. Ten percent resolving gel was prepared and mixed thoroughly by pipetting and poured into glasses immediately. Distilled water was placed on the gel solution to prevent drying. The resolving gel was kept at room temperature for at least 2 hours. Three percent spacer gel was prepared and mixed thoroughly and poured in the resolving gel. The comb was inserted into spacer gel solution immediately and tapped several times to remove the air bubbles under the comb. The spacer gel with comb was left for at least 1 hour. (Gel size: 12 X 15 cm/cm). Forty microlitre of each sample was loaded in a single well on the gel and electrophoresis was carried out at 100V of constant current for 18-20 hours.
3.4.6 Silver Staining

The gels were stained using a modification of the method described by Herring et al (1982). The gel was fixed in 40 % ethanol with 10 % acetic acid for 40 min. The gel was then soaked in 11 mM silver nitrate solution for 40 min before being washed in distilled water. Finally, the gel was reduced in a solution of 0.75 M sodium hydroxide containing 0.3 % formaldehyde. The reaction was stopped with 5 % acetic acid.

The gel was removed from the plates and the bottom right hand corner was cut to orientate the gel. The first fixing solution was added to the gel and incubated for 30 minutes on an orbital shaker. The fixing solution was drained and replaced with the second fixing solution and incubated for 30 minutes on orbital shaker. The second fixing solution was drained off and silver nitrate staining solution was prepared just before use and was then added and incubated for 30 minutes on the orbital shaker. Developing solution was prepared by adding the Sodium Hydroxide (NaOH) to previously prepared formaldehyde and water solution. The silver nitrate solution was drained and the gel was washed twice with distilled water for 2 minutes each time. Approximately 50 ml of developing solution was added to the gel and agitated for 30 seconds. This removes any black precipitate. This was drained off and the remaining developing solution approximately 200 ml was then added and incubated for approximately four minutes or until RNA bands could be seen. The developing solution was drained off and the stopping solution added to prevent further colouring and then incubated for 5 minutes.
before rinsing in distilled water. The gel was dried on the Easy Breeze Gel Dryer overnight.

3.4.7 VP 6 Subgroup ELISA

The presence and specificity of the two subgroup antigens, I and II, was determined by a solid-phase enzyme immunoassay using monoclonal antibodies raised against the subgroup I and II antigens that are found on the surface of rotaviruses. A rabbit antirotavirus serum was used as capture antibody and a monoclonal antibody against the rotavirus group A antigen was used as described by Beards et al., (1984).

A 1:50 dilution of coating antibody (rabbit anti-human rotavirus) in 0.05M-carbonate/bicarbonate buffer was prepared. 100 μl of the diluted antibody was placed into each well of a 96-well flat bottom microtitre plate and the plate was incubated overnight at 4°C. The plates were washed 4-6 times in PBS/T. The plate was drained of fluid. One hundred microlitre PBS/T/EDTA was added to each well and 50 μl of the previously prepared stool suspension was dispensed into the consecutive wells. Distilled water was used as the negative control and standard rotavirus strains as the positive controls. The plates were incubated overnight at 4°C. The plates were then washed 4-6 times in PBS/T. The plate was drained of fluid. A 1:5000 dilution in PBS/T/BSA of the monoclonal antibodies was prepared. One hundred microlitre of the solutions was dispensed into the correct microtitre wells and the plates incubated at 37°C for 3 hours. The plates were washed 4-6 times in PBS/T and drained of fluid. Horseradish peroxidase conjugated goat anti-mouse IgG 1:1000 was conjugated with PBS/T/BSA. One hundred microlitre of this
mixture was dispensed into each well and the plates incubated at 37°C for 2 hours. The plates were subsequently washed 4-6 times in PBS/T and then drained of fluid. One hundred microlitre hydrogen peroxide plus TMB was dispensed to each well and incubated for 10 minutes in the dark. The plates were read visually and 5% sulphuric acid (stopping solution) was added. The plates were read spectrophotometrically at 450 nm on a Microplate Reader to obtain an optical density (OD) reading. The ratio between the optical density (OD) reading obtained for the two subgroup monoclonal antibodies was determined. Results were interpreted as follows: Rotavirus Group A antigen OD > 1.0 indicated a positive result A sample is Subgroup I if the OD SGI: SGII > 1.7 and Subgroup II if the OD SGII: SGI > 2.0

3.4.8 Extraction and Purification of ds-RNA for PCR

TRIzol Extraction

Purified ds-RNA was extracted from stool using Trizol solution and chloroform. The ds-RNA was then precipitated out using ice-cold isopropyl alcohol (Gibco).

Previously prepared stool suspension was centrifuged at 5000 rpm for 5 minutes at room temperature. Stool supernatant (200μl) was transferred to a clean eppendorf tube and 500μl of Trizol added. The tubes were vortexed for 30 seconds and incubated at room temperature for 5 minutes. One hundred microlitre of chloroform was added and vortexed for 30 seconds and incubated at room temperature for 3 minutes. This was then centrifuged at 4°C at 12,000 rpm for 15 minutes to separate the phases. The aqueous
phase was carefully transferred to a clean eppendorf tube. Approximately 700 µl of ice-cold isopropyl alcohol was added and mixed gently by turning the tubes 4-6 times. This was then incubated at room temperature for 20 minutes and centrifuged at 4°C at 12,000 rpm for 15 minutes to pellet the ds-RNA. The supernatant was discarded and the pellets allowed to air dry. The pellet was then resuspended in 15 µl DEPEC treated water.

3.4.9 Reverse Transcription-polymerase chain reaction (RT-PCR)

3.4.9.1 VP7 Genotyping

Different RNA electropherotypes were characterised using a method described by Gouvea et al., (1990). This is a reverse transcriptase polymerase chain reaction (RT-PCR). In brief, the VP7 specificity of the strains is determined by a nested RT-PCR reaction using a cocktail of VP7 specific primers to the six human serotypes (G1, G2, G3, G4, G8 & G9).

The first round PCR amplified the whole length of the VP7 gene (1062 bp) using outer primers, Beg9/sBeg9 and End9/EndA (Appendix 3)(Gouvea et al., 1990; Gault et al., 1999). The second round typing PCR was a multiplex PCR and incorporated the primer RVG and the G-type specific primers aBT1 (G1 specific), aCT2 (G2 specific), aET3 (G3 specific), aDT4 (G4 specific), aAT8 (G8 specific), and aFT9 (G9 specific) (Appendix 3). After denaturation at 94°C for 5 minutes, 30 PCR cycles each consisting of 94°C for 1 min, 42°C for 2 min, and 72°C for 3 min. were performed, followed by an extension at 72°C for 7 min. The second round PCR was performed using the same protocol but with 1
μl of the first round reaction product as the template. All amplified products were examined by gel electrophoresis in 1% agarose gels containing 4 μg of ethidium bromide/ml.

**3.4.9.2 VP4 Genotyping**

Genomic types P4, P6, P8, P9 and P10 were identified using the RT-PCR method described by Gentsch *et al.*, (1992). This method also uses a nested PCR reaction with a mixture of VP4 specific primers.

The first round PCR amplified an 876-bp fragment (VP8 subunit) of the gene 4 of group A rotaviruses by using two outer primers, con2 and con 3 (Appendix 4) (Gentsch *et al.*, 1992). The second-round PCR incorporated con3 and the P-type-specific primers1T-1 (P[8] specific), 2T-1 (P[4] specific), 3T-1 (P[6] specific), 4T-1 (P[9] specific), 5T-1 (P[10] specific (Appendix 4). After denaturation at 94°C for 5 minutes, 30 PCR cycles each consisting of 94°C for 1 min, 42°C for 2 min, and 72°C for 3 min. were performed, followed by an extension at 72°C for 7 min. The second round PCR was performed using the same protocol but with 1 μl of the first round reaction product as the template. All amplified products were examined by gel electrophoresis in 1% agarose gels containing 4 μg of ethidium bromide/ml under standard conditions.
CHAPTER FOUR

4.0 RESULTS

4.1 ELISA Method

Stool specimens from the chosen hospitals were screened by an ELISA method, which is able to detect group A rotaviruses causing gastroenteritis in children. A total of 285 rotavirus positive strains were detected with this method and were further subjected to rotavirus characterization procedures that included electrophoresis of the rotavirus genome, rotavirus subgroup determination using ELISA method, serotype and genotype determination using RT-PCR technique.

4.2.1 Polyacrylamide gel electrophoresis of the Rotavirus genome

The sizes of the segments in figure 2 are as follows in base pairs (bp), Segment 1 (3302 bp); Segment 2 (2690 bp); Segment 3 (2591 bp); Segment 4 (2362 bp); Segment 5 (1581 bp); Segment 6 (1356 bp); Segment 7 (1104 bp); Segment 8 (1059 bp); Segment 9 (1062 bp); Segment 10 (751 bp); Segment 11 (667 bp).

Figure 2 shows the two main group A rotavirus families based on genome RNA electrophoretic analysis that were detected. Lane A shows a known rotavirus positive control of the short type seen in Kenya previously. Lane D shows a known positive control of the long type that was also seen in Kenya previously. Lane B and C were the representative of the short and long patterns respectively identified in the study. The distribution of the eleven segments into the four size classes is seen using polyacrylamide gel electrophoresis (PAGE) of RNA are as shown in Fig. 2. These RNA segments are
numbered in order of migration during PAGE, with the slowest RNA segment designated gene segment 1 and the fastest gene segment 11. The difference between “long” pattern and “short” pattern is determined at the class IV region. In the “short” pattern family, segment 11 is heavy and migrates at a slower rate than segment 10 and is found to lie above segment 10 (Lane B in Figure 2). In the “long” pattern family, segment 11 migrated fastest and is seen to migrate beyond segment 10 (Lane C) as seen in figure 2.

![Figure 2](image)

**Figure 2.** 10% SDS-PAGE Electrophoretic analysis of group A rotavirus genome RNA. Large segments (class I), medium segments (class II), smaller segment (class III) and smallest segments (class IV).

C(S): Control (short pattern); C(L): Control (long pattern); S: Long pattern; L: Short pattern
4.2.2 The distribution of Rotavirus electropherotypes

A total of 285 rotavirus positive stool samples were subjected to SDS-Polyacrylamide gel electrophoresis to determine the electropherotypes. It was possible to determine the electropherotypes in 214 (75%) of the samples. One hundred and fifty five strains (72.4%) belonged to the long pattern and 56 (26.2%) to the short pattern. Additionally three samples (1.4%) were showing mixed infections, of both long and short patterns (Figure 3).

Figure 3: The distribution of the long, short and mixed pattern rotavirus electropherotypes in Kenya.

4.2.3 The different RNA electropherotype patterns observed

Based on the migration pattern of the 11 RNA segments by polyacrylamide gel electrophoresis, five electropherotypes of the long type were detected in the study samples as shown in Figure 4. All the five electropherotypes are long, but they differ with
different segments migrating at different destinations. The difference between L1 and L2 is seen at class IV region where the second last segment at the bottom of the gel in L1 has migrated slower than its counterpart in L2. In L3 there is an additional segment at class II which is not present in L1 and L2. In L4, all the segments in class III are merged and appear as one segment. In L5 the difference is at class I were the gap between segment 2 and 3 is wider as compared to the others. L1 electropherotype was found to be the most predominant in this study (61%). L5 electropherotype was only found in samples from Kisumu city (3.5%). L4 electropherotype was a unique electropherotype since it was found in only one stool sample that was collected from Aga Khan hospital within Nairobi city.

Figure 4. The different “long” RNA electropherotypes (L1, L2, L3, L4 and L5)

The L1 (61%) pattern was found to be the main long type circulating in the study areas, followed by L2 (32%), L5 (3.5%), L3 (2.8%) and L4 (0.7%) respectively (Figure 5).
According to the migration pattern of the eleven RNA segments by polyacrylamide gel electrophoresis, five different electropherotypes of the short type (S1, S2, S3, S4 and S5) were detected in the study samples as seen in figure 6. The difference between S1 and S2 is at the class III where in S1 two segments are merged together and appear as one while in S2 the three segments are clearly seen. In S3 the difference is seen at class I where segment 2 and 3 are clearly separated while in S1 and S2 they are merged together. In S4 the difference is at class III where the upper two segments are merged together and in S5 the lower two segments at class III are also merged together. Also looking at S4 and S3 the migration of the second last segment in class IV is more as compared to S1, S2 and S3 making them look like strains which are slightly different from the other short strains. Electropherotype S2 was only found in Nairobi area. S4 and S5 were only found in Kisumu area.
Figure 6. The different “short” RNA electropherotypes (S1, S2, S3, S4 and S5).

The S3 (42%) pattern was found to be the main short type circulating in the study areas, followed by S1 (30%), S2 (15%), S5 (7.6%) and S4 (5.7%) respectively as indicated in figure 7.

Figure 7. The distribution of the “short” electropherotype patterns
The majority of rotavirus positive samples were from Gertrude’s children hospital (n=163) and the least number from Kisumu district hospital (n=11). In all the three hospitals, the long electropherotype was more abundant than the short one. Samples with mixed infection of both the long and the short pattern were only three and all were collected from Gertrude’s hospital (Table 1).

### Table 1. Distribution of the electropherotypes in the three study hospitals in Kenya

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of RNA Positive samples</th>
<th>Electropherotypes</th>
<th>Long</th>
<th>Short</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gertrude’s Hospital</td>
<td>163</td>
<td>119</td>
<td>41</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Aga Khan Hospital</td>
<td>40</td>
<td>29</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kisumu Hospital</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

#### 4.3 Rotavirus subgroup distribution

This study analysed human rotavirus isolates using an ELISA method that employed monoclonal antibodies directed at the sub-group specific antigens of the virus. Subgroup II viruses occurred more commonly than subgroup I viruses (69% vs 22%). It was also found that 2% of the samples were expressing both subgroup I and II antigens (Figure 8).
Figure 8. Distribution of the rotavirus subgroup antigens in the study.

4.3.1 Rotavirus subgroup distribution in the hospitals

The rotavirus subgroup could be determined for 201 (71%) of the samples. Majority of the samples were from Gertrude’s children hospital (n=156), then Aga Khan hospital (n=34). Not many rotavirus positive samples were found in Kisumu city (n=11). 139 (69%) were subgroup II, 58 (29%) subgroup I and 4 (2%) exhibited both I and II (Table 2).

Table 2. Relative frequency of subgroup I and II rotaviruses in the study hospitals

<table>
<thead>
<tr>
<th>Hospital</th>
<th>Subgroup determined</th>
<th>Subgroup I</th>
<th>Subgroup II</th>
<th>Subgroup I &amp; II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gertrude’s Hosp.</td>
<td>156</td>
<td>42</td>
<td>111</td>
<td>3</td>
</tr>
<tr>
<td>Aga Khan Hosp.</td>
<td>34</td>
<td>12</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Kisumu Hosp.</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>201</td>
<td>58(29%)</td>
<td>139(69%)</td>
<td>4(2%)</td>
</tr>
</tbody>
</table>
4.5 Combination of electropherotype and subgroup

One hundred and fourteen rotavirus samples, which were of “long” electropherotype had subgroup II antigens and 42 samples which were of “short” electropherotype had subgroup I antigens. One sample which has a “long” electropherotype had subgroup I antigens (Table 3). This shows that long electropherotype is associated mainly with subgroup II and short electropherotype associated with subgroup I.

Table 3. Rotavirus electropherotype and subgroup distribution

<table>
<thead>
<tr>
<th>Electropherotypes</th>
<th>Subgroup Long(L)</th>
<th>Subgroup Short(S)</th>
<th>Mixed(L&amp;S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgroup I</td>
<td>1</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>Subgroup II</td>
<td>114</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Subgroup I &amp; II</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

4.6 VP7 G serotypes of human rotavirus

A representative of all the samples studied by RT-PCR to determine the rotavirus serotypes were run on an agarose gel as shown on figure 9.

The agarose gel picture was taken under ultra violet (UV) light. The ethidium bromide added into the gel binds with the DNA making it possible to view the DNA bands under UV light. Lane A shows the 100 base pair ladder that shows the rang from 100 base pairs to greater than a 1000 base pairs. The smallest being 100 base pair and the largest
segment 1000 base pairs. This ladder helped to determine the size of sample DNA. Lane B shows the size of the full length VP7 gene. The band lies just above the 1000 base pair. Lane C shows the G1 rotavirus serotype, since the band lies between 700 and 800 base pairs. The real size of G1 is 749 base pairs. Lane D shows serotype G2 as the band lies between 600 and 700 base pairs. The real size of G2 is 652 base pairs. Lane E shows serotype G3 as the band lies between 300 and 400 base pair ladder. The real size of G3 is 374 base pairs. Lane F shows serotype G4 as the band lies between 500 and 600 base pair ladder. The real size of G4 is 583 base pairs. Lane G shows an example of a G8 strain since the band lies between 800 and 900 base pair ladder. The real size of G8 is 885 base pairs. No G9 rotavirus serotypes were detected in the study.

Figure 9. RT-PCR 1% agarose gel VP7 genotyping with RVG/Gouvea primers: A, 100 base pair ladder marker (Promega); B, full length VP7 gene (1062bp); C, amplified serotype G1 rotavirus (749bp); D, amplified G2 rotavirus (652bp); E, amplified G3 rotavirus (374bp); F, amplified G4 rotavirus (583bp) and G amplified G8 rotavirus
A total of 114 strains from the three study hospitals were subjected to VP7 RT-PCR to detect the rotavirus serotypes. Twenty-five strains were found to be G1, then followed by G2 which were 10 in number. G8 was the third next common with 9, and then G3 and G4 having one each. Two strains had mixed infections. One having G1 and G3 and the other one having G1 and G2. The G9 strains were not detected in this study (Table 4).

Table 4. Distribution of VP7 G serotypes of human rotaviruses in the study hospitals

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of strains tested</th>
<th>No. with given serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>Gertrude’s</td>
<td>78</td>
<td>16</td>
</tr>
<tr>
<td>Aga Khan</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Kisumu</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>114</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

In this study it was found that 57% of the strains could not be typed (NT) by the primers used. This study demonstrated that VP7 serotype G1 predominated (22%), followed by G2 (9%), then G8 (8%). G3 and G4 were each found in 1% of the samples. Two percent of the samples had mixed infections (Figure 10).
A representative of all the samples studied by RT-PCR to determine the rotavirus serotypes were run on an agarose gel as shown on figure 11. The agarose gel picture was taken under ultra violet (UV) light. The ethidium bromide added in to the gel binds with the DNA making it possible to view the DNA bands under UV light. Lane A shows the 100 base pair ladder showing the range from 100 to 1000 base pairs. The smallest being 100 base pair and the largest segment 1000 base pairs. This ladder helps to determine the size of sample DNA. Lane B shows the size of the amplified subunit of the full length VP4 gene. The band lies between 800 and 900 base pair ladder. Lane C shows the P[8] rotavirus genotype, since the band lies between 300 and 400 base pairs. The real size of P[8] is 345 base pairs. Lane D shows genotype P[6] as the band lies between 200 and 300 base pairs. The real size of P[6] is 267 base pairs. Lane E shows genotype P[4] as the

**Figure 10.** Distribution of VP 7 (G) Serotypes

### 4.7 VP4 (P) genotypes
band lies between 400 and 500 base pair ladder. The real size of P[4] is 483 base pairs. Rotavirus genotypes P[9] and P[10] were not detected in the study.

![Agarose gel VP4 genotyping with Con3/Gentsch primers](image)

**Figure 11.** 1% Agarose gel. VP4 genotyping with Con3/Gentsch primers: A, 100bp ladder marker (Promega); B, Amplified VP8* subunit of the VP4 gene (876bp); C, Amplified P[8] rotavirus (345bp); D, Amplified P[6] rotavirus (267bp); E, Amplified P[4] rotavirus (483bp).

A total of 53 specimens (47%) were typed by VP4 specific primers. Of these, 25 of the strains were of the P8 genotype (Table 5). The P4 genotype was identified in 16 specimens, while the P6 genotype was observed in 10 specimens. Strains bearing the P9,
P10 and P12 VP4 genotypes were not identified in this study. Two specimens had dual genotypes. One strain had P4 and P8, while the other had P8 and P6.

Table 5. Distribution of VP4 (P) genotypes in the study hospitals

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of strains</th>
<th>No. with given genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P8</td>
<td>P4</td>
</tr>
<tr>
<td>Gertrude’s</td>
<td>76</td>
<td>18</td>
</tr>
<tr>
<td>Aga Khan</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>Kisumu</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>114</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

It was shown in this study that 53% of the strains could not be typed (NT) by the primers used. The majority of the isolates were genotype P8 which were found in 22% of the isolate. P4 was the next most common genotype, which was detected in 14% of the samples. The least detected genotype was P6, which was found in 9% of the strains. Two percent of the strains were found to have mixed infections (Figure 12).
Figure 12. Distribution of the VP 4 (P) Genotypes

4.8 Combinations of VP7 and VP4 identified

Table 6 shows the correlation of the VP4 genotype with the VP7 serotype identified in the same specimen. In every case of a VP7 G2 serotype strain, the P4 VP4 genotype was identified. The P8 genotype was found to occur predominantly with serotype G1 strains, although an association occurred with G3 strains (Table 6). The P6 genotype was found to occur predominantly in serotype G8 strains and also slightly with serotype G1. P4 genotypes were found to occur in both serotype G2 and G8 strains.

Table 6 shows the combinations of both G and P. Majority of the strains (n=17) were found to be G1P[8], then followed by G2P[4] which were 6 in number. Four strains were G8P[4] and another four G8P[6]. One strain each were G1P[6] and G3P[8].

It was found that 52% of the strains were G1P[8], and 18% of the strains were G2P[4]. The G8P[4] and G8P[6] were each detected in 12% of the strains. The least identified were G1P[6] and G3P[8], which were each detected in 3% of the strains (Figure 13).
Table 6. Combinations of VP7 and VP4 identified in single rotavirus strains.

<table>
<thead>
<tr>
<th>VP4 genotype</th>
<th>VP7 serotype (no. of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>P8</td>
<td>17</td>
</tr>
<tr>
<td>P4</td>
<td>0</td>
</tr>
<tr>
<td>P6</td>
<td>1</td>
</tr>
</tbody>
</table>

A total of 258 rotavirus strains were studied for VP7 and VP4 antigenic combinations in stools. The samples were electrophoresed on polyacrylamide gel electrophoreses. Electrophoresis of both families of electrophoretotypes, long and short types, of the samples studied for viral RNA migration by PAGE, 214 strains which yielded a typical RNA profile. Not all rotavirus positive samples showed an RNA profile. The basis for a viral strain is shown in the table. This table was derived from a study done in India and South Africa (Hendrix et al., 1999) as well as other parts of the world. In conclusion, 12% of the strains belonged to the long pattern and 52% to the short pattern.}

Figure 13. Distribution of the combinations of G and P strains.
A total of 285 rotavirus positive samples were analysed in this study. These stool samples were all positive after the diagnosis by an ELISA method for detecting group A rotaviruses in stool. The samples were studied for viral RNA migration by polyacrylamide gel electrophoresis. Electrophoretic analysis revealed the presence of both families of electropherotypes, long and short types. Of the 285 rotavirus positive samples studied for viral RNA migration by PAGE, 214 (75%) were rotavirus positive which yielded a typical RNA profile. Not all rotavirus positive samples by ELISA would show an RNA profile by PAGE because, the RNA profile depends on the amount of RNA extracted from the virus particles. If the sample has very few virus particles, then the sample will generate a very small amount of RNA that would not be visible on PAGE gels or would appear as very faint bands.

Information on the rotavirus electropherotype distribution in the study, revealed that 72.4% of the samples belonged to the long pattern and 26.2% of the samples, the short pattern, showing that isolates with a long RNA electropherotype occurred more often than strains with a short pattern. This observation has also been made in other similar studies in other parts of the world. In a study undertaken in Rio de Janerio, Brazil (De Silva Vaz et al., 1999), among the rotavirus strains tested for dsRNA by PAGE, 70% belonged to the long pattern and 30% the short pattern. A similar result was also found in a study done in India and South Africa (Broor et al., 1993; Fiona et al., 1992).
Three samples in the study (1.4%) were found to have mixed infections with both the long and short electropherotypes. This is not a very common occurrence. A prerequisite for gene reassortment is the simultaneous infection of one individual by two different rotavirus isolates. Considering the different RNA electropherotype patterns observed, five electropherotypes (L1, L2, L3, L4, L5) of the long type were detected in the study samples. L1 electropherotype was found to be the most predominant long pattern, while L2 was the next most common, then L5, L3 and L4.

In addition five different short patterns were detected in the study (S1, S2, S3, S4, S5). The S3 pattern was the most predominant among the short patterns followed by S1, S2, S5 and S4 in that order. The electropherotype pattern L1 being the most abundant type in the study and was detected in both areas of the study, that is Nairobi and Kisumu area. This shows that the main strains are not confined in certain localities but are transmitted freely within localities. This could be attributed to the free movement of people between various localities and in the process spreading the infection.

The results of the distribution between the long and the short electropherotype patterns within the three study hospitals, the long electropherotype pattern was found to be the most commonly isolated pattern in the three hospitals. However, mixed infections between long and short electropherotypes were very rare. Only three samples from Gertrude’s hospital had mixed infections. No mixed infections were observed in the other two hospitals. The RNA electropherotype of a particular strain of rotavirus, while not
indicative of a specific serotype, is a useful indication of the strain most prevalent in the population at that time.

The genomic variation in rotaviruses is probably brought about by both antigenic drift (sequential point mutations) and antigenic shift (gene reassortment between different virus isolates). The group A human rotaviruses have been classified into two subgroups based on an antigen on the inner capsid of the viral particle. This study has shown that subgroup II rotaviruses were found to be more prevalent than subgroup I strains (69% and 29% respectively).

Epidemiological studies investigating the relative frequency of rotavirus subgroup I and II strains world-wide have demonstrated the increased prevalence of subgroup II isolates. In a study undertaken in Brazil (Houly et al., 1986), 96% of the RNA positive samples were classified as subgroup II while 2% were classified as subgroup I. Also in two other studies carried out in India and South Africa (Anand et al., 2000; Mnishi et al., 1992) subgroup II strains were found to be the most prevalent.

The two subgroups were further characterized by the association of the subgroup antigen specificity and the electropherotypes. It was found that all short RNA electropherotypes except one were subgroup I and all the long RNA electropherotypes except one were subgroup II. Of the two exceptions, one was a long pattern exhibiting subgroup I antigens and the other was a short electropherotype exhibiting subgroup II antigens. Recent reports have described human isolates that do not show what has been observed in the
majority of the strains, with most of the divergent strains showing subgroup I specificity with long electropherotypes (Jagannath et al., 2000). In this study the strain that was found to exhibit the characteristics of long RNA pattern and subgroup I specificity is likely to be an animal rotavirus that has infected a child, as most animal strains have subgroup I specificity and a long RNA profile. This kind of finding was reported in a study in India (Jagannath et al., 2000) where two isolates from two children suffering from severe, acute dehydrating diarrhea showed that both isolates exhibited long RNA pattern and subgroup I specificity suggesting the likelihood of animal origin.

Global surveillance of rotavirus G and P types have been extensively performed and indicate that types G1 to G4 and P[8] and P[4] are most frequently distributed worldwide (Estes, 1996; Kapikian et al., 1996). In addition, four frequent combinations of VP7 and VP4, G1P[8], G2P[4], G3P[8], and G4P[8], are common worldwide (Griffin, et al., 2000). In contrast, unusual G types, P types, or G-P combinations have also been detected. These epidemiological surveys on VP7 and VP4 types are important for developing efficient rotavirus vaccines and for elucidating rotavirus ecology and evolution. Genotyping studies conducted in developing countries have shown that the genetic diversity of rotavirus P and G types is much greater than previously believed. Thus, studies have found that strains of genotype P[6] previously thought to be restricted to neonates may be common in children with diarrhea in some countries (Ramachandran et al., 1996). In this study of rotavirus G and P characterization in Kenya, it was not possible to determine the G type in 57% of the strains that were analysed by RT-PCR. Serotype G1 were the predominant strain detected (22%), followed by G2 (9%) and then
G8 with 8%. Serotype G3 and G4 were detected at very low levels (1% for each). The high number of strains that could not be typed, could be an indication that they are probably new serotypes that can not be detected with the primers that were used or that these strains could be having nucleotide substitutions at the primer binding sites, making it difficult for the primers to bind. The aspect of G1 being the predominant serotype has also been found in other studies. In a study done in the U.S.A, G1P[8] was the most predominant strain (66.4%) and G2P[4] the next common (8.3%) (Ramachandran et al., 1998). This aspect was also reported in South Africa (Steele et al., 1995) where G1 was reported in 51% of the rotavirus strains tested and also in Tunisia (Trabelsi et al., 2000). This result differs from what was reported in Kenya in a previous study (Nakata et al., 1999) where Serotype G4 was the most prevalent, accounting for 41.6 % followed by G1 (23.3%), G2 (17%), and serotype G3 was rarely isolated. This study shows that the dominant serotype has now shifted from G4 to G1 in Kenya. The study has further demonstrated that G8 is appearing as the third most important serotype, ahead of G3 and G4. Although serotype G8 was reported for the first time in Kenya by Nakata et al., (1999), the numbers were very low (2.2%). Serotype G8 is now emerging as an epidemiologically important serotype.

This finding has very important implications since the vaccine studies that are on going, have been targeting serotypes G1, G2, G3, G4 as the main serotypes that are circulating worldwide. This trend seems to be changing. A study in Blantyre, Malawi serotype G8P[6] was the most abundant serotype (Cunliffe et al., 1999). Also in a small study in Ghana (Armah et al., 2001) rotavirus with G8 specificity were identified for the first
time. The origin of G8 strains in the human population is unknown, but interspecies transmission from a bovine source has been suggested (Browning et al., 1992).

From this study, half the G8 strains were found to be G8 P[6] and the other half G8 P[4]. All the G8 P[6] and G8 P[4] isolates had short electrophoretic pattern and typed as subgroup I rotaviruses. These strains could be similar to the novel isolates detected in Blantyre, Malawi (Cunliffe et al., 1999). The report of the novel strain G8 P[4] is significant since strains with P[4] genotype typically are G2. Rotaviruses readily undergo reassortment in vivo and in vitro (Graham et al., 1987), and this is one mechanism whereby rotaviruses may evolve (Taniguchi and Urasawa, 1995). This could be an indication of the spread of these strains in this region and also the possibility of these strains becoming the dominant strains circulating in this region in future. The finding of a high proportion of G8 strains in this study may have important implications for rotavirus vaccine development. Looking at the distribution of VP4 (P) genotypes, it was also not possible to determine the P type in 53% of the strains that were analysed by RT-PCR. P8 genotypes were the most common (22%), followed by P4 genotypes (14%) and then P6 (9%). Genotype P6 strains have been well described in association with asymptomatic rotavirus infections of neonates (Gorziglia et al., 1988). In recent studies just like in this study, genotype P6 is being found in infants with diarrhea. In a recent multicenter study 43% of infants with diarrhea in India had P[6] infections (Ramachandran et al., 1996). In addition, P[6] infections have now been reported at high frequency in infants with diarrhea in Nigeria (Adah et al., 1997) and in the United States (Ramachandran et al., 1998).
Considering the combination of VP7 and VP4 identified in single rotavirus strains in this study, it was found that GI P[8] were the most predominant strains isolated (52%), then G2P[4] found in 18% of the strains. G8P[4] and G8P[6] were detected at similar levels (12%). This aspect is consistent with other studies carried out in other parts of the world (Ramachandran et al., 1998; Steele et al., 1995; Trabelsi et al., 2000). This study also observed cases of dual infectivity within both the G serotypes and the P genotypes. Within the VP7 G serotype, two samples had dual reactivity (G1/G3 and G1/G2). Also within the VP4 P genotypes, two samples had dual reactivity (P4/P8 and P8/P6). The existence of dual infectivity increases the possibility of natural reassortment and the creation of novel strains, with serious implications for vaccine design and development.
CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The study has shown an emergence of serotype G8 as an important serotype in Kenya. It was the third most commonly detected serotype after G1 and G2. Future rotavirus vaccines will need to provide adequate protection against disease caused by serotype G8 rotaviruses.

2. The study has also demonstrated that genotype P[6] is becoming an important cause of symptomatic infections in Kenya and has further demonstrated the existence of rotavirus strains with unusual G and P combinations.

3. The potential for reassortment to occur between viral strains with different VP7 and VP4 gene segments has been identified due to the mixed infections that were detected. This may have important implications for future vaccine development because reassortant viruses may arise in any region where a rotavirus vaccine is implemented.
6.2 Recommendations and scope for further work

1. Since many strains could not be G and P determined when typed with conventional primers, variation in the sequence of the primer binding region of common serotypes, as well as the occurrence of uncommon serotypes, should be considered when nontypeable rotaviruses are identified in strain characterization studies.

2. It will be important to further characterize the strains in order to determine antigenic and genetic changes over time and initiate studies in other regions of Kenya to detect the actual distribution of strains across the country. This will be important in providing information on the choice of vaccines to be used in Kenya.

3. Similar studies in other African countries will provide crucial information for future vaccine programs in a continent where the need for an effective rotavirus vaccine is probably greatest.
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acute diarrhea in Blantyre, Malawi, from 1997 to 1998: predominance of novel

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**Gibco-Brl/ Life Technologies.** TRizol method for RNA isolation.


<table>
<thead>
<tr>
<th>Segment</th>
<th>Base pairs</th>
<th>Protein products</th>
<th>Nucleotides/polyprotein (kDa)</th>
<th>Matrix protein modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1001</td>
<td>VP1</td>
<td>125, 005 (105k)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2890</td>
<td>VP2</td>
<td>1024, 012 (94k)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2591</td>
<td>VP3</td>
<td>95120 (88k)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2362</td>
<td>VP4, (VP5*, VP6*)</td>
<td>86792 (88k)</td>
<td>Cleared by VP5* and VP2*</td>
</tr>
<tr>
<td>5</td>
<td>1581</td>
<td>NSP1</td>
<td>7564 (55k)</td>
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<td>6</td>
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<td>NSP2</td>
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<tr>
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<td>37365 (33k)</td>
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<tr>
<td>10</td>
<td>751</td>
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<td>30290 (28k)</td>
<td>Signal sequence, polyprotein modification and processing</td>
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<tr>
<td>11</td>
<td>667</td>
<td>NSP5</td>
<td>21722 (20k)</td>
<td>Polyprotein modification and processing</td>
</tr>
</tbody>
</table>
## Appendix 1

### Rotavirus genome segments (Adopted from Estes, 1996)

<table>
<thead>
<tr>
<th>Segment</th>
<th>Base pairs</th>
<th>Protein products</th>
<th>Nascent polypeptide (M)</th>
<th>Mature protein modified</th>
<th>Location in the viral particle</th>
<th>Function</th>
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<td>3302</td>
<td>VP1</td>
<td>125005(125k)</td>
<td>-</td>
<td>Core</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>2</td>
<td>2690</td>
<td>VP2</td>
<td>102431(94k)</td>
<td>Cleaved</td>
<td>Core</td>
<td>RNA binding</td>
</tr>
<tr>
<td>3</td>
<td>2591</td>
<td>VP3</td>
<td>98120(88k)</td>
<td>-</td>
<td>Core</td>
<td>Guanylytransferase</td>
</tr>
<tr>
<td>4</td>
<td>2362</td>
<td>VP4 (VP5*, VP8*)</td>
<td>86782(88k)</td>
<td>Cleaved to VP5* and VP8*</td>
<td>Outer capsid spike</td>
<td>Haemagglutinin, cell attachment, virulence protease-enhanced infectivity, neutralization</td>
</tr>
<tr>
<td>5</td>
<td>1581</td>
<td>NSP1</td>
<td>58654(53k)</td>
<td>-</td>
<td>Nonstructural</td>
<td>Zinc finger, RNA binding</td>
</tr>
<tr>
<td>6</td>
<td>1356</td>
<td>VP6</td>
<td>44816(41k)</td>
<td>-</td>
<td>Inner capsid</td>
<td>Subgroup antigen, trimer, hydrophobic</td>
</tr>
<tr>
<td>7</td>
<td>1104</td>
<td>NSP3</td>
<td>34700(34k)</td>
<td>-</td>
<td>Nonstructural</td>
<td>RNA binding</td>
</tr>
<tr>
<td>8</td>
<td>1059</td>
<td>NSP2</td>
<td>36700(35k)</td>
<td>-</td>
<td>Nonstructural</td>
<td>RNA binding</td>
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<tr>
<td>9</td>
<td>1062</td>
<td>VP7</td>
<td>37368(38k)</td>
<td>Signal sequence, glycosylation and trimming</td>
<td>Outer capsid</td>
<td>RER integral membrane glycoprotein, neutralization antigen, 2 hydrophobic regions</td>
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<tr>
<td>10</td>
<td>751</td>
<td>NSP4</td>
<td>20290(28k)</td>
<td>Signal sequence, glycosylation and trimming</td>
<td>Nonstructural</td>
<td>RER integral membrane glycoprotein, viral enterotoxin, role in morphogenesis</td>
</tr>
<tr>
<td>11</td>
<td>667</td>
<td>NSP5</td>
<td>21725(26k)</td>
<td>Phosphorylated O-glycosylated</td>
<td>Nonstructural</td>
<td>RNA binding, rich in serine and threonine</td>
</tr>
</tbody>
</table>
Appendix 2

Sero-typic and geno-typic classification of group A rotavirus VP7 and subgroup specificities
(adapted from Hoshino et al, 1994)

<table>
<thead>
<tr>
<th>VP7 (G) Serotypes</th>
<th>Strains</th>
<th>Subgroup</th>
<th>Strain</th>
<th>Subgroup</th>
<th>Species</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Wa, KU, D, M37, RV-4, W179, K8</td>
<td>II</td>
<td>C60, C86, T449</td>
<td>I</td>
<td>Pig, Cow</td>
</tr>
<tr>
<td>2</td>
<td>DS-1, S2, KUN, RV-5, 1076</td>
<td>I</td>
<td>C134</td>
<td>I</td>
<td>Pig</td>
</tr>
<tr>
<td>3</td>
<td>P, MO, YO, RV-3, Ito, Nemoto, W178, McN, AU-1, AU228, Ro1845, HCR3, 0264</td>
<td>II</td>
<td>SA11</td>
<td>I</td>
<td>Vervet Monkey</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I and II</td>
<td>MMU18006</td>
<td>I</td>
<td>Rhesus Monkey</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>CU-1, K9</td>
<td>I</td>
<td>Dog</td>
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<tr>
<td></td>
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<td></td>
<td>TAKA, Cat2</td>
<td>I</td>
<td>Cat</td>
</tr>
<tr>
<td></td>
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<td>H-2</td>
<td>I</td>
<td>Horse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F1-14</td>
<td>I and II</td>
<td>Pig</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>CRW-8, C176</td>
<td>I</td>
<td>Rabbit</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>R-2</td>
<td>I</td>
<td>Rabbit</td>
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<td></td>
<td></td>
<td>ALA, C11</td>
<td>I</td>
<td>Mouse</td>
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<td></td>
<td></td>
<td>Eb</td>
<td>Not I or II</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
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<td>EW</td>
<td>Not I or II</td>
<td>Mouse</td>
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<td>ST3, ST4, VA70, Hosekawa, Highi, 57M</td>
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<td>Pig</td>
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<td>Pig</td>
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<td>SB-2</td>
<td>?</td>
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<td>5</td>
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<td>I</td>
<td>Horse</td>
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<td>NCDV, UK, RF, WC3, Q17, OK, ID B641, C486</td>
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<td>Cow</td>
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<td>PO-13</td>
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<td>Pigeon</td>
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<td>Cow</td>
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<td>8</td>
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<td>678, J2538, A5</td>
<td>I</td>
<td>Cow</td>
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<tr>
<td>9</td>
<td>W161, F45, 116E, Me323</td>
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<td>ISU-64</td>
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<td>Pig</td>
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<tr>
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<td>B223, V1005</td>
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</tr>
<tr>
<td></td>
<td>Me35, 1321</td>
<td>I</td>
<td>Lp14</td>
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<td>Sheep</td>
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<tr>
<td>11</td>
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<td>YM, A253.1</td>
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<td>Pig</td>
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<td>12</td>
<td>L26</td>
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<td>None</td>
<td>-</td>
<td>-</td>
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<td>13</td>
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<td>L338</td>
<td>I</td>
<td>Horse</td>
</tr>
<tr>
<td>14</td>
<td>None</td>
<td>-</td>
<td>F123</td>
<td>I</td>
<td>Horse</td>
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</tbody>
</table>
Appendix 3

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Position (nt)</th>
<th>Strain (genotype)</th>
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<tbody>
<tr>
<td>sBeg9</td>
<td>GGCTTTAAAGAGAGAATTTC</td>
<td>1-21</td>
<td>Group A</td>
</tr>
<tr>
<td>Beg9</td>
<td>GGCTTTAAAGAGAGAATTTCCGTTCTGG</td>
<td>1-28</td>
<td>Group A</td>
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<tr>
<td>End9</td>
<td>GGTCACATCATACAAATTCTAATCTAAG</td>
<td>1062-1036</td>
<td>Group A</td>
</tr>
<tr>
<td>EndA</td>
<td>ATAGTATAAAATACTTGCCAACCA</td>
<td>922-944</td>
<td>Group A</td>
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<tr>
<td>aAT8</td>
<td>GTCACCATTTGTAAATTCG</td>
<td>178-198</td>
<td>69M (G8)</td>
</tr>
<tr>
<td>aBT1</td>
<td>CAAGTACTCAAATCAATGATGG</td>
<td>314-335</td>
<td>Wa (G1)</td>
</tr>
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<td>aCT2</td>
<td>CAATGATATTAACACATTTTCTGTG</td>
<td>411-435</td>
<td>DS-1 (G2)</td>
</tr>
<tr>
<td>aDT4</td>
<td>CGTTTCTGGGAGGAGTTG</td>
<td>480-498</td>
<td>ST-3 (G4)</td>
</tr>
<tr>
<td>aET3</td>
<td>CGTTTGAGGAAGCTGCAACAG</td>
<td>689-709</td>
<td>P (G3)</td>
</tr>
<tr>
<td>aFT9</td>
<td>CTAGATGTAACCTACACTAC</td>
<td>757-776</td>
<td>W161 (G9)</td>
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<tr>
<td>RVG9</td>
<td>GGTCACATCATACAAATTCT</td>
<td>1062-1044</td>
<td>Group A</td>
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</tbody>
</table>
Oligonucleotide primers for P genotype PCR typing as designed by Gentsch et al., 1992.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Position (nt)</th>
<th>Strain (genotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1T-1</td>
<td>ACTTGGATAACGTGC</td>
<td>339-356</td>
<td>KU (P8)</td>
</tr>
<tr>
<td>2T-1</td>
<td>CTATTGTTAGAGTGGTAC</td>
<td>474-494</td>
<td>RV5 (P4)</td>
</tr>
<tr>
<td>3T-1</td>
<td>TGTTGATTAGTTGGATTCAA</td>
<td>259-278</td>
<td>1076 (P6)</td>
</tr>
<tr>
<td>4T-1</td>
<td>TGAGACATGCAATTGGAC</td>
<td>385-402</td>
<td>K8 (P9)</td>
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<tr>
<td>5T-1</td>
<td>ATCATAGTTAGTGTCG</td>
<td>575-594</td>
<td>69M (P10)</td>
</tr>
<tr>
<td>con3</td>
<td>TGGCTTCGCCATTTATAGACA</td>
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<td>con2</td>
<td>ATTCGGACCATTATAGACA</td>
<td>868-887</td>
<td>Group A</td>
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</table>
Appendix 5

POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents

1. **30% Acrylamide Stock (100ml).**

   30g Acrylamide + 0.8g NN’ methylene bis-acrylamide.

   Dissolve in 50ml dH₂O and then make up to 100ml.

   Filter before use.

   **Caution!!!!!!!** Acrylamide is a potent neurotoxin and is absorbed through the skin. Always wear gloves when working with acrylamide and methylenebisacrylamide.

2. **Resolving Gel Buffer (pH 8.9)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1NHCl</td>
<td>48ml</td>
</tr>
<tr>
<td>Tris</td>
<td>366.3g</td>
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</tbody>
</table>

   Dissolve and make up to 100ml with dH₂O. pH 8.9.
3. **Spacer Gel Buffer (pH 6.7)**

Dissolve 5.98g Tris and adjust pH to 6.7 with 1NHCL. Make up to 100ml with dH₂O.

4. **1NHCL**

Add 86mlconc. HCl to 910ml dH₂O.

5. **10% (w/v) Ammonium persulphate.**

0.1g APS in 1ml dH₂O. Store at 4°C for up to 3 days.

NB!!!!!! Prepare ONLY the amount that you need.

6. **5 x Tris-Glycine Running Buffer**

25mM Tris base 15.1g.

250mM Glycine 94g

Dissolve and make up to 1000ml with dH₂O.

**Note:** 1 x running buffer must be used for running PAGEs.

Therefore, dilute 200ml 5 x Running buffer in 800ml dH₂O.
### Gels

#### 1. 10% Resolving Gel

<table>
<thead>
<tr>
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<th>2x</th>
<th>1x</th>
<th>2x</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH₂O</td>
<td>15.8ml</td>
<td>31.6ml</td>
<td>9.9ml</td>
<td>19.8ml</td>
</tr>
<tr>
<td>300% Acrylamide Stock</td>
<td>10.0ml</td>
<td>20.0ml</td>
<td>6.3ml</td>
<td>12.5ml</td>
</tr>
<tr>
<td>Resolving Buffer (pH8.9)</td>
<td>3.75ml</td>
<td>7.5ml</td>
<td>2.4ml</td>
<td>4.8ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15μl</td>
<td>30μl</td>
<td>10μl</td>
<td>19μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>450μl</td>
<td>900μl</td>
<td>282μl</td>
<td>564μl</td>
</tr>
</tbody>
</table>

#### 2. 3% Spacer Gel

<table>
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<th>2x</th>
<th>1x</th>
<th>2x</th>
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</thead>
<tbody>
<tr>
<td>DH₂O</td>
<td>6.8ml</td>
<td>13.6ml</td>
<td>5.1ml</td>
<td>10.2ml</td>
</tr>
<tr>
<td>30% Acrylamide Stock</td>
<td>1.6ml</td>
<td>3.2ml</td>
<td>1.2ml</td>
<td>2.4ml</td>
</tr>
<tr>
<td>Spacer Buffer (pH 6.7)</td>
<td>1.25ml</td>
<td>2.5ml</td>
<td>0.9ml</td>
<td>1.9ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>5μl</td>
<td>10μl</td>
<td>4μl</td>
<td>8μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>15l</td>
<td>300μl</td>
<td>112μl</td>
<td>225μl</td>
</tr>
</tbody>
</table>

**NOTE:** Thick spacers use recipe for 1.5 gels.

Thin spacers use recipe for 0.75 gels.
SILVER STAINING

1. Remove gel from glass plates (NB!!!!! Cut off corner for orientation).
   Soak gel in 200ml of 40% EtOH, 5% acetic acid in dH₂O.
   Shake gently on an orbital shaker for 30min.

2. Drain off fixing solution. Replace with 200ml of 10% EtOH, 0.5% acetic acid in dH₂O.
   Shake for 30min.

3. Drain off 2nd fixing soln. Add 11mM silver nitrate (i.e 0.37g AgNO₃ in 200ml dH₂O).
   NB!!!!!!! Make up AgNO₃ just before use!.
   Be careful when weighing out AgNO₃, as it stains hands, bench tops etc.
   Shake for 30 min.

4. Rinse gel 2 x 2 minutes in dH₂O.
   NB!!!!!! Rinsing time is very important.

5. Prepare developing solution (7.5g NaOH dissolved in 250ml dH₂O, add 2ml of 36% formaldehyde soln).

6. Add 50ml of developing soln. and shake for 30 sec (the soln turns a dark brown).
Drain off the 50ml and add the remaining 200ml of developer.

After 1-2min the gel background turns yellow. After approx. 5 min the bands have turned from a pale brown to dark brown or black.

7. Drain off the developer and add 5% acetic acid for 1-2min to stop the reaction.

Drain off the stopping soln. And replace with a storage soln. Of 0.5% acetic acid and 5-10% EtOH. Shake in storage soln. For 5 min.

8. Drain off storage soln. And replace with 200ml dH₂O. Shake for 5 min.

9. To keep the gel, dry on the gel dryer.

10. Destaining of gels: Use: 5% methanol, 0.5% acetic acid.

Leave shaking in soln for ±60 min.

**Solutions**

<table>
<thead>
<tr>
<th>1 Gel</th>
<th>2 Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 80ml EtOH</td>
<td>1. 160ml EtOH</td>
</tr>
<tr>
<td>110ml dH₂O</td>
<td>220ml dH₂O</td>
</tr>
<tr>
<td>10ml Ac acid</td>
<td>20ml Ac acid</td>
</tr>
</tbody>
</table>
2. 20ml EtOH  
   180ml dH₂O  
   1 ml Ac acid

3. 0.37g AgNO₃  
   2000ml dH₂O

4. Rinse 2 x 2 min in dH₂O

5. 7.5g NaOH  
   250ml dH₂O  
   2ml 36% Formaldehyde

6. 10ml Ac acid  
   200ml dH₂O  
   Stop soln!

7. 1ml Ac acid  
   20ml EtOH  
   1800ml dH₂O

2. 40ml EtOH  
   360ml dH₂O  
   2ml Ac acid

3. 0.74g AgNO₃  
   400ml dH₂O

4. Rinse 2 x 2min in dH₂O

5. 15g NaOH  
   500ml dH₂O  
   4ml 36% Formaldehyde

6. 20ml Ac acid  
   4000ml dH₂O  
   Stop soln!

7. 2ml Ac acid  
   40ml EtOH  
   360ml dH₂O
Appendix 6

Reagents for running agarose gels

1. **1.2% Agarose gel (20ml)**

0.24g agarose in 20ml 1 x TAE buffer (pH 7.9)

Heat to dissolve. Check volume – make up to 20ml with dH$_2$O if needed.

After solution has cooled add 2µl ethidium bromide (Stock concentration – 5mg/ml).

2. **Ethidium bromide**

**Caution!!!** Ethidium bromide is a powerful mutagen and is moderately toxic!

Wear gloves when working with solutions containing this dye.

10mg Ethidium Bromide

1 ml dH$_2$O

**OR** Dissolve 1 x 100mg Et Br tablet in 10ml dH$_2$O.

Store solution in a dark bottle, as it is light sensitive.

3a. **20x TAE (pH 7.9)**

0.4M Tris 500ml 1000ml
0.05M NaCl  24.22g  48.44g  
0.01M EDTA  1.86lg  3.72g  

Dissolve in dH₂O and adjust pH to 7.9 with glacial acetic acid. Make up the solution up to the final volume.

3b.  **1 x TAE (pH 7.9)**

To make up 1 litre:

50ml 20x TAE is added to 950ml dH₂O.

4. **Bromophenol Blue Tracking Dye**

Make a 0.1% bromophenol blue (BFB) stock solution in dH₂O. If it does not dissolve in water add 0.1% NaCl.

Add 1ml BFB stock soln to 9ml of 40% sucrose made up in 1 x TE. Filter the final solution.

5. **Molecular Weight Markers (Boehringer Mannheim)**

19-1114bp

Mixture of pUCBM21 DNA, cleaved with Hpa II and pUCBM21 cleaved with Dra I and Hind III.
72-1353bp

X174 DNA cleaved with Hae III.

100-1500bp

Prepared by restriction digests of a specifically constructed plasmid.

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