Burden of Rotavirus Disease and Molecular Characterization of Rotaviruses at Dr George Mukhari Hospital from 2003-2005

by

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February 2010
DECLARATION

I Luyanda Mapaseka SEHERI, hereby declare that the work presented in this thesis is original and it has not been submitted before for any degree or examination at this or any other University.

The work is being submitted for the fulfilment of the degree of PhD (Medical Virology) in the MRC Diarrhoeal Pathogens Research Unit, Department of Virology, University of Limpopo (Medunsa Campus).

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LM SEHERI

-----------------------
DATE
DEDICATION

This work is dedicated to my grandparents Mr Joshua and Mrs Stella Seheri.
ACKNOWLEDGEMENTS

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Finally, a very special thanks to my son Phomello, my mother, my brother † Tumelo and my sisters Tiisetso and Patricia for their constant support, understanding and encouragement so that I could achieve my goals.
PUBLICATIONS AND PRESENTATIONS EMANATING FROM THIS THESIS

Publications


INTERNATIONAL CONFERENCE: PAPERS AND POSTERS


4. Seheri LM, Nermarude L, Jere K, Geyer A, Bos P, Dewar JB, Mphahlele MJ, Steele AD. Diversity of circulating rotavirus strains detected At Dr George Mukhari Hospital, South Africa during the


8. Seheri L, Dewar J, Steele A, Esona M. Geyer A, Bos P, Tumbo J Prospective hospital-based burden of rotavirus disease at Dr George
Mukhari and Brits Hospital 2003-2005. *Medunsa Academic day, University of Limpopo 2006*


SUMMARY

Background: Rotavirus infection remains a significant clinical problem throughout the world, infecting almost every child younger than 5 years of age, despite socio-economic status or environmental conditions. Rotavirus is the most common cause of severe dehydrating gastroenteritis in infants and young children. Implementation of an effective vaccine programme could reduce the incidence and severity of rotavirus disease. Decisions about new candidate rotavirus vaccines require reliable data on disease impact in both developed and developing countries. The aim of this study was to assess the burden of rotavirus associated disease at the tertiary care Dr George Mukhari Hospital, Ga-Rankuwa and the secondary care hospital Brits Hospital, Madibeng and to describe the genetic diversity of rotavirus strains circulating in Ga-Rankuwa and Brits communities over a similar time period as the testing of Rotarix® vaccine. The broad objectives included; to perform a hospital-based burden of rotavirus disease in two different hospitals in the North West of Pretoria area, to conduct molecular characterization of rotaviruses circulating in the Pretoria region and lastly to devise an alternative molecular typing method to detect rotavirus VP6 subgroups.

Materials and Method: To investigate the hospital-based burden of rotavirus disease, diarrhoeal stool samples were collected at Dr George Mukhari and Brits Hospitals from children less than 5 years of age. Group A rotavirus antigen was detected from the samples using commercially available rotavirus enzyme immunoassay IDEIA™ Rotavirus test (DAKO, Dakocytopation, Denmark). Genetic analyses of rotavirus strains were determined by polyacrylamide gel electrophoresis (PAGE) to characterize the electrophoretic patterns followed by analysis of the P and G genotypes by RT-PCR and multiplex PCR amplification of specific sequences of VP7 and VP4 genes. To devise an alternative molecular typing method to detect rotavirus VP6 subgroups, with subgroup specificities determined by both VP6 monoclonal antibodies and restriction fragment length polymorphism using restriction endonuclease Acil, Ddel and Rsa I.
Selected PCR amplicons (VP7 and VP6 genes) were purified, cloned and sequenced. Consensus sequences of the VP7 and VP6 genes were aligned and analysed manually with Chromaslite and BioEdit software packages. Multiple sequence alignment was implemented by Mafft software packages. The nucleotide and deduced amino acid sequences of the VP7 and VP6 genes were compared with reference strains available from GenBank. Multiple methods were used to construct phylogenetic trees and included neighbor-joining, maximum parsimony analysis and maximum likelihood distance. Bootstrap values were computed using 1000 replicates with Phylip and the MEGA softwares. The graphic representation of each phylogenetic tree was displayed with the Treeview program.

Results: Between 2003 and 2005, a total of 2 514 diarrhoeal stool samples were collected. Of these, 527 (21%) were positive for group A rotavirus and the majority of children hospitalized were less than 2 years of age. The annual peak prevalences of group A rotavirus were 56%, 59% and 56% for 2003, 2004 and 2005, respectively and were observed during the autumn and winter months. The estimated incidence of gastroenteritis associated with rotavirus indicates that one in every 50 to 70 children in the area is likely to be hospitalized with rotavirus diarrhoea between birth and 2 years of age. During the three-year study period, ten, six and seven different RNA electrophoretic patterns were identified in 2003, 2004 and 2005, respectively. The VP6 genes of the representative strains (G1, G2, G3, G9, G8 and G12) were analysed with restriction endonuclease Acil, Ddel and Rsal. The restriction endonucleases produced 11 unique restriction profiles (A-K). The VP6 RFLP results correlated well with strains displaying long RNA electropherotypes and VP6 subgroup II specificity and also with strains displaying short RNA electropherotypes and exhibiting VP6 subgroup I specificity as determined with VP6 monoclonal antibodies.

The consensus VP7 nucleotide sequences, exhibited the greatest homology and identity (97-99%), when compared against corresponding international reference strains. The nucleotide sequence datasets were closely related to strains from South Africa, Vietnam, Bangladesh, East India, Republic of Congo, China, Russia, Thailand and Japan. The phylogenetic tree revealed the South African strains (G1-G3, G8-G9 and G12) clustered with international strains whereas the G1 strains clustered into two different lineages. Phylogenetic analysis of the VP6 gene revealed four lineages with international reference strains. The VP6 gene showed 97-99% identity at the deduced amino acids level with strains from Taiwan, Bangladesh, the United States and Brazil.

**Conclusion:** This is the first study to estimate the disease burden associated with rotavirus diarrhoea in South Africa. The overall results confirm that rotavirus is the most common cause of severe diarrhoea. The epidemiology of rotavirus diarrhoea in South Africa correlates well with what has been reported in other countries. The proportion of hospitalization of rotavirus infection in children less than 5 years was estimated to an annual prevalence of 22.8% (95%CI 21.2%, 24.5%) at Dr George Mukhari Hospital, while at Brits Hospital was estimated at 18.2% (95%CI 14.9%, 22.1%). Rotavirus genotypes circulating at Dr George Mukhari Hospital showed a high degree of diversity and the emergence of uncommon rotavirus strains such as G12. The emergence of novel rotaviruses in the region needs to be taken into account where vaccine efficacy is concerned. It is, thus, important to continue with such studies to monitor the rotavirus strains associated with severe gastroenteritis in a hospital setting before and after the introduction of a rotavirus vaccine. Results also indicated that RFLP analysis of VP6 amplicons might be a simple and reliable, alternative to MAb subgrouping. The sequence analysis of the partial length VP6 gene confirmed the location and the recognition sites of the restriction enzymes The RFLP analysis proved to have more potential to accurately detect different rotavirus subgroups.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>micromolar</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACIP</td>
<td>Advisory Committee on Immunization Practices</td>
</tr>
<tr>
<td>AGMK</td>
<td>African green monkey kidney</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus reverse transcriptase</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>Bo</td>
<td>bovine</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CVP</td>
<td>Children’s Vaccine Program</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleoside-5’ triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylprocarbonate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded Ribonucleic acid</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
</tr>
<tr>
<td>EaggEC</td>
<td>enteroaggregative E.coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EHEC</td>
<td>enterohaemorrhagic E.coli</td>
</tr>
</tbody>
</table>
EIA  enzyme immunoassay
EIEC  enteroinvasive E.coli
ELISA  enzyme-linked immunosorbent assay
EM  electron microscopy
EPEC  enteropathogenic E.coli
EPI  Expanded Program on Immunization
ER  endoplasmic reticulum
et al.  et alii (and others)
ETEC  enterotoxigenic E.coli
eIF4Gi  eukaryotic initiation factor 4 gamma 1
FBK  fetal bovine kidney cells
FDA  Food and Drug Administration
ffu  fluorescent focus units
G  guanine
GAVI  Global Alliance for Vaccines and Immunization
h  hour
HIV  human immunodeficiency virus
HRP  horseradish peroxidase
IgA  Immunoglobulin A
IgG  immunoglobulin G
IgM  immunoglobulin M
IRF3  interferon regulator factor 3
IPTG  isopropyl β-D-thiogalactopyranoside
kb  kilobase pairs
kDa  kilodalton
LA  latex agglutination assay
lac Z  beta-galactosidase
LB  Luria-Bertani
LCR  ligase chain reaction
Leu  leucine
MAbs  monoclonal antibodies
Medunsa  Medical University of Southern Africa
MRC  Medical Research Council of South Africa
mg  milligram
<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>millimoles</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NaAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>NCDV</td>
<td>Nebraska calf diarrhoea virus</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NSP</td>
<td>nonstructural protein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>ORT</td>
<td>oral rehydration therapy</td>
</tr>
<tr>
<td>OPV</td>
<td>oral polio vaccine</td>
</tr>
<tr>
<td>PABP</td>
<td>poly (A) binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PATH</td>
<td>Program for Appropriate Technologies in Health</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS/T</td>
<td>phosphate buffered saline/ Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>Po</td>
<td>porcine</td>
</tr>
<tr>
<td>RE</td>
<td>restriction enzyme</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RRV</td>
<td>rhesus rotavirus</td>
</tr>
<tr>
<td>RRV-TV</td>
<td>rhesus-human reassortant tetravalent rotavirus vaccine</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RV</td>
<td>rotavirus</td>
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<tr>
<td>SA11</td>
<td>simian rotavirus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
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1 CHAPTER 1: GENERAL INTRODUCTION AND EXPERIMENTAL PROPOSAL

1.1 Diarrhoeal disease

Acute diarrhoeal disease or gastroenteritis continues to be a significant clinical problem throughout the world. Gastroenteritis is one of the most common illnesses of humans and is associated with high mortality and morbidity worldwide, especially among infants and young children. In addition, elderly and immunocompromised individuals are also at greater risk of diarrhoeal disease. In developing countries, gastroenteritis is considered to be the third most common cause of death (Figure 1:1) with an estimate of one child in 40 dying of diarrhoea and 8-10 episodes of gastroenteritis experienced per child each year during early childhood or before their 5th birthday (Black et al., 1989). In developed countries, diarrhoeal disease is the second most significant cause of paediatric morbidity. For example, in the United States (US) children will experience 1.5-2.5 episodes of gastroenteritis per year with an estimate of 2 million doctor or clinic visits and 160 000 hospitalizations (Tucker et al., 1998).

Over the years there has been a worldwide decline in diarrhoeal mortality, with a relatively stable morbidity rate. In a review of literature in 1982, Snyder and Merson estimated that 2.2 episodes of diarrhoea occurred per person annually which resulted in 4.6 million deaths. In 1986, another review by the Institute of Medicine, estimated 3.5 million diarrhoeal deaths per year. Bern et al. (1992) estimated diarrhoeal mortality to be approximately 3.3 million per year whereas Murray et al. (2001) estimated that diarrhoea accounted for 13% of all childhood deaths, leading to 1.4 million deaths annually. Kosek et al. (2003) estimated the diarrhoeal incidence at 21% with 2.5 million deaths per year in children less than five years of age. In order to reduce both the diarrhoeal mortality and morbidity rate, preventive efforts have focused mainly on promoting oral rehydration therapy (ORT), breast-feeding, interventions to improve water quality and sanitation as well as measles immunization and
vitamin A supplementation. Control programmes have been successful in reducing mortality by 3 million since the early 1980’s. However, recent data from the World Health Organisation (WHO) indicate much higher mortality rates than previously estimated stating that diarrhoeal disease is responsible for 18% of 10.6 million deaths in children less than 5 years of age (Bryce et al., 2005), a figure higher than the 2001 estimates (Figure 1:1).

Diarrhoeal disease is caused by a variety of viral, bacterial and parasitic enteropathogens. According to Black et al. (1989), prevention efforts have decreased the mortality rate associated with bacterial and parasitic infections. Glass et al. (2001) stated that the larger proportion of diarrhoeal disease in developing countries is caused by bacterial and parasitic enteropathogens, whereas in developed countries the majority of diarrhoea can be attributed to viruses. Furthermore, diarrhoeal disease in children can have a significant impact on families and society at large as a result of increased medical expenditure, indirect medical cost and loss of productivity (Glass et al., 2001).

![Figure 1:1 Percentage of diarrhoeal mortality in children less than 5 years (WHO World Health Report 2006 annual estimates 2000 – 2003)](image-url)
In Africa, early exposure to diarrhoeal disease is the major cause of childhood mortality and morbidity due mainly to poor sanitation, lack of healthcare facilities, and lack of life saving interventions. Sub-Saharan Africa is a developing region that faces unique health challenges, particularly because of increasing numbers of people infected with human immunodeficiency virus (HIV). In South Africa, there is a high prevalence of HIV/AIDS with an estimated 5.7 million people infected and deaths of almost 1 000 people every day (UNAIDS/WHO, 2006, UNAIDS/WHO 2008 Report on the global AIDS epidemic). Many diseases and conditions co-exist in immunocompromised patients and in children infected with HIV. These include diarrhoeal disease, malnutrition, pneumonia, and failure to thrive - the leading causes of death (Yigael et al., 2004). There are, however, other social factors that contribute to the high incidence of acute gastroenteritis or diarrhoea other than HIV/AIDS. These include urbanization, which is often associated with overcrowding and poor living conditions within informal settlements, particularly adjacent to the city. In these settings, lack of adequate safe water supply and sanitation facilities as well as poor nutrition exacerbates health problems.

In South Africa, acute diarrhoeal diseases are ranked as the third major cause of childhood mortality in children less than 5 years of age (Bradshaw et al., 2003) where the majority of deaths are among black and coloured children < 5 years. An estimated 160-200 children die each day (Wittenberg, 1997; Westaway and Viljoen, 2000) with a diarrhoeal morbidity at 1.5 million cases per annum in children less than 5 years of age (von Schirnding et al., 1993).

Vaccination and improvement in sanitation, access to clean water and adequate nutrition measures would have the greatest impact in reducing both mortality and morbidity associated with diarrhoeal disease worldwide. However, improvements in sanitation have only decreased bacterial diarrhoea, while viral gastroenteritis has remained unaffected (Vesikari, 1997). Progress has been made in the development of candidate vaccines against
major enteric pathogens (Svennerholm and Steele, 2004). Some vaccines are still under development including those against norovirus, *Campylobacter*, *Shigella*, enterotoxigenic *Escherichia coli (E.coli)*, while some have been licensed – including those against rotavirus, cholera and *Salmonella typhi* strains (Svennerholm and Steele, 2004). The vaccines against diarrhoeal disease will have to be effective, safe, affordable and sustainable in both developed and developing countries (Vesikari, 1997).

1.2 The most common bacterial agents

*Escherichia coli (E. coli)* are the most important enteropathogen throughout the world. Five distinct categories cause diarrhoea and their pathogenesis and clinical presentations differ (Levine, 1987). Thus, enteropathogenic *E.coli* (EPEC) causes watery diarrhoea associated with nursery and community outbreaks. Enteroinvasive *E.coli* (EIEC) is genetically related to *Shigella* and may cause dysentery and watery diarrhoea. Enterohemorrhagic *E.coli* (EHEC) causes a bloody diarrhoea and haemolytic–uremic syndrome. Enteroaggregative *E.coli* (EaggEC) is associated with persistent diarrhoea i.e. longer than 14 days. Enterotoxigenic *E.coli* (ETEC) is both the agent of traveller's diarrhoea as well as weaning diarrhoea in developing countries.

Other bacterial pathogens associated with gastroenteritis include; *Shigella, Salmonella, Vibrio* species and *Campylobacter jejuni*. *Shigella* causes inflammation and enterocyte necrosis. Clinically, *Shigella* dysentery is associated with frequent passage of small loose stools with blood and mucus, abdominal cramps and tenesmus (Niyogi, 2005). The clinical presentation following infection with *Salmonella* varies from mild to severe diarrhoea (sometimes bloody) with headache, abdominal cramps, nausea, vomiting and a febrile systemic disease (Rubin and Weinstein, 1977). *Vibrio* species include *Vibrio parahemolyticus, V. vulnificas, V. cholerae* and others. Infection with *Vibrio cholerae* results in severe diarrhoea due to production of cholera toxin
Campylobacter jejuni, on the other hand, causes watery diarrhoea and invasive dysentery (Blaser and Reller, 1981).

1.3 Parasitic diarrhoea

Parasites that cause diarrhoeal disease include Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum, Microsporidia and Cyclospora cayetanesis. The most common protozoan pathogens that cause opportunistic infections of the gut in HIV patients are: Cryptosporidium parvum, Microsporidia and Isospora beli (Lanjewar et al., 1994). These parasites enter the human intestine through oral ingestion of contaminated food or water.

1.4 Viral gastroenteritis

Common viruses that cause acute gastroenteritis include rotaviruses, noroviruses, sapoviruses, adenoviruses (type 40 and 41) and astroviruses. Other viral agents associated with diarrhoeal diseases are coronaviruses, toroviruses, picobirnaviruses, aichi viruses and bocaviruses (Conner and Ramig, 1997; Yamashita et al., 2000; Vicente et al., 2007). The main symptoms of virus-associated gastroenteritis are non-bloody watery diarrhoea, fever and vomiting which usually persist for more than three days (Bern and Glass, 1994). Other symptoms include headache, fever and abdominal cramps. In general, the clinical spectrum of disease ranges from asymptomatic infections to mild or severe disease leading to dehydration that can be fatal. Viruses causing gastroenteritis are extremely contagious due to the small infective dose (of 10 plaque-forming units (pfu)) required to cause infection among susceptible individuals. The viruses are spread through close contact with infected persons or eating or drinking contaminated foods or beverages. Other transmission routes include environmental routes, such as hospital work surfaces, floors, blankets/sheets, medical equipment, medical
staff/nurses, light switches, taps or door handles (Parashar and Monroe, 2001; Matson and Szücs, 2003; Green, 2007).

Worldwide, people of all ages are susceptible to viral gastroenteritis. Children aged 6-24 months, premature infants, and immunocompromised individuals are prone to severe symptoms or prolonged viral excretion (Chrystie et al., 1982; Pedley et al., 1984; Eiden et al., 1985; Cunningham et al., 1988). Rotavirus and norovirus infections are the most common cause of gastroenteritis in infants and young children less than 5 years old. Adenoviruses and astroviruses cause diarrhoea mostly in young children, but older children and adults can also be infected (Mendez and Arias, 2007). Noroviruses are, however, more likely to cause diarrhoea in older children and adults and are the major cause of outbreaks of gastroenteritis in adults in semi-closed institutions that include hospitals, old age homes, cruise ships and other settings. The peak incidence of viral gastroenteritis varies in different countries. Each virus has its own seasonal distribution; i.e. in the United States, rotavirus and astrovirus infections occur during the cooler months of the year, whereas adenovirus infections occur throughout the year (Matson and Szücs, 2003; Green, 2007).

1.5 Rotavirus infections

Rotavirus is the most common pathogen that has been identified in children hospitalized with diarrhoea worldwide. Rotavirus infections are frequently associated with severe diarrhoea and dehydration in young children and are estimated to account for one third of all diarrhoeal cases (Cunliffe et al., 1998; Kane et al., 2004; Bresee et al., 2004).

The burden of rotavirus disease is significant in both developed and developing countries where almost all children are infected with rotavirus
during their first few years of life (Parashar et al., 1998a). Improvement in sanitation and access to clean water are unlikely to reduce the rate of rotavirus infection. In a review of studies, de Zoysa and Feachem, (1985) estimated that rotavirus-associated disease worldwide accounts for 20-70% of all hospital cases of diarrhoea as well as 20% of diarrhoeal deaths in children less than 5 years of age. In 1986, the Institute of Medicine estimated that 130 million children are infected with rotavirus each year while 18 million experience moderate to severe diarrhoea resulting in 873 000 deaths annually. Data from more recent reviews have estimated that rotavirus infections cause approximately 25-55% of all diarrhoeal hospital admissions (Cunliffe et al., 1998; Kane et al., 2004; Bressee et al., 2004). Annually, throughout the world, rotavirus infection is estimated to cause 111 million episodes of gastroenteritis requiring home care, 25 million clinic visits, 2 million hospitalizations and approximately 600 000 deaths in children under 5 years of age. By the age of 5, nearly every child will have had an episode of rotavirus gastroenteritis, approximately 1 in 5 will visit the clinic, 1 in 65 will have been hospitalized and approximately 1 in 293 will die. In developing countries, 82% of paediatric deaths result from rotavirus infections (Parashar et al., 2006).

1.6 Interventions to prevent rotavirus diseases

Rotaviruses are very infectious and relatively resistant to inactivation by chemical disinfectants and antiseptics. Control and prevention of rotavirus infection are difficult because the virus is stable on environmental surfaces and are shed in high concentrations in the faeces of infected patients. These viral particles are then easily transmitted in hospital settings and day care facilities to susceptible hosts. Infection control measures are, thus, the primary keys to prevent rotavirus infection. These include, among others; isolation of infected children, hand-cleaning agents containing alcohol used before and after contact with infected children and disinfecting environmental surfaces with effective agents (Ward et al., 1991; Dennehy, 2000).
Based on the critical assessment of the burden of rotavirus disease in many countries, a rotavirus vaccine was considered to be the most cost effective public health intervention in both developed and developing countries to reduce mortality and morbidity associated with rotavirus disease. Multiple approaches to develop safe and effective vaccines have been considered. These include the use of live attenuated human and animal rotavirus strains, use of animal-human reassortants, virus-like particles produced in insect cells, DNA vaccines and cloning of rotavirus genome with expression of viral proteins in different vectors, and the production of synthetic peptide vaccines (Vesikari et al., 1983; Kapikian et al., 1986; Clark et al., 1996; Rennels et al., 1996; Estes et al., 1997; Chen et al., 1998; Kapikian et al., 2001).

Early epidemiological studies suggested that naturally acquired rotavirus infection provides protection against severe disease, but it does not prevent subsequent infection (Bishop et al., 1983; Velázquez et al., 1996). Therefore, an oral live, attenuated vaccine could simulate natural infection and could induce local intestinal immunity. To be effective, the rotavirus vaccines should be administered to very young children since the natural rotavirus infection occurs during the first 2 years of life causing mild to severe symptomatic infections (Bishop et al., 1983; Rodriguez et al., 1977).

### 1.7 Recommendation for a rotavirus vaccine

During 1997 and 1998, the World Health Organization (WHO), Global Alliance for Vaccines and Immunization (GAVI), the Children’s Vaccine Program (CVP) at the Program for Appropriate Technologies in Health (PATH) and the Centers for Disease Control (CDC) and Prevention, identified rotavirus vaccine as a global public health priority due to the tremendous burden of rotavirus disease. This consortium outlined plans to expedite the availability and introduction of rotavirus vaccines in developing countries where rotavirus-associated mortality was very high.
The plans included:

(1) Reviewing the epidemiology and burden of rotavirus disease and rotavirus strain surveillance in developing countries (including Africa and Asia).

(2) Conducting clinical trials for immunogenicity and effectiveness of available rotavirus vaccines, to assess factors related to introduction of rotavirus vaccines in the Expanded Programme on Immunization (EPI).

(3) Identifying important areas for research in order to expedite the introduction of rotavirus vaccine in developing countries and addressing regulatory and vaccine supply issues related to the introduction of vaccines in those settings.

In 2001, GAVI considered rotavirus vaccines as number one priority for development, evaluation and introduction in developing countries. A rotavirus vaccine was identified as the most promising approach to achieve Target 4 of the United Nations Millennium Development Goal (MDG) i.e. to “Reduce by two thirds between 1990 and 2015, the under five mortality rate”.

Rotavirus vaccines have been developed to provide protection against four epidemiologically important rotavirus serotypes G1, G2, G3 and G4 (Hoshino and Kapikian, 2000). The first licensed live, oral rhesus-human rotavirus reassortment-tetravalent vaccine (RRV-TV; Rotashield, Wyeth Laboratories, Philadelphia USA) was developed in the USA and licensed for routine use in August 1998. The vaccine demonstrated high efficacy in developed countries and lower efficacy in developing countries (Hanlon et al., 1987; Santosham et al., 1991). In October 1999, however, the Advisory Committee on Immunization Practices (ACIP) and the CDC withdrew its recommendation because the vaccine was associated with increased risk of intestinal intussusception (CDC, 1999a).
Since the withdrawal of Rotashield, two additional vaccines have been licensed: Rotarix® and RotaTeq™. Rotarix®, developed by GlaxoSmithKline Biologicals, (Rixensart, Belgium), is a live human attenuated monovalent P1A[8] G1 rotavirus vaccine (De Vos et al., 2004) and its efficacy is based on heterotypic immunity. RotaTeq™, developed by Merck (Blue Bell, PA, USA), is a live attenuated bovine-human vaccine containing five reassortant strains with G1P[7], G2P[7], G3P[7], G4P[7] and G6P[8] specificity (Heaton et al., 2005) and its efficacy is based on homotypic immunity. Both these vaccines have demonstrated high efficacy rates in preventing severe rotavirus diarrhoea among children in middle and high-income countries and neither has been associated with an increased risk of intussusception (De Vos et al., 2004; Ruiz-Palacios et al., 2006; Vesikari et al., 2006). The widespread use of these two vaccines should, therefore, have a significant impact in reducing deaths, hospitalization and medical visits associated with rotavirus diarrhoea globally.

There is a significant difference in the natural history and acquisition of protective immunity to rotavirus disease between the developed and developing countries (Glass et al., 1996; Vesikari, 1999). In anticipation of vaccine introduction, accurate and reliable estimates of rotavirus disease burden in developing countries are crucial in order to assess the need for rotavirus vaccines. It was, therefore, important that rotavirus vaccines be evaluated in both developed and developing countries and all future vaccine trials should monitor vaccine adverse events to include intestinal intussusception. Setbacks, as previously mentioned, have broadened the knowledge and development of safe and effective rotavirus vaccines usage.

With regard to future directions for rotavirus vaccine in developing countries, researchers believe that alternative factors may potentially affect the effectiveness of a new candidate vaccine including:
1. Relatively high maternal antibodies among vaccinees, which may decrease the immune response to specific vaccine proteins and restrict replication of live attenuated virus vaccine.

2. Breastfeeding at the time of vaccination.

3. Micronutrients as well as malnutrition.

4. The presence of other microorganisms in the intestine.

5. HIV and malaria, which could inhibit the immune response of the vaccinees by lowering the effective titre of the oral vaccines or interfering with viral replication (Cunliffe et al., 1998; Glass et al., 2006).

Alternative effective rotavirus vaccine development and testing should continue in order to prepare for future vaccine implementation.

1.8 Experimental proposal

The burden of rotavirus disease is significant in both developed and developing countries. Regardless of public health interventions and good hygiene practices, almost all children will be infected with rotavirus in early childhood. Therefore, rotavirus vaccine will be the only viable measure that may prevent severe disease, since there are no antiviral drugs to treat severe diarrhoea (Bresee et al., 1999). The severity, high incidence and fatality of rotavirus gastroenteritis can be reduced through the implementation of an effective vaccine programme.

South Africa is a country of diverse culture, socioeconomic status, geographical and population settings. Presently, there are several disease (e.g. polio virus, measles) eradication efforts initiated in the country that have been successful in increasing the immunization coverage rate to more than 80%. Following global prevention of severe rotavirus disease, Rotarix® and RotaTeq™ have been licensed in South Africa (South Africa Dept. of Health, MCC, 2006). As of 1 August 2009, the South African Department of Health
included the Rotarix® in the Expanded Programme on Immunization (EPI-SA). Inclusion of rotavirus vaccines in EPI-SA could potentially reach more than 80% of children and could prevent clinical visits, hospital admissions and deaths due to rotavirus gastroenteritis.

Estimates for the South African burden of rotavirus disease are, therefore, crucial for targeting healthcare interventions that have a significant impact on disease prevention. These estimates will provide an excellent framework for the Department of Health to prioritize on such health interventions that prevent childhood morbidity and mortality in South Africa.

1.9 Problem Statement

In many countries in Africa, including South Africa, the disease burden and epidemiology of rotavirus are unknown because of lack of adequate data. Dependable information on rotavirus infections is essential and the country needs accurate information to identify important features in the local epidemiology of the disease and to provide baseline epidemiology and up-to-date estimates of the extent to which severe rotavirus diarrhoea has an impact on the South African population. The burden of disease estimates will create awareness of rotavirus disease within the health care system, among paediatricians, the community at large and policy makers including the health minister. These results will aid in defining strategies for diagnosis and the need to include rotavirus in the national EPI of the country.

In an attempt to quantify the burden of rotavirus disease in monetary terms, a cost analysis study should also be conducted to focus not only on the morbidity expenses in the country but also on the mortality rate caused by gastroenteritis associated with rotavirus infection. Policy makers will have to make decisions regarding the economic burden of disease including direct
cost of health care and indirect cost to individuals and the benefit of vaccination on health and economic outcomes.

1.10 Aim of the study

The aim of the study was firstly, to follow the WHO generic protocol (WHO/V&B/02.15) to assess the burden of rotavirus disease in children less than 5 years of age and secondly to determine circulating rotavirus strains in selected communities. Such information could ultimately be used to monitor the impact of the rotavirus vaccines.

Thus, the broad objectives of the study were:

1) To perform a hospital-based burden of rotavirus disease in a tertiary and secondary health care facilities in the Pretoria area, South Africa.
2) To conduct molecular characterization of rotaviruses circulating in the Pretoria region.
3) To devise an alternative molecular typing method to detect rotavirus VP6 subgroups

Rationale for broad objective 1:

Development, evaluation and introduction of an effective rotavirus vaccine requires the evaluation of burden of rotavirus disease in South Africa. This study evaluated prospective hospital based surveillance to estimate the rotavirus disease burden in the North West (secondary hospital setting) and Gauteng provinces (tertiary hospital settings) of South Africa.
Specific Objectives

1. To estimate the incidence of hospitalizations associated with rotavirus in children less than 5 years of age.
2. To determine the age and seasonal distribution of hospitalization associated with rotavirus in children less than 5 years of age.
3. To estimate the proportion of diarrhoea hospitalizations in children under 5 years of age, that could be attributable to rotavirus infections
4. To monitor temporal trends in the incidence of hospitalization, age distribution, seasonality and strains.

Rationale for broad objective 2:

Anticipating the introduction of an effective rotavirus vaccine, the study documented the rotavirus serotypes in circulation during rotavirus vaccine trials and before the implementation of such a rotavirus vaccination programme. In addition, to determine whether uncommon strains not represented in the vaccines might be circulating in South Africa, which could emerge to become more prevalent following widespread use of the vaccine in children and to identify novel strains that may pose challenges for vaccines. The effectiveness of the rotavirus vaccines against novel or rare circulating rotavirus serotypes remains an open question and cannot be predicted. Thus, research to examine strain diversity of rotavirus and the emergence of new or previously uncommon strains in the population is required, particularly in areas conducting rotavirus vaccine studies. Again, understanding the diversity of rotavirus strains circulating in the country will provides insight as to which vaccines might prove to be efficacious or which rotavirus strains should be targeted by vaccines.

Specific objectives

1. To characterize the genetic diversity of rotavirus strains.
2. To describe in detail the molecular epidemiology of rotavirus infections within communities in the North West and Gauteng Provinces, during consecutive rotavirus seasons, through genotyping of rotavirus-positive samples collected throughout each year from 2003-2005.

3. To monitor any changes in the prevalence of common rotavirus strains circulating in such communities.

4. To identify novel rotavirus strains that may fall outside of the immune response to candidate rotavirus vaccines.

**Rationale for broad objective 3:**

In addition to determining the G and P types of rotavirus strains circulating in the identified communities, a comprehensive analysis of both genetic and antigenic diversity of the VP6 subgroup specificity be carried out by VP6 enzyme-linked immunosorbent assay (ELISA) and by the development of a restriction fragment length polymorphism (RFLP) analysis method using the restriction endonucleases Acil, Ddel and Rsal. The aim of this is to devise an alternative technique that can be widely used as a diagnostic assay for the VP6 subgroup and the technique should be highly sensitive, specific, simple to perform and inexpensive.

**Specific objectives**

1. To perform ELISA to determine rotavirus VP6 antigenic subgroup specificity.

2. To assess genetic diversity by RT-PCR and amplification of VP6 gene (379 bp) and sequencing. The deduced nucleotide and amino acids sequence of the representative strains was compared with reference strains from the Genbank.
To establish genetic lineages between different VP6 subgroup sequences.

4. To compare VP6 monoclonal antibodies results with RFLP analysis using three restriction endonucleases Aci I, Dde I and Rsa I.

1.11 Thesis outline

Chapter 2 provides a literature review of rotavirus infection including history, characterization of rotavirus, epidemiology, evolution, genotypes, natural history of rotavirus infection, pathogenesis, immune response, laboratory diagnosis and rotavirus vaccine.

Thereafter, three individual scientific publications are presented which share results obtained among various section of the study.

Chapter 3 - first paper: Prospective hospital-based burden of rotavirus disease in South Africa from 2003-2004. The study estimated the incidence of rotavirus disease-associated with hospitalizations, the results showed age, gender and seasonal distribution of rotavirus infection in the country.

Chapter 4 - second paper: Molecular epidemiology of rotavirus strains recovered in 2003 to 2005 during the rotavirus vaccine trials. The paper was able to determine the genetic diversity of rotavirus strains recovered from children attending Dr George Mukhari Hospital.

Chapter 5 - third paper: The use of restriction fragment length polymorphism analysis to determine human group A rotavirus subgroups. The genetic diversity of rotavirus subgroups circulating within communities attending Dr George Mukhari Hospital was determined by three restriction endonucleases Aci I, Dde I and Rsa I on selected known nucleotide sequences.
Chapter 6 – General discussion and conclusion:

These data indicate that a substantial number of children who are treated for diarrhoea are infected with rotavirus and most are less than 2 years of age. The study highlights numerous essential factors of rotavirus epidemiology. Firstly, the data demonstrated: (1) that rotavirus infection is the most common cause of hospitalization for severe diarrhoea during autumn and winter months; (2) the rotavirus burden of disease in the Ga-Rankuwa and Madibeng area, South Africa; approximately 22.8% of hospital admissions was due to rotavirus diarrhoea. (3) the incidence of rotavirus associated with hospitalization in both tertiary and secondary hospital settings; the annual incidence of rotavirus was estimated to be one in every 50 to 70 children in the area is likely to be hospitalized with rotavirus diarrhoea between birth and 2 years of age (4) the genetic diversity of rotaviruses circulating in the region and the emergence of uncommon rotavirus strains G12.

It should be noted that accuracy and reliability of information presented in different papers relies extensively on the hospital data. The information can be used as a valuable aid for future use of vaccines. The data suggest that diarrhoeal treatment and rotavirus infection place a significant strain on the South African health care system and support the general introduction of a rotavirus vaccine.
2 CHAPTER 2: LITERATURE REVIEW

2.1 Historical perspective

Bishop and colleagues first discovered human rotavirus in 1973, when rotavirus was observed by electron microscopy in thin sections of duodenal mucosa in the faeces of children with gastroenteritis. The appearance of the viruses in the electron micrograph gave rise to the name, “rota” (a Latin word meaning wheel), with the 70 nm particles displaying short spokes and a well-defined smooth outer rim (Estes, 2001). As a result, laboratories worldwide started to detect the virus in the stools of paediatric patients with gastroenteritis. Within 5 years, rotavirus was considered to be the most important aetiological agent associated with severe diarrhoea in infants and young children, accounting for approximately one third of all severe diarrhoea worldwide (de Zoysa and Feachem, 1985). Almost every child throughout the world, both in industrialized and less industrialized countries, will be infected with rotavirus in the first 5 years of age. This implies that even improvement in sanitation, water resources and food supply will not effectively reduce disease incidence. According to Parashar et al. (2006) rotavirus-associated deaths are significant, with approximately 611 000 deaths annually, occurring mainly in developing countries.

2.2 Characterization of rotaviruses

Early in 1980, rotaviruses were first cultivated in tissue culture (Wyatt et al., 1980). The cultivation of human rotavirus, however, was only successful after trypsin treatment. In combination, these techniques provided more information about the structure of rotavirus particles. Rotaviruses have three important antigenic and genetic specificities based on group, subgroup and serotype/genotypes (Estes and Kapikian, 2007). The rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA) surrounded by a triple-layered capsid (Figure 2:1). An inner capsid consists of viral protein (VP) 6, which is the major determinant of group and subgroup antigen reactivity. Bridger
(1994) concluded that there are seven major rotavirus groups (A-G) based on the VP6 antigen. Epidemiological studies have shown that rotavirus groups A, B, C infect both humans and animals whereas groups D, E, F and G have to date mainly been found in animals (Saif et al., 1994; Geyer et al., 1996; Estes, 2001). Globally, group A rotavirus is most prevalent and is also the most extensively studied rotavirus that infects humans. This group is further subdivided into subgroup I, subgroup II, subgroup I+ II and subgroup non- I non- II specificity, based on the subgroup epitopes on VP6 (Bishop, 1994). Rotaviruses are additionally classified based on the VP7 and VP4 genes and associated outer capsid proteins in order to specify the G and P types, respectively, leading to the development of a binary classification system (Estes, 2001; Kapikian et al., 2001). RNA-RNA hybridization studies have identified three distinct human rotavirus genogroups; represented by Wa (subgroup II, long electropherotype), DS-1 (subgroup I, short electropherotype) and AU-I (subgroup I, long electropherotype) (Nakagomi et al., 1989), respectively.

Group B rotaviruses are less common and are mainly endemic in rats and pigs. However, these viruses have been associated with major waterborne epidemics of diarrhoeal disease in adults and children in China (Donelli and Superti, 1994; Christensen, 1989). Group C rotaviruses, on the other hand, have been associated with sporadic cases of diarrhoeal diseases worldwide in children and adults (Von Bonsdorf and Svensson, 1988).

2.3 Molecular characterization of rotavirus

2.3.1 Particle structure and genome arrangement

Rotaviruses are classified as a genus in the family *Reoviridae*. The genome consists of 11 segments of double stranded RNA (dsRNA) surrounded by a non-enveloped icosahedral structure. The genome codes for six structural viral proteins (VP1-VP4, VP6 and VP7) found in the virus particle, and six
non-structural proteins (NSP1-NSP6) that are found in infected cells. Viral particles possess a triple-layered capsid composed of outer layers made up of VP4 and VP7 proteins as well as an intermediate layer of VP6 protein and an inner core layer composed of shell protein VP2 and enzymes VP1 and VP3 (Figure 2.1). The complete virion is about 102 nm in diameter that includes the spikes (Estes, 2001). The molecular weights of rotavirus proteins range from $2 \times 10^5$ to $2.2 \times 10^6$ daltons, the size of the gene fragments range from 0.6-3.3 kilobase pairs (kbp) and the genome size totals approximately 18kbp (Kalica et al., 1978).

The RNA segments are numbered according to their sizes from largest to smallest and produce four electropherotype patterns when separated by polyacrylamide gel electrophoresis (PAGE). The RNA segments of group A rotaviruses are designated by four large segments, two medium sized segments, three smaller segments and lastly two smallest segments giving rise to a 4-2-3-2 pattern. The segments range from 3 302 base pairs (segment 1) to 667 base pairs (segment 11) (Shaw and Greenberg, 1999).

The viral core contains its own RNA-dependent RNA polymerase that transcribes the individual RNA segments into active mRNA. Each RNA segment contains a 5’ methylated cap consisting of a sequence of $m^7$GpppG”GPy followed by a 5’ nontranslated sequence, followed by an open reading frame (ORF) coding for the protein product, and another set of noncoding sequences as well as 3’ terminal cytosine nucleotides (nt). No poly (A) tract is found in the RNA segments. In each RNA segment, the 5’ and 3’ ends consist of terminal consensus sequences of about 7-9 nt, that are highly conserved and are unrelated, which means they have different functions. The 5’ and 3’ terminal consensus sequences are important in packaging as well as the regulation of rotavirus gene expression at the levels of transcription, replication and translation (Shaw and Greenberg, 1999).
2.3.2 Rotavirus structural proteins

2.3.2.1 Core and inner capsid proteins

VP1

VP1 is the product of RNA gene segment 1 and is a minor component of the central core. VP1 comprises only 2% of the viral mass (Liu et al., 1988) and its molecular weight in group A and group C rotaviruses is 125 000 daltons and for group B is 136 000 daltons. The group C VP1 gene sequence is well conserved. VP1 is less important from a structural point of view and is associated with RNA transcription with viral transcriptase and replicas function. VP1 is one of the three proteins (VP1, VP2, and VP3) that make up the rotavirus core particle. VP1 and VP3 form part of the RNA-dependent RNA polymerase activity associated with double-shelled particles (Patton, 1995).

The following evidence has suggested that VP1 has transcriptase and replicas activity.
• A: Si: SA11 its C mutants mapping to gene segment 1 have a sera and dsRNA negative phenotype (Gambol et al., 1987).

• All VP1 sequences of groups A, B and C share the four common motifs conserved in the sequences of all RNA-dependent RNA polymerases (Pooch et al., 1989; Eiden and Hirsh on, 1993), and the conserved region is probably important for the structural or functional activity of the polymerase.

• When using the photoreactable nucleotide analogue azido-adenoside triphosphate, it must be cross-linked with VP1 of transcriptionally active VP6 particles (Valenzuela et al., 1991). Cross-linking blocks the activity of the transcriptase, indicating that VP1 is a nucleotide-binding protein and implies that VP1 is the RNA polymerase. When the photoreactable reagent is not used, single-shelled particles use azido-ATP as substrate for the synthesis of viral mRNA (Valenzuela et al., 1991).

• VP1 specifically binds to the 3' end of viral mRNA (Patton, 1995).

• Co-expression of A: Bo: RF: VP1 and A: Si: SA11: VP3 in the baculovirus system provides evidence of polymerase activity as determined by a poly (U)-oligo (A) polymerase assay.

**VP2**

VP2 is a product of RNA gene segment 2, and forms the major protein component of the rotavirus central core. The core is estimated to contain approximately 200 molecules of VP2 and 120 molecules are organised as 60 dimers on a T=1 symmetry. VP2 is the third most abundant protein in double-shelled viral particles. The VP2 protein is immunogenic and serum antibodies to VP2 protein are good indicators of previous infection (Conner et al., 1988). Amino acid (aa) sequence analysis of gene segment 2 demonstrated leucine at every seventh residue starting at amino acid 536 and 665. The leucine zipper is thought to represent a part of protein that interacts with a target site on DNA (Kumar et al., 1989). In addition, an RNA overlay-protein assay experiment has shown that the VP2 protein has nucleic acid-binding activity.
(Boyle and Holmes, 1986). VP2 is found to be myristylated (Clark and Desselberger, 1988) and lacks the NH$_2$-terminal sequence Gly-X-X-X-Ser/Thr present in many other myristylated proteins (Chow et al., 1987). The VP2 gene sequence of rotavirus strains such as simian SA11, human Wa and bovine RF has been determined and show similarity at the nucleotide and protein levels (Estes and Cohen, 1989).

VP2 plays an important role in the structure and function of the central core. For example:

- VP2 is a component of viral replication linked to the ability of VP2 to bind to the mRNA template for minus strand synthesis. This was suggested by the results of replicas assays performed with mutant VP2 (Patton et al., 1997).
- Each VP2 protein is rich in proline and these residues are highly conserved and also contain leucine zipper motifs, which have been suggested to be involved in dimerization of nucleic acid binding proteins (Mattoin et al., 1994).
- The two internal VP1 and VP3 proteins interact within the inner surface of VP2 at the fivefold axes (Labbe et al., 1991).
- VP2 interacts with the trimers of VP6 and are perforated by 132 aqueous channels transporting metabolites in and nascent RNA out during transcription (Taniguchi et al., 1986).
- In the absence of other viral genes, the expression of VP2 in the baculovirus system has shown that VP2 assembles into particles that have similar morphology to the core (Labbe et al. 1991).
- In the absence of RNA, VP2 presents some sites (Gln-92 and Lys-93) that are susceptible to protease or VP2 has autoproteolytic activity under certain circumstances (Mattoin et al., 1994).
VP3

VP3 is a product of RNA gene segment 3 and is the minor sub core protein of 835 amino acids with a predicted molecular weight of 98,000 daltons. The deduced amino acids sequence of gene segment 3 contains multiple repeats of amino acids. Baculovirus system expression of VP3 in the absence of other viral proteins has shown a covalent bond to GTP, which shows that VP3 has viral guanyltransferase activity. The VP3 was shown to covalently bind $\alpha^{32}\text{P}\text{-GTP}$ (Liu et al., 1992) and has methyltransferase activity. Although produced in small quantities, the main function of these enzymes is to produce the capped mRNA transcripts. Adenoside 5'-triphosphate 2',3'-dialdehyde and guanine 5'-triphosphate 2',3' dialdehyde inhibit in vitro transcription (Pizarro et al., 1991). Therefore, VP3 could have an impact on transcription or an association with phosphatase or ATPase activity. The VP3 sequence has high homology with RNA polymerase from other viruses, which suggests that this protein is involved in RNA replication (Estes and Cohen, 1989).

VP6

VP6 is the product of RNA gene segment 6 and is the major structural protein of the inner viral capsid, which constitutes 51% of the virion and has a molecular weight of 44,816 daltons (Liu et al., 1988). During the infection processes, VP6 plays important structural, immunological and morphogenic functions. VP6 is a trimeric protein that is stabilized by noncovalent bonds on the surface of viral particles (Prasad et al., 1988). Studies have shown that the removal of VP6 from single shelled particles results in transcriptase activity failure, while addition of VP6 again restores transcriptional activity (Kohli et al., 1992). VP6 is, thus, associated with viral RNA transcriptase activity although it has no polymerase activity by itself. It is unknown whether VP6 functions as a structural component, keeping VP1, VP2, VP3 and RNA segments in the proper conformation to permit transcription, or participates actively in the transcription process (Shaw and Greenberg, 1999).
VP6 contains epitopes that have been used to classify the group A viruses into subgroup I, subgroup II, subgroup I + II and subgroup non-I non-II, based on the presence or absence of two distinct epitopes reactive with one, both or neither of the monoclonal antibodies described by Greenberg et al. (1983). The subgroup specific epitopes on VP6 have been used as an epidemiological tool because they define and identify different virus strains. Human and animal group A rotaviruses share antigenic determinants on VP6 as well as epitopes which specify subgroup I and II. In Group A rotavirus, the immunodominant sites of the VP6 that contain the group specific epitopes are found in four regions (aa residues 32-64, 155-167, 208-294 and 380-397) (Holmes, 1983). The aa residues comprising subgroup specific epitopes are located at aa 172, 305 and region 296-299, respectively, whereas VP6 has a single ORF encoding a protein of 397 amino acids.

2.3.2.2 Outer capsid proteins

VP4

VP4 is the product of gene segment 4. It is the minor outer capsid non-glycosylated protein, which constitutes only 2% of the viral mass and has a molecular weight of 88 000 dalton (Liu et al., 1988). VP4 forms 60 surface projections of about 10-12 nm in length with a knoblike structure at the distal end on the smooth outer capsid of VP7. VP4 is the viral attachment protein (Ludert et al., 1996). As an important outer capsid protein, VP4 performs various functions that have been identified by genetic and immunologic analysis including (1) VP4 has a haemagglutinating activity (Kalica et al., 1983), (2) VP4 carries neutralization-specific epitopes (Hoshino et al., 1985b), (3) VP4 is a cell attachment protein and (4) VP4 is an important determinant of virulence.

The proteolytic cleavage of VP4 enhances viral infectivity. The cleavage of the VP4 gene with proteases such as trypsin, results in two polypeptides, VP5* and VP8*. This is necessary for the virus to efficiently infect the cells and facilitates
virus entry into the cell (Estes et al., 1981; Kaljot et al., 1988). The molecular weight of VP8* is approximately 60 000 while VP5* is 28 000 daltons (Graham and Estes, 1980). VP8* is responsible for the binding of sialic acid and for haemagglutinating activity found in many animal rotavirus strains (Fiore et al., 1991). Antibodies against VP8* neutralize the virus by inhibiting viral attachment (Kaljot et al., 1988). The function of VP5* is for viral attachment that does not require sialic acid for viral infectivity (Estes et al., 1981) and it also plays an important role in cellular entry (Denisova et al., 1999). Proteolytic cleavage is found at a conserved region of arginine residues 241 and 247 (Lopez et al., 1985). Other conserved cysteine residues are found in positions 216, 318, 380 and 774 in all rotavirus strains (Gorziglia et al., 1988). Most animal rotaviruses have an additional cysteine residue at position 203 (Mackow et al., 1988).

Variable sites were found close to the amino terminus in the region of VP8* at aa 140-160 and aa 180-210 and between aa 580-610 in the VP5* product (Mattion and Estes, 1991). Baculovirus expression of VP4 proteins showing binding to erythrocytes and haemagglutinating activity; the VP4 protein generated induces antibodies that inhibit haemagglutination of homologous strains and neutralizes viral infectivity (Estes et al., 1981). In vitro, VP4 determines growth and plaque formation (Greenberg et al., 1983; Kalica et al., 1983), directs virulence in mice (Offit et al., 1986) and influences pathogenesis in humans (Flores et al. 1986).

VP4 induces neutralizing antibodies that define P (protease–susceptible) serotypes. The neutralizing activity is serotype specific. Various studies indicate that in protection against rotavirus disease, both VP4 and VP7 appear to be type specific (Offit et al., 1986; Hoshino et al., 1988; Bernstein et al., 1995; Rennels et al., 1996; Santosham et al., 1997).
The sequence analysis of VP4 shows a long ORF of 776 aa in animal rotaviruses and 775 aa in human strains. Interactions between VP4 and structural proteins and non-structural proteins have been observed within the endoplasmic reticulum (ER) and membrane complexes of VP4, NSP4 and VP7 have been detected. The function of such complexes is to participate in the budding of the single-shelled particles into the lumen of the ER, where maturation to double-shelled particles occurs (Mattion and Estes, 1991).

VP7

VP7 is the product of (1) RNA gene segment 7 in rhesus rotavirus (RRV) serotype 3, (2) RNA gene segment 8 in bovine rotavirus serotypes 6 and (3) of RNA gene segment 9 in simian rotavirus (SA11) serotype 3. The VP7 protein is encoded by gene segment 7, 8 or 9, depending on the strain. VP7, the major outer capsid protein, is glycosylated and constitutes 30% of the viral mass (Liu et al., 1988). VP7 is composed of 780 molecules arranged as 260 trimers, perforated by 132 channels and each VP7 molecule has a molecular weight of 34 000 (Kapikian et al., 2001). VP7 contains N-linked high mannose oligosaccharide residues that are modified by trimming (Estes and Cohen, 1989). VP7 is an integral protein that is translated by ER ribosomes and translocated into the lumen of the ER.

Sequence analysis of the VP7 gene segment reveals two ORF’s encoding polypeptides of 326 and 286 aa. The ORFs are in frame and start with hydrophobic sequences that could be signal sequences directing the nascent VP7 to the ER. The glycosylation site at aa 69 is present in nearly all VP7 molecules except calf rotavirus strains NCDV and RF, whereas other glycosylation sites are located at aa 146, 238 and 318 (Desselberger and McCrae, 1994). Conserved cysteine residues are found in aa 82, 135, 165, 191, 207, 244 and 249, while conserved proline residues are found at aa 58, 86, 112, 131, 167, 197, 254, 275 and 279 (Desselberger and McCrae, 1994).
VP7 is a highly immunogenic protein that induces neutralizing antibodies specific to the G serotypes of group A rotavirus (Estes and Cohen, 1989). According to the reactivity of VP7-specific MAbs and the results of experiments done by Sabara et al. (1985) and Fukuhara et al. (1988), VP7 is the viral cell attachment protein. Serotype specific MAbs in an enzyme-linked immunosorbent assays (ELISA) were widely used in early epidemiological studies for detection of rotavirus serotypes. Currently, there are 23 VP7 serotypes that have been identified and shared between humans and animals. Serotypes G1-G4 and G9 are the most prevalent serotypes circulating globally in humans (Santos and Hoshino, 2005; Solberg et al., 2009; Ursu et al., 2009).

2.3.3 Nonstructural proteins

NSP1

NSP1 is the product of gene segment 5, with a molecular weight of 58 400 in SA11 strains. The protein is found in infected cells and in a cell free system, NSP1 is synthesized in large amounts relative to other viral proteins (Ericson et al., 1982). Results from hybridization studies have indicated that the sequence of the NSP1 gene is considered the least conserved of all group A rotavirus genes (Mitchell and Both, 1990). A region rich with cysteines near the amino terminus of the NSP1 protein is highly conserved. This cysteine rich sequence C-X2-C-X8-C-X2-C-X3-H-X-C-X2-C-X5-C forms a zinc (Zn) finger motif, where X indicates any other amino acids. In vitro studies have shown that the conserved zinc finger motif is responsible for binding zinc to the 5’ end of the viral mRNA (Hua et al., 1994).

Indirect immunofluorescence indicates that NSP1 is found throughout the cytoplasm and can also be associated with the cytoskeleton when analysed by cellular fractionation studies. This indicated that NSP1 co-purifies with the
cytoskeletal matrix. In addition, NSP1 binds to all rotavirus mRNAs near the 5’ end (Hua et al., 1994). The functional role of the NSP1 to bind to the viral mRNAs and cytoskeleton is not known. Using a vaccinia virus expression system in mammalian cells, NSP1 binds to interferon regulator factor 3 (IRF3) and induces degradation during a proteasome-dependent pathway (Piña-Vázquez et al., 2007). Previous studies described by Graff et al. (2002) using yeast two hybrid and Barro and Patton, (2005) using rotavirus wild type, also indicates that NSP1 down-regulates the interferon (IFN) response, because it interacts with IRF3, resulting in the proteasome-mediated degradation of IRF3. A subsequent study by Barro and Patton, (2007) suggests that rotaviruses have a mechanism to suppress the IFN signalling pathway during early stages of infection. Strains encoding defective NSP1 grow poorly in some cell lines because of failure to suppress IFN expression. The study indicated that rotaviruses encoding wild-type NSP1 subvert IFN signalling by inducing the degradation of IRF3 and IRF7 (Barro and Patton, 2007). During viral replication, NSP1 plays a role in regulating the efficiency of viral gene expression or in modulating host cell responses. Thus, in the infected cells NSP1 is expressed at very low levels, predominantly soon after infection (Ericson et al., 1982), however, in mature infected cells the NSP1 is synthesized at high levels. This suggests that NSP1 might play a regulatory role in the viral replication cycle.

Defective NSP1 has been identified that contains rearrangements in genome segments 5 which can grow in cell culture (Hundley et al., 1985; Patton et al., 2001). Both in vitro and in vivo studies have indicated that reassortment of gene segments 5 occurs during mixed infections (Allen and Desselberger, 1985). Defective NSP1 viruses yield smaller plaques when plated as compared to their parent (Tian et al., 1993). These suggest that NSP1 may play a regulatory role during RNA replication, genome reassortment or are involved in packaging of genomic RNA segments (Ericson et al., 1982; Patton et al., 2001)
NSP2

NSP2 is encoded by gene segment 8 of SA11 rotavirus strains and is located in the viroplasms of infected cells where viral replication is taking place (Petrie et al., 1984). NSP2 is involved in packaging of viral mRNA. Analyses of temperature sensitive rotavirus mutants indicate that NSP2 is important for the formation of viroplasm and genome replication (Ramig and Petrie, 1984; Gambol et al., 1985). At nonpermissive temperatures, the empty virions are found in tsE-infected cells, indicating a role for NSP2 in coordinating the packaging and replication of viral RNA with virion morphogenesis. These results suggest that NSP2 plays a major role in RNA replication or sera packaging into subviral particles (Patton, 1995).

NSP2 have multiple activities; it binds single-stranded RNA non-specifically, it has Mg\(^{2+}\)-dependent nucleoside triphosphate activity (NTP), in vitro studies with purified recombinant and radiolabelled NTPs have shown that hydrolysis results in the phosphorylation of NSP2, it has helix-destabilizing activity with purified bacterially expressed proteins and it is the component of replication intermediates (Taraporewala and Patton, 2001). This suggests that NSP2 might unwind and help package RNA and bind to and work with VP1 to create its functionality. Jayaram et al. (2002) has shown the atomic structure of NSP2 with the probable site involved in NTP binding and hydrolysis, and RNA binding is distinct with small overlap.

NSP2 and NSP5 appear to be responsible for the replication of virus RNA and for its incorporation into new virions and NSP5 seems to work with NSP2 and VP2 to delay the assembly of the outer capsid long enough to allow RNA replication to complete (Berois et al., 2003).
**NSP3**

NSP3 is the product of gene segment 7, 8 or 9 depending on the virus strain. In SA11 it is from genome segment 8, RRV genome segment 9 and bovine UK virus genome segment 7 (McCrae and McCorquodale, 1982; Mason et al., 1983; Gambol and Ramig, 1986). NSP3 has been found in large amounts in infected cells with complexes containing replicas activity (Patton and Gallegos, 1988).

NSP3 is responsible for helping to regulate the synthesis of mRNA from the virus dsRNA genome (Patton et al., 2001). Using co-immunoprecipitation assays, NSP3 has been found to interact with eukaryotic initiation factor 4 gamma 1 (eIF4GI) complexes. It regulates circularization of mRNA by binding simultaneously to the 3' consensus sequence and the cap binding protein eIF4G (Piron et al., 1998). During rotavirus infection, the competition between NSP3, eIF4GI complexes and poly (A) binding protein (PABP) are responsible for the shut off of cellular protein synthesis (Piron et al., 1998). Therefore, NSP3 is involved in new viral mRNA translational regulation.

**NSP4**

The NSP4 is a nonstructural transmembrane protein of the rough ER-specific glycoprotein. NSP4 is the product of gene segment 10 and is 175 aa long with molecular weight of 28 000 (Mattion et al., 1994). There are five known NSP4 genogroups: KUN (A) includes the human DS-1 genogroup, bovine, equine and SA11 rotaviruses, Wa (B) includes the humans Wa genogroup and porcine rotaviruses, AU-1 (C) includes human AU-1 genogroup, rhesus, feline, canine and lapine rotaviruses, EW (D) includes murine rotaviruses, and avian (E)-like, and, at least in non-human species, they appear to cluster based on the species from which rotavirus was isolated, suggesting a conserved pattern of evolution within species (Cunliffe et al., 1997; Ciarlet et al., 2000). The NSP4 of human rotavirus strains are
clustered into two, i.e. genotypes A and B of the five known genotypes (Iturriza-Gómar et al., 2003).

NSP4 acts as a transmembrane protein of the rough ER and its amino terminus is maintained in the luminal side and carboxy terminus is extended into the cytoplasm (Chan et al., 1988). Rotavirus NSP4 plays an important role in morphogenesis in infected cells. Its function is to act as intracellular receptor by mediating the conversion of double-layered particle in the cytoplasm to triple layered particle in the ER that transports subviral particles across the membrane of the ER and therefore, NSP4 acts as a molecular chaperone. NSP4 acts in viral replication including packaging and particle assembly (Silvestri et al., 2005). NSP4 also functions as an enterotoxin, i.e. when the NSP4 protein of simian rotavirus expressed as a baculovirus recombinant is administered by intraperitoneal or intraluminal route, it acts as an enterotoxin by inducing age-dependent diarrhoea in mice (Estes and Morris, 1999). This suggests that rotavirus virulence can be associated with certain NSP4 sequences. The other function of NSP4 lies in its modulation of intracellular calcium levels and chloride secretion (Silvestri et al., 2005). Amino acid residues 114-134 of NSP4 SA11 strain is capable of inducing diarrhoea in suckling mice, and tyrosine at residue 131 is associated with toxigenic activity of NSP4 (Kapikian et al., 2001). Vaccination against NSP4 in mice induces both homotypic and heterotypic protection against rotavirus (Estes et al., 2001). In humans, NSP4 induce humoral and cellular immune responses (Johansen et al., 1999a).

**NSP5**

NSP5 is a product of gene segment 11 of SA11 with molecular weight of 26 000. Gene segment 11 has been found to have two ORFs (Mitchell and Both, 1988) that code for NSP5 protein of 198 aa and NSP6 protein of 92 aa, both of which accumulate in viroplasm (Petrie et al., 1984). NSP6 interacts with
NSP5 during the replication process, but the precise function of NSP6 remains unclear. NSP6 appears to change the three-dimensional structure of NSP5 in order to allow NSP5 to function more effectively (Torres-Vega et al., 2000). It has been suggested that NSP6 has a regulatory role in the self-association of NSP5 (Torres-Vega et al., 2000). In any event, it seems clear that NSP6 is involved in the replication of the viral genome.

NSP5 is an O-glycosylated phosphoprotein rich in serine (Ser) and threonine (Thr) residues that self assemble into dimers and have non-specific RNA-binding activity (Gonzalez and Burrone, 1991). Co-transfection experiments involving NSP2 and NSP5 indicate that NSP2 upregulates the phosphorylation of NSP5. In addition, a co-immunoprecipitation study indicated the interaction of NSP5 with NSP2 from extracts of UV-treated rotavirus infected cells (Afrikanova et al., 1998). Both NSP5 and NSP2 are nonstructural viroplasmic proteins that are found to be associated with precore replication intermediates that are precursors of the viral core (Berois et al., 2003). Defective expression of NSP2 or NSP5 inhibits the formation of viroplasms, genomic replication and viral assembly (Silvestri et al., 2004). Therefore, the function of NSP5 may be to regulate the binding of RNA by NSP2 during replication and packaging (Pesavento et al., 2006). Afrikanova et al. (1998) has shown that NSP5 can form protein-protein cross-linking of infected lysates with VP1 and NSP2.

2.4 Epidemiology

2.4.1 Geographic and seasonal distribution

The peak of rotavirus infection varies in different countries and from year to year (Cook et al., 1990), and during a single year, the prevalence of rotavirus infections can be variable across regions within the same country.
Climatic conditions have a major influence on the incidence of rotavirus infections, more especially in regions where there is seasonal change. For example, in temperate climates (mostly developed countries), rotavirus disease occurs during epidemic peaks in the cooler, drier months. Many studies have shown significant winter seasonality of rotavirus infection, although endemic and sporadic cases may occur during other months. Rotavirus infections in South Africa display a distinct seasonal pattern with epidemic peaks occurring predominantly in the cooler winter months of the year and a low rate of rotavirus infection in the summer months of the year (Steele et al., 1986). In the United States, Europe, India, Asia, Republic of Korea and Japan, hospitalization rates of rotaviruses peak during winter months (Koopmans and Brown, 1999; MMWR Recommendations and Reports, 1999; Rivest et al., 2004; Bresee et al., 2004; Kim et al., 2005; Nakagomi et al., 2005; Gleizes et al., 2006). During the winter season, nearly 50% of all paediatric gastroenteritis may be due to rotavirus. There is no clear explanation for winter epidemic peaks. The proposed mechanism underlying seasonal variation probably involves the interplay of many factors including survival of virus in the environment, low indoor relative humidity, higher airborne transmission, physiological effects on the host and the degree of crowding during the winter season (Bishop, 1996).

In tropical areas, seasonal rotavirus gastroenteritis patterns are not as pronounced and rotavirus infection occurs throughout the year with no marked increase in the disease (Cook et al., 1990) and in some countries, a minor increase may be observed during the dry season. In sub Saharan Africa, increased cases of rotavirus infection are usually experienced during the drier months of the year and a low rate of rotavirus infection during the wet season (Cunliffe et al., 1998, Binka et al., 2003). The recent seasonal rotavirus infection data in the tropical regions of Africa showed a marked increase of rotavirus diarrhoea during the dry season (AfrRSN unpublished data).
2.4.2 Transmission

Rotavirus is highly infectious and the primary mode of transmission is via the faecal-oral route. Ingestion of virus aerosol has also been suggested but has not been proven (Santosham et al., 1983; Stals et al., 1984). Fragoso et al. (1986) indicated that rotavirus was detected in respiratory tract secretions. Co-morbidity of rotavirus and upper respiratory infections has been shown by another study (Goldwater et al., 1979). It is difficult to control rotavirus transmission because the virus is stable on environmental surfaces and is relatively resistant to chemical disinfectants (Ward et al., 1991). Rotavirus spreads easily in circumstances of overcrowding and poor hygiene where transmission can occur by drinking contaminated water, or ingestion of contaminated food that do not require further cooking like fruits, vegetables and salads. Further transmission can also occur by touching contaminated hands or contaminated surface (Dennehy, 2000).

The presence of high concentrations (e.g. \(10^{11}\) viral particles per gram faeces) of rotaviruses in the stools of infected children leads to successful spread of the virus since the virus can survive for days to weeks on surfaces. Rotavirus is viable on hands for at least for 4 hours but Morris et al. (1975) has shown that rotavirus in calf faeces can retain infectivity for up to 7 months at room temperature. Rotavirus infections are most common during the cooler months of the year and may be spread more easily due to low indoor humidity, which may contribute to aerosol transmission (Brandt et al., 1982).

Transmission of rotavirus can be easily facilitated in children’s’ day care centres or family day care homes through frequent exposure of susceptible hosts. Outbreaks of rotavirus infections are numerous in day care centres where large numbers of young children are found. Common sources of rotavirus contamination in homes and day care centres are amongst other,
diaper disposal containers, diaper-changing rooms, toys, hand washing areas, telephones, water fountains and water-play tables (Butz et al., 1993).

In addition, rotavirus infection in younger children may be acquired from older siblings or alternatively from a parent with an asymptomatic infection (Engleberg et al., 1982). Animal-to-human rotavirus transmission has also been suggested due to the fact that certain animal rotaviruses share neutralization antigens with human rotaviruses and naturally occurring strains may infect humans or form reassortants with human rotaviruses (Nakagomi and Nakagomi, 1993).

2.4.3 Nosocomial rotavirus gastroenteritis

Studies have shown that nosocomial rotavirus infections in paediatric wards and hospital nurseries are quite frequent (Steele et al., 1987; Gerna et al., 1990; Bishop, 1994; Das et al., 1994; Kilgore et al., 1996; Gianino et al., 2002). Nosocomial rotavirus infection is estimated to cause 40-50% asymptomatic cases within 72 hours of hospital admission (Kilgore et al., 1996). In European countries including France, Germany, Italy, Poland, Spain and the United Kingdom, nosocomial infection is responsible for 31-87% (Gleizes et al., 2006) of rotavirus cases. A study by Middleton et al. (1977) demonstrated that approximately one in every five children with rotavirus infections appeared to be acquired in hospitals. Nosocomial rotavirus infection causes a significant economic burden due to prolonged hospital stays that also serves as a virus reservoir resulting in additional cases of rotavirus infection (Frühwirth et al., 2001).

Nosocomial infection of rotavirus in newborn babies often appears to be avirulent and asymptomatic. These mild or asymptomatic clinical outcomes may possibly be explained by the presence of secretory IgA and trypsin
inhibitor in breast milk as well as a delay in the appearance of proteolytic enzymes in the neonatal gut (White and Fenner, 1986).

### 2.4.4 Community outbreaks of rotavirus

Community outbreaks of rotavirus infection occur infrequently and may be caused by either food-borne or water-borne contamination. In water-borne epidemics, the virus is resistant to chlorination (Ward et al., 1991). This leads to the detection of rotavirus in both untreated and treated sewage water (Dubois et al., 1997). In the event of food-borne contamination, it is possible that oysters and mussels can be heavily contaminated by rotavirus (Christensen, 1989). Outbreaks of rotavirus infection in school children as well as adults, has been reported (Griffin et al., 2002a; Mikami et al., 2004).

Because rotaviruses are highly contagious and can be transmitted easily, the primary means to prevent rotavirus transmission involves stringent hygiene measures. We know that rotaviruses are relatively resistant to chemical disinfectants as well as antiseptics commonly used in the hospital, but chemical disinfectants containing chlorhexidine gluconate, quaternary ammonium compounds with a high alcohol content as well as Lysol disinfectant sprays can successfully prevent rotavirus infection (Ward et al., 1991). Effective disinfections of contaminated materials, careful attention to hand washing and disposal of contaminated material is, therefore, an important measure to contain rotavirus infection, more especially in nurseries and hospitals where nosocomial infections occur frequently.

### 2.4.5 Animal rotavirus

Rotaviruses have been described in avian (e.g. chickens, turkey, pigeons, ducks) and mammal (e.g. simian, bovine, ovine, caprine, equine, murine,
feline, porcine, lapine and canine) species (Woods et al., 1976; McNulty et al., 1976; 1978; Saif et al., 1994). In both mammalian and avian species, the clinical presentation of rotavirus infections vary from sub clinical to symptomatic severe diarrhoea, associated with weight loss and increased mortality (McNulty et al., 1980). In the United States, the economic impact of animal rotavirus infections, especially in cattle is significant. Since calf deaths occur at the rate of 5-20%, these results in a loss of $3.1 and $8.7 million annually (House, 1978). In other countries, the economic impact of animal rotavirus has not been investigated but the epidemiology of animal rotavirus has been confirmed throughout the world including the US, United Kingdom, Hungary, Brazil and Australia (Gouvea et al., 1994b; Saif et al., 1996; Palombo et al., 2000).

2.5 Evolution of rotavirus

2.5.1 Genetic variations

There is great genetic diversity of rotaviruses that has evolved from three mechanisms: (1) Point mutations (2) Gene reassortants (3) Gene rearrangement. These mechanisms have provided major challenges in epidemiological studies of rotavirus (Blackhall et al., 1996).

2.5.1.1 Point mutations

A point mutation refers to a spontaneous change of a single base pair caused by chemical modification of DNA. RNA-dependant RNA polymerases, similar to those found in rotaviruses, do not have proofreading activity leading to high mutation rates during replication. Studies have revealed that rotavirus mutation rates of \(<5 \times 10^{-5}\) mutations per nucleotide per viral replication have been found. This mutation rate suggests that the average rotavirus genome differs from its parental genome by at least one mutation (Blackhall et al., 1996). Antigenic drift, thus, results from the accumulation of point mutations, caused by deletions, substitutions and insertions during replication.
The mutations may be conserved and accumulate in progeny viruses, defining genetic lineages and sublineages (Iturriza Gómara et al., 2001). In addition, mutations in antigenically important sites may lead to the generation of monotypes able to escape detection by antibodies in laboratory essays and in the human host (Coulson et al., 1996).

2.5.1.2 Reassortment

The segmented nature of the rotavirus genome may facilitate reassortment between different strains during mixed infection leading to a variety of phenotypes (Kapikian et al., 2001). Both in vitro and in vivo studies have indicated that reassortment of rotavirus gene segments occurs at high frequency during mixed infections of two or more rotavirus strains. The ability of rotavirus to reassort during mixed infections causes an exchange of genetic material between animal and human viruses (Midthun et al., 1986; Gulati et al., 1999; Maunula et al., 2002) thus generating novel virus strains. Rotaviruses may evolve through accumulation of point mutations (genetic drift) and through gene reassortment (genetic shift) during dual infections (Itturiza-Gómara et al., 2003). Earlier studies have shown that genetic reassortment occurs only among viruses of the same genogroup (Yolken et al., 1988). For example, serotype G1, G3, G4 or G9 associated with P[8] strains have the ability to cross hybridize with other genogroup member for all genes except the VP7 gene. Whereas the G2P[4] strains does not cross-hybridize any gene segment with Wa genogroup (Gentsch et al., 2005). Recent epidemiological studies have indicated the existence of inter genogroup reassortants with gene segment exchange between the Wa and Ds-1 strains (Medici et al., 2007; Mc Donald et al., 2009). Phylogenetic sequence analysis of co-circulating G1 and G4 strains with long electrophoretic patterns associated with the P[8] strain has revealed two distinct genetic lineages according to the G-types, demonstrating that reassortment occurs. However, partial sequence analysis of all 11 RNA segments revealed genetic diversity among gene segments other than VP4 and VP7 gene (Maunula and von Bonsdorff, 2002).
Interspecies transmission of animal rotavirus genes to human rotavirus genes has been documented through reassortment (Nakagomi et al., 1989). These provide a large pool of genes and allow substantial diversity of circulating rotaviruses. For example, serotypes G6 and G8 are frequently detected in ruminants whereas serotype G5 is commonly found in pigs. In addition, these serotypes have also been identified in humans (Alfieri et al., 1996; Cunliffe et al., 2000; Park et al., 2006).

2.5.1.3 Rearrangement of viral genome

Genome rearrangement occurs when RNA segments are altered from the characteristic 4-2-3-2 electrophoretic pattern and replaced by differently migrating bands of dsRNA (Pedley et al., 1984). The new segments usually represent concatemeric forms of dsRNA containing sequences specific for the missing RNA segments whereas the fast migrating segments represent deletions (Pedley et al., 1984). Genome rearrangement was observed on RNA segment 5, 6, 8, 10 and 11, with a frequent genomic rearrangement detected in segment 5 and 11 (Pedley et al., 1984; Hundley et al., 1985; Tanaka et al., 1988; Kojima et al., 1996; 2000). During mixed infection, replication or gene packaging, segments can be exchanged between two different strains.

Genome rearrangements have been isolated from chronically infected immunodeficient patients as well as from asymptomatic immunocompetent children and animals such as pigs, calves and rabbits (Pedley et al., 1984; Besselaar et al., 1986; Pocock, 1987; Mattion et al., 1988; Tanaka et al., 1988). Hundley et al. (1985) described the genome rearrangement obtained during multiple passage of tissue culture of adapted bovine and simian rotavirus at high multiplicity of infection. In vitro studies have shown that genomically rearranged segments are not defective (Hundley et al., 1985) but
can reassort to replace normal RNA segments both structurally and functionally (Allen and Desselberger, 1985).

2.6  Antigenic and genetic composition

2.6.1  Serotypes

Rotaviruses are classified into different P and G types based on the two outer capsid proteins, VP4 and VP7. Similar to other viruses, rotavirus-neutralizing antibodies appear to play an important role in protection against disease. Both VP4 and VP7 are important targets for vaccine development because these proteins independently elicit protective neutralizing antibodies (Estes and Cohen, 1989; Hoshino and Kapikian, 2000).

Neutralizing assays using hyperimmune serum against distinct rotavirus strains, including both monoclonal antibodies (MAbs) and polyclonal antibodies were used to classify rotavirus serotypes. Using such assays, 15 G (VP7) serotypes have been defined and most are shared between humans and animals. Monoclonal antibodies to VP4 will not react clearly in reciprocal neutralization assays with hyperimmune antiserum. Although several common P serotypes were identified cross-reactivity was shown between the different P-types (Coulson et al., 1987). Therefore, MAbs specific for the diversity of P serotypes were excluded for routine use. Instead, molecular characterization methods have been used to identify P genotyping of rotavirus (Gouvea et al., 1990; Gentsch et al., 1992).

Epidemiological studies of rotavirus infections have shown that there is great diversity of rotavirus strains circulating throughout the world. Currently, there are 23 G genotypes and 28 P genotypes identified in humans and animals (Estes, 2001; Kapikian et al., 2001; Liprandi et al., 2003; McNeal et al., 2005; Rahman et al., 2005a; Khamrin et al., 2007a; Matthijnssens et al., 2008;
Various human rotavirus serotypes have been identified - thus far, at least 10 G types [G1-G6, G8-G10 and G12] (Santos and Hoshino, 2005) and 11 P-types {P[3]- P[6], P[8]- P[11], P[14] and P[19]} have been detected (Hoshino and Kapikian, 2000, Liprandi et al., 1990; Okada et al., 2000). Surveillance of rotavirus genotypes or serotypes have shown five human group A rotavirus strains which are frequently detected globally, i.e. serotypes G1-G4 and G9 strains in association with P[8] and P[4] genotypes. The most common G-P combinations of rotavirus genotypes were found to be G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] and G9[6] (Hoshino and Kapikian, 2000; Kapikian et al., 2001; Iturriza-Góñara et al., 2003; Santos and Hoshino, 2005; Van Damme et al., 2007). Globally, rotavirus strains carrying G1P[8], G2P[4], G3P[8] and G4P[8] specificity are accountable for 88.5%, and in North America, Europe and Australia, represent over 90% of infections. In South America and Africa, strains with G1P[8], G2P[4], G3P[8] and G4P[8] specificity represent 68% and 50%, respectively (Santos and Hoshino, 2005). In Africa, studies were conducted in South Africa, Tunisia, Nigeria, Ghana, Guinea-Bissau and Kenya (Trabelsi et al., 2000; Pager et al., 2000; Pennap et al., 2000; Steele et al., 2003; Fischer et al., 2003). In Africa, however, G8 genotypes associated with both long and short electrophoretic patterns are also considered epidemiologically important, rivalling the predominance of G3 strains in many settings (Adah et al., 1997; Cunliffe et al., 1999; Steele et al., 1999; Armah et al., 2001). In Latin America, Africa and Asia there is a higher incidence of P[6] strains identified compared to those detected in industrialized countries such as Europe and North America (Gorziglia et al., 1986). Previously, serotypes G5, G6, G10 and G11 were exclusively isolated from animals, but now have also been detected in humans (Gouvea et al., 1994c, Timenetsky et al., 1997; Santos et al., 1998; Araujo et al., 2002; Esona et al., 2004).

A large majority of rotavirus infections in developing countries also display mixed infections with global common strains (Gouvea et al., 1994c; Timenetsky et al., 1994; Bresee et al., 1999). However, developed countries
for example, Ireland, reported an increased incidence of mixed infections (Ready et al., 2005). Mixed infections can potentially lead to formation of novel reassortant strains. In addition to mixed infections, strains that could not be G or P typed (untypeables) have been identified and in Europe make up 18% (Koopmans and Brown, 1999) and in Brazil 21% of untypeables (Gouvea et al. 1994c; Timenetsky et al. 1994).

2.6.1.1 Serotype G1

Studies of rotavirus serotypes have been published worldwide; including the UK, Spain, Sweden, USA, Hungary, Italy, Finland, Germany, Africa, (Santos et al., 1994; Ramachandran et al., 1998; Iturriza-Gómar et al., 2000a; van Damme et al., 2007; Mas Marques et al., 2007) and G1 was the most prevalent strain co-circulating with other strains. Thus, studies of rotavirus diversity in Europe during 1981-1990 showed that serotype G1 was the most prevalent strain, ranging between 44-84% (Koopmans and Brown, 1999). Between 1996 and 1999, African serotyping studies showed a similar pattern of rotavirus diversity with serotype G1 predominating (Trabelsi et al., 2000; Pennap et al., 2000; Cunliffe et al., 1999; Steele et al., 1999; Armah et al., 2001). Phylogenetic analysis of VP7 gene of G1P[8] strains in Italy showed six lineages and seven sublineages (Arista et al., 2006).

2.6.1.2 Serotype G2

Serotype 2 has been found worldwide in the number of studies, where there is a season of high prevalence followed by a season of low prevalence (Iturriza-Gómar et al., 2000a; Araujo et al., 2002; Lin et al., 2006). During 1996-1999, studies in Africa including South Africa, Ghana and Burkina Faso showed a significant number of serotype 2 rotavirus strains in association with P[4] (Yeats et al., 1999; Armah et al., 2001; Steele and Ivanoff, 2003; Steele et al., 2003). In Nigeria, Adah et al. (2001) and in Ghana, Armah et al. (2001)
identified G2P[6] human rotavirus strains among asymptomatic diarrhoea in hospitalized children. In the UK, serotype 2 made up a significant proportion during the 1995-1996 and 1997-1998 seasons (Iturriza-Gómar et al., 2000a) and in Taiwan, the temporal distribution of G types from 2000 to 2002 showed a high prevalence of serotype 2 during 2000 (Lin et al., 2006). According to Page and Steele (2004a), the phylogeny of serotype 2 from Africa revealed two lineages (I and II) and four sublineages.

2.6.1.3 Serotype G3

Most recent studies found that serotype 3 is the second most common genotype in the world (Kirkwood et al., 2006; Phan et al., 2007a; Trinh et al., 2007). Recent studies reported a decline in G1 strain prevalence over time while G3 increased continuously. Natural rotavirus infection is not restricted to humans and G3 serotypes have also been recovered in animal host species including rabbits, pigs, birds, cats, dogs, monkeys, cows and horses (Estes and Cohen, 1989; Gerna et al., 1992). The G3 rotavirus strains isolated in China, Russia, Thailand and Vietnam in 2001-2004 clustered into the same branch of the phylogenetic tree (Trinh et al., 2007). In Japan, Phan et al. (2007a) demonstrated an emergence of new variant rotavirus G3 strains. A G3 strain of rotavirus was identified commonly in Zimbabwe between 1996-1999 (Steele and Ivanoff, 2003).

2.6.1.4 Serotype G4

Rotavirus serotype G4 has been detected at very high frequency and showed marked fluctuation in many countries such as Italy, France, United Kingdom, Paraguay, Germany and Slovenia (Gault et al., 1999; Arista et al., 2005; Parra et al., 2007). In Italy, genetic variability of serotype 4 showed three lineages of strains, belonging to lineage Ia, Ib and Ic (Arista et al., 2005). Previous data
shows that serotypes 4 were subdivided into two subtypes i.e. subtype A [ST3-like] and subtype B [VA70-like] (Gerna et al., 1988).

### 2.6.1.5 Serotype G9

Since the mid-1990’s, G9 serotypes associated with P[6] and P[8] have emerged as a significant cause of rotavirus diarrhoea, spreading efficiently throughout the world including USA, UK, Australia, Taiwan, India, France, Belgium, Italy, Hungary, Bangladesh, Ghana, India, Brazil, Yugoslavia, Thailand, Portugal, Latin America, Africa and Japan (Cunliffe et al., 2001; Kirkwood et al., 2002; Steele et al., 2002a; Armah et al., 2003; Gentsch et al., 2005; Rahman et al., 2005b; Santos and Hoshino, 2005; Khamrin et al., 2006b; Lin et al., 2006; Rodrigues et al., 2007a; van Damme et al., 2007).

Due to the predominance of G9s, the serotype is considered the fifth most common global rotavirus strain in circulation. In Latin America, G9 was associated with severe gastroenteritis (Linhares et al., 2006) and in Portugal; G9 strains were responsible for 90% of all rotavirus cases. This seems a much higher figure when compared to previous reports from other countries (Rodrigues et al., 2007a).

### 2.6.1.6 Serotype G8

G8 strains exhibiting super short electropherotypes were first reported in Indonesia during 1979 and again in 1981 from children with gastroenteritis (Hasegawa et al., 1984). Since then, serotypes G8 in association with P[4], P[6] and P[8] have been isolated from patients with diarrhoea in various African countries (Adah et al., 1997; Cunliffe et al., 1999; Steele et al., 1999; Armah et al., 2001). Serotype G8 has been found as the third most frequently detected strain in cattle, after G6 and G10, and has been found to be associated with a variety of P types (P[1], P[5], P[11] or P[14] (Adah et al., 1997; 2003; Fukai et al., 1999; 2002; Hoshino et al., 2006). Interestingly, in
Malawi, novel serotype G8 human rotavirus strains were detected that exhibited the greatest homology to human and animal serotype G8s. These might represent human/bovine reassortant viruses (Cunliffe et al., 2000). Hybridization experiments by Browning et al. (1992) also showed that human and bovine serotype G8 might derived from reassortment.

### 2.6.2 Novel rotavirus serotypes

Unusual strains have been reported frequently worldwide. In South Korea, a novel human rotavirus strain G11P[4] was isolated from a one year old patient with severe diarrhoea (Hong et al., 2007). Human strains with short electrophoretic patterns generally exhibit subgroup I specificity while long electrophoretic patterns exhibit subgroup II specificity. In India, a human rotavirus strain G8P[1] was isolated that had a long RNA electropherotype with subgroup I (Jagannath et al., 2000).

In Thailand, an uncommon human rotavirus strain with a AU-1-like G3P[9], subgroup I and displaying a long RNA electrophoretic pattern was isolated from a one year old child with acute gastroenteritis (Kitaoka et al., 1987; Khamrin et al., 2007b). In 2005, the human G11P[25] was the first to be reported in Bangladesh and subsequent to that, G11P[25] strains were also isolated in India and Nepal (Rahman et al., 2005b; Uchid et al., 2006). G3P[3] and G10P[11] rotavirus strains were isolated from asymptomatic children in India and the G11P[25] was associated with interspecies transmission (Iturriza-Gómara et al., 2004b; Banerjee et al., 2007a). According to Khamrin et al. (2006a), the G3P[3] human rotaviruses demonstrated multiple human-animal interspecies transmission. Uncommon strains isolated in Brazil included G2P[8], G5P[8], G10P[6] and G9P[4] (Araujo et al., 2001).
Bovine rotavirus strains have long, short and super short electropherotypes and are restricted to serotype 6 and serotype 10. The G5 genotype, however, is a novel G genotype in cattle, which has resulted from a natural reassortment between bovine and porcine rotavirus strains (Park et al., 2006). The study done by Alfieri et al. (1996) showed sequence homology of Brazilian G5P[8], human (P1A[8]G1 (Wa), P[8]G3 (AU17) and porcine P9[7]G5 (OSU) rotavirus strains, suggesting that Brazilian G5 strains are natural occurring reassortants between human P[8] (Wa) and porcine G5 OSU strains.

Interspecies transmission of rotaviruses occurs in nature at a relatively high frequency, particularly in developing countries where animals and humans often live in close proximity. Two novel bovine G5 genotypes in association with bovine P[1], P[5] and VP3 were detected in South Korea from 2004 to 2005. Other genes, which belong to porcine rotaviruses, were also identified (Park et al., 2006). The G5 serotypes have also been described in children with acute gastroenteritis in Argentina (Bok et al., 2001), Paraguay (Coluchi et al., 2002), Brazil (Araujo et al., 2002) and Cameroon (Esona et al., 2004).

2.7 Natural history of rotavirus infection

Rotavirus is a “democratic” virus, as nearly every child becomes infected by the age of 3 years, in both developed and developing countries (Gurwith et al., 1981). Infections after 3 months of age are more likely to be symptomatic (Bresee et al., 1999). There is no significant difference between the rotavirus infections rate in industrialized as opposed to developing countries. The natural or primary rotavirus infection occurs during the first 2 years of life and the first infections are usually associated with acute diarrhoea that ranges from mild to severe (Rodriguez et al., 1977). However, more than 50% of acute rotavirus diarrhoea patients require hospital admission (Barnes et al., 1998). The peak incidence rate of symptomatic rotavirus infection in
industrialized countries is from 6 to 18 months whereas in developing countries children tend to become infected earlier, i.e. below 6 months of age (Glass et al., 1996).

The natural history of rotavirus infection indicates that children develop an immune response after the first infection, with immunity improving with each subsequent infection (Bishop et al., 1983; Velázquez et al., 1996). Rotavirus infection in neonates is occasionally asymptomatic (Bishop et al., 1983; Hoshino et al., 1985a; Flores et al., 1986; Gorziglia et al., 1986; Pager et al., 2000; Steele et al., 2002b). Since the peak incidence rate of rotavirus infection usually occurs during the time that acquired maternal antibody is waning, rotavirus vaccines should be administered early in life to mimic the protection conferred by maternal antibodies. After 2 years of age, the incidence of rotavirus infection decreases which indicates that acquired immunity results in protection against this disease.

Adult rotavirus infections are occasionally also found in the elderly and in caretakers of sick children. Neonatal rotavirus infection, however, usually occurs within the first week after birth and appears to be mostly of nosocomial origin.

2.8 Clinical features of infection

Rotavirus infections produce a clinical spectrum of symptoms that varies from asymptomatic to severe with occasional fatal dehydrating illness. The incubation period of rotavirus infections in young children is 24-78 hours after infection whereas in adults it is between 1-4 days (Bishop, 1994).
Acute gastroenteritis associated with rotaviruses is characterized by either the onset of vomiting, which lasts for 1-2 days accompanied by or preceding a fever ($\geq 37^\circ C$), followed by the onset of watery diarrhoea, which persists for approximately 3-8 days (Staat et al., 2002). Rotavirus infections cause severe diarrhoea and are more likely to be associated with dehydration with up to 10-20 bowel movements per day. This is often accompanied by abdominal cramps and sometimes children may experience a cough and runny nose (Christensen, 1989).

Severity of disease and duration of symptoms vary widely, however, rotavirus infections in children aged 6 months to 2 years tend to be severe (Kim et al., 2005). Severe illness and dehydration may require a hospital stay, which can range in duration from 2-14 days, with an average stay of 4 days. The outcome of infection depends on viral pathogenicity factors as well as host immune status. Factors associated with severe and prolonged diarrhoeal disease in children are primarily due to immunodeficiency, malnutrition and co-infection with parasites. More specifically severe rotavirus infections may exacerbate malnutrition by damaging the intestinal mucosa, which results in absorption becoming deficient over an extended period (Yao et al., 2006). Rotavirus can cause severe and chronic gastroenteritis in children who are immunodeficient since rotavirus shedding is prolonged (Cunningham et al., 1988). Some patients may develop antigenemia (Gilger et al., 1992) and extra intestinal spread of rotavirus has reportedly resulted in viremia.

Rotavirus infections can be fatal if left untreated with the major cause of death due to dehydration and electrolyte imbalance. Dehydration is associated with the loss of sodium and chloride in the stool causing compensated metabolic acidosis. Signs of dehydration include: thirst, irritability, restlessness, lethargy, sunken eyes, sunken fontanelles, a dry mouth and tongue, dry skin, dry nappy for several hours in infants and in young children, reduced urination is also observed.
Asymptomatic rotavirus infections are common mainly in children under 3 months. Mild to asymptomatic infections occur in older children from 3 years of age and in adults. Hoshino et al. (1985a) suggested that neonatal rotavirus strains causing asymptomatic infection are naturally attenuated. However, infection with the same strains after the neonatal period would be symptomatic.

Serotypes P[6] in association with a variety of G types (G1-G4) were reported in asymptomatic neonates in Australia, Venezuela and England, and G9 India (Das et al., 1994). In the late 1980's, Gorziglia et al. (1988), Flores et al. (1986) and Hoshino et al. (1985b), showed that the P[6] human rotavirus strain was associated with neonatal infections but was considered to be less virulent and caused symptom-free infections. Subsequently, the two human rotavirus strains M37 and RV-3 were proposed to be candidate rotavirus vaccines. Nevertheless, subsequent studies indicated that P[6] rotavirus strains were also associated with symptomatic infections (Timenesky et al., 1994; Santos et al., 1994; Steele et al., 1995; Adah et al., 1997; Cunliffe et al., 1999). The relationship between rotavirus strains bearing P[6] with asymptomatic and symptomatic infection remains unknown. However, Hoshino et al., (2003) reported that the P[6] allele from asymptomatic and symptomatic strains have shown similar if not identical VP4 neutralization specificities.

Unusual clinical manifestations associated with rotavirus gastroenteritis include rash, gastrointestinal bleeding, chronic diarrhoea, elevated serum transaminases, pneumonia, febrile and afebrile convulsions, necrotizing enterocolitis in neonates, hemorrhagic gastroenteritis in neonates, intussusception, haemolytic uremic syndrome, encephalitis and disseminated intravascular coagulation (Maldonado and Yolken, 1990; Pazdiora et al., 1990).
2.9 Pathogenesis

After ingestion of rotavirus particles by animals or humans, the virus is not neutralized by gastric acid but attaches to the proximal small intestine. During the incubation period of 18–36 hr, the virus enters the mature enterocytes at the tip of the small intestinal villi. Replication occurs in the mature enterocytes and large numbers of new virus particles are released by transcytosis into the small intestinal lumen to infect other cells. During the incubation period and before detectable viral antigen in the stool, shortening and blunting of intestinal villi and vacuolation of enterocytes may be observed (Estes, 2001).

Histopathology studies from the small intestine of infants and young children have shown that mononuclear cell infiltration of the lamina propria, distended cisternae of endoplasmic reticulum, mitochondrial swelling and denuding of microvilli also occur. Virus particles have been identified in columnar epithelial cell, goblet cells, phagocytic cells and M cells in the small intestine and impaired D-xylose absorption and depressed levels of disaccharides was also noted in some patients (Shaw and Greenberg, 1999; Kapikian et al. 2001).

The following changes may cause malabsorptive diarrhoea:

- The destruction of specialized absorptive brush border apical epithelial cells result in a loss of intestinal lactase, consequently failure to hydrolyse lactose to monosaccharides. The lactose is then converted to lactic acids in the large bowel by bacteria causing irritant large bowel diarrhoea.
- The destruction of specialized absorptive brush border apical columnar cells, subsequently replaced with non-specialized secretory crypt cell.
- An inflammatory response in the small intestine with perturbation of electrolytes and fluid transfer function as a result of prostaglandin production.
These changes contribute to the profuse watery diarrhoea symptoms with shedding of massive quantities of virus (>\(10^{11}\) per g) in the stool (Estes, 2001). In addition, sodium and chloride salts are lost. Studies have shown that the NSP4 protein has viral enterotoxin activity and its expression is associated with an alteration in intracellular calcium homeostasis and reduction in cell viability (Tian et al., 1994; Newton et al., 1997). Recovery from rotavirus infection occurs when new epithelial cells repopulate the lumen surface.

### 2.10 Rotavirus immune response

Rotavirus infection induces both innate and specific adaptive immunity. Innate immunity occurs early in infection and plays an important role in pathogenesis and/or protection against rotavirus disease in children. Specific adaptive immunity appears later during infection by stimulation of humoral and cell mediated immunity (Jiang et al., 2003; Dharakul et al., 1990). Many studies have shown that natural rotavirus infection protects children against development of severe rotavirus diarrhoea upon reinfection (Velazquez et al., 1996; Bishop et al., 1983). A study conducted in Mexico monitoring children from birth to 2 years of age and collecting weekly stool samples and monthly blood samples, indicated that the first infections are generally the most severe and severity of disease decreases after subsequent infections. The initial rotavirus infection elicits a homotypic neutralizing antibody response to the virus whereas subsequent infection may elicit heterotypic responses (Clark et al., 1990; Bernstein et al., 1990).

During rotavirus infection, the antibodies produced in the intestinal lumen are the most important in passive protection against rotavirus infection and are considered to be markers of the immune response. Anti-rotavirus IgM and IgA are the most common isotopes found in the duodenum, saliva and faeces of infants less than 3 months of age (Sonza and Helmes, 1980; Davidson et al.,
Anti-rotavirus-specific IgG, IgA and neutralizing antibodies can also be detected in serum and intestinal secretions of infected rotavirus patients. Various studies have indicated the correlation between serum IgA and IgM and mucosal IgA and IgM against rotavirus infection (Davidson et al., 1983; Coulson et al., 1986; Hjelt et al., 1986; Grimwood et al., 1988). Several days after primary rotavirus infection, IgM antibodies are the first to appear, followed by IgA antibodies. Serum IgA antibodies are well maintained, whereas in mucosal secretions they do not persist for very long (Davidson et al., 1983). Rotavirus-specific IgA, rotavirus-specific T helper cells and neutralizing antibody response can still be detected in three to five months after acute symptomatic rotavirus infection in children less than 2 years of age (Offit, 1994). While the mechanism of cell mediated protection is not entirely clear, in the adult mouse model it appears that CD8+ T lymphocytes are responsible for clearance of rotavirus particles (Dharakul et al., 1990).

2.11 Laboratory diagnosis

2.11.1 Sample collection, transportation and storage

For direct detection of rotavirus, stool specimens should ideally be collected during the acute phase of the illness, preferably within the first 3-5 days of illness even though shedding of rotavirus may continue for up to 3 weeks. Nevertheless, virus shedding coincides with the duration of the symptoms. The specimens should be placed directly into clean screw cap containers and immediately transported to the laboratory and stored at 4°C. For long-term storage, stool specimens should be kept at −20°C. Avoid repeated freezing and thawing that could interfere with detection limits of the virus particles (Christensen, 1999).
2.11.2 Electron microscopy

Electron microscopy (EM) is a reliable method used to detect rotaviruses, mainly due to their distinctive morphology. Examination by EM of a negatively stained stool specimen is a relatively rapid diagnostic method when only small numbers of specimens are tested. It can detect both group A and non-group A rotavirus as well as other enteric viruses, and can be used as a confirmatory test when any discrepancies arise with other techniques (Christensen, 1999; Estes and Kapikian, 2007). Testing using EM has been replaced by more readily available solid-phase immunoassays. The disadvantages of EM are: (1) the technique requires access to the electron microscope and skilled personnel capable of interpreting results, (2) the method requires an expensive instrument, (3) it may be less sensitive than EIAs with a detection limit of $10^6$ particles/ml of stool (Desselberger, 1996b) and (4) when a large number of specimens are investigated simultaneously electron microscopy may be slower than EIAs.

2.11.3 Serological assays

Laboratory diagnosis of rotavirus infection may involve detection of viral antigens, viral nucleic acids (dsRNA) or a serological response. For rotavirus antigen detection, kits are commercially available. These include: enzyme immunoassay (EIA) and latex agglutination assay (LA) kits (Christensen, 1999). These two diagnostic assays are antibody-based tests that utilize an antigen-antibody reaction. EIA are highly sensitive, specific, and convenient, require at least $10^5$ virions to produce positive results and require a small amount of stool for testing (Desselberger, 1996b). A large number of samples can be processed simultaneously and the kits utilize microtitre plates that are coated with polyclonal antibodies. The LA assays utilize a rapid slide test, in which latex particles coated with specific antibody react with rotavirus antigen in the stool, and then agglutinate. The results are rapid (within 15-20 minutes of receiving the specimens) and the unaided eye can visualize the agglutination reaction. The test is generally used to screen a small number of
specimens (Christensen, 1999; Estes and Kapikian, 2007). However, LA assays are the least sensitive method for detection of rotaviruses and are not recommended for use. Rotavirus antigen detection with antibody-based kits are practical, inexpensive, are easy to perform, allow rapid diagnosis, and do not require specialised laboratory equipment. Other serological techniques to measure rotaviruses include complement fixation, immunofluorescence, immune-adherence haemaglutination, enzyme-linked immunospot assays, radioimmunoassays and neutralization tests (Estes and Kapikian, 2007).

Early serological studies to classify rotavirus VP7 and VP4 utilized serotype-specific MAb-based ELISA. The serotype-specific MAbs to neutralizing epitopes on the VP7 protein were easy to isolate, whereas polyclonal antibodies were used for VP4 serotypes. The assays were widely used in early epidemiological studies but are less common today. The MAb assay has shown unreliable results due to antigenic drift through the accumulation of point mutations and reassortment in the rotavirus genome. Therefore, the viruses can possess heterologous neutralizing antigens for VP4 or VP7 (Flores et al., 1985).

Subgroups defined by epitopes on the VP6 protein can be detected using subgroup-specific monoclonal antibodies in an ELISA (Greenberg et al., 1983). There are four subgroups that have been described: subgroup I, subgroup II, subgroup I + II and subgroup non -I non -II. The subgrouping ELISA has been largely used in epidemiological studies to determine the subgroups of animals and humans strains. The most commonly found human strains appear to be subgroup II while subgroup I tend to be more common among animal rotaviruses (Lopez et al., 1994).
2.11.4 Polymerase chain reaction

Molecular techniques such as polymerase chain reaction (PCR) amplification have been developed as an alternative approach to typing the outer capsid proteins and widely utilized (Gouvea et al., 1990). The method correlates well with viral antigenic specificity. PCR is highly sensitive and specific with the detection limit of $10^2$ viral particles per ml of stool samples (Desselberger, 1996b). Using group-specific primers, the technique can be used to detect group A, B and C rotaviruses isolated from stool samples as well as typing for both G and P genotypes. The viral RNA is extracted from the stool sample and the resultant RNA is used in a reverse transcriptase reaction to produce complementary DNA (cDNA), which is then subjected to semi nested PCR typing assays with a set of oligonucleotide primers to identify both animal and human rotavirus genotypes (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Gouvea et al., 1994a; Gouvea et al., 1994b; Mphahlele et al., 1999; Cunliffe et al., 1999; Iturriza-Gómar et al., 2000b; Iturriza-Gómar et al., 2003a; Banerjee et al., 2007b; Simmonds et al., 2008). Reverse transcription polymerase chain reaction (RT-PCR) and genotyping techniques were used in a number of epidemiological studies to show that rotavirus genotypes G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] or G9P[6] were the most common cause of rotavirus disease in humans.

2.11.5 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis has been used to determine the genomic diversity of rotaviruses by displaying distinct migration patterns of the 11 segments of dsRNA. The resulting RNA migration pattern is termed an electropherotype. Polyacrylamide gel electrophoresis with silver staining has become the most useful technique for rotavirus detection and for molecular epidemiological studies to monitor rotavirus outbreaks and transmission. The technique has shown that rotaviruses have distinct RNA patterns. The groups A, B and C can be identified by their specific genomic migration pattern. The genomic pattern of group A rotavirus consists of four larger molecular weight
segments (segments 1-4), two middle segments (segments 5-6) followed by three small segments (segments 7-9), and lastly the two smallest segments (segments 10-11). The group A RNA pattern is 4-2-3-2 whereas the groups B and C RNA migration pattern is 4-2-2-3 and 4-3-2-2, respectively (Figure 2.2). The genomic segments are numbered according to their molecular size from largest to smallest (Estes and Kapikian, 2007).

The analysis of group A rotavirus electrophoretic migration patterns can also demonstrate two major RNA migration patterns, which include the short or super short electropherotypes and long electropherotypes based on the migration rate of gene segment 11. The slowly migrating 11\textsuperscript{th} RNA segment is located between the 9th and 10th segments in strains with short and super short electrophoretic patterns. This has been confirmed by hybridization analyses where gene 11 probes from strains with long electropherotypes bind to “segment 10” in strains with short electropherotypes. It is, therefore, clear that gene 11 of the short RNA profile has a larger molecular size than gene 11

![Figure 2.2](image-url) Polyacrylamide gel electrophoresis for group A, B and C rotavirus (Geyer et al., 1996)
of the long RNA profile. In general, human rotavirus with long electropherotypes represents the rotavirus subgroup II with serotypes 1, 3, 4 and 9, whereas short electropherotypes represents subgroup I with serotype 2 (Estes and Kapikian, 2007).

A metanalysis of the PAGE patterns of the rotaviruses have demonstrated that the electrophoretic pattern of the same gene in different rotavirus strains often shows heterogeneity. The same serotypes have, thus, shown significantly different RNA migration patterns and the same electrophoretic migration pattern can be exhibited by strains with different genotypes (Beards, 1982; Flores et al., 1982). In addition, many studies have shown that human rotaviruses exhibit a variety of electropherotypes (Kalica et al., 1981; White et al., 1984; Steele et al., 1988; Geyer et al., 1996). Therefore, the RNA profiles of group A rotavirus cannot be used as the only classification method. The major benefits of PAGE, according to Kaga et al., (1994) are: (1) to observe multiple strains that co-circulate in the same epidemic season, (2) to determine whether only one or two strains predominate in each season, (3) lastly to determine which different strains emerged in each epidemic season.

For viral RNA, stool samples are extracted with phenol and chloroform (1:1) and RNA is precipitated by ice-cold ethanol. The resulting RNA fragments are then resolved by PAGE and visualized by staining the gels with silver nitrate.

2.11.6 Cell culture

Rotaviruses were initially difficult to propagate in vitro. The African green and cynomolgus monkey cell cultures and the human colon carcinoma line CaCo-2 and HT-29 are more efficient than MA104 cultures and are, therefore, commonly used for rotavirus isolation. When cells are exposed to human rotavirus strains, cytopathic effects, plaque or focus reduction and viral
interference are noted (Hoshino and Kapikian, 1996). The cultivation of human rotavirus requires pre-treatment of the specimens with trypsin (5-10 μg/ml of growth medium) (Christensen, 1989; Donelli and Superti, 1994). Propagation of rotavirus in M104 cell culture is not routinely used by most diagnostic virology laboratories.

2.12 Rotavirus vaccines

Bishop et al. (1983) reported that in neonates, rotavirus infection provides significant protection against severe rotavirus infection but not to subsequent re-infection. Therefore, an effective rotavirus vaccine should be administered in early childhood. Potentially, the vaccine is used to prevent severe rotavirus diarrhoea (which may lead to dehydration or death) by inducing a protective response that is similar to the one induced by natural infection. The initial site of rotavirus infection is intestinal mucosa and almost all rotavirus vaccines developed were live attenuated oral vaccines. Therefore, oral vaccines have been considered to be the best to induce a protective immune response against rotavirus.

2.12.1 Rationale for vaccines

The development of effective rotavirus vaccines is a high priority. It has been estimated that an effective rotavirus vaccine could: (1) Reduce all diarrhoeal deaths by 30% in the age group 3-24 months in developing countries; (2) Reduce the deaths of ~ 2.5 million children by 2025 (Atherly, 2007); (3) Reduce the number of diarrhoeal related hospitalizations and (4) Reduce high medical and indirect costs of rotavirus disease (WHO, 2007).
2.12.2 History of vaccination: Monovalent “Jennerian” vaccines

Human and animal rotavirus strains share a common group antigen (VP6). The VP6 is the most immunogenic viral protein and carries group and subgroup specific epitopes, and various epidemiological studies using animal models have demonstrated good heterotypic protection, supporting a Jennerian approach to vaccine development (Wyatt et al., 1975; Zheng et al., 1988). The Jennerian approach involved the use of a live, attenuated animal rotavirus strain as the vaccine candidate (Kapikian, 1994). Efforts to develop live attenuated, oral vaccines for human rotavirus diarrhoea have resulted in five candidates: bovine, rhesus, rhesus-human reassortant, bovine-human reassortant and lamb rotavirus vaccines (Table 2:1).

Bovine vaccines

The first two bovine rotavirus vaccines candidates to be developed using the Jennerian approach were RIT4237 and WC3.

2.12.2.1 RIT4237 oral rotavirus

The bovine rotavirus vaccine strain RIT4237 was derived from Lincoln isolate bovine rotavirus NCDV (Nebraska calf diarrhoea virus) with serotype G6P[6] (Mebus et al., 1971). The strain was cloned after 147 passages in fetal bovine kidney cells (FBK) and after 149 passages the virus was grown in Cercopithecus monkey kidney cells up to 154 passages (Delem et al., 1984).

Safety, immunogenicity and efficacy: Nine efficacy trials of bovine rotavirus vaccine RIT4237 were conducted in 5 countries including Finland, Arizona, Gambia, Rwanda and Peru (Midthun and Kapikian, 1996). The protective efficacy rates were highly variable ranging from 0-58% against rotavirus gastroenteritis and 0-100% against severe rotavirus diarrhoea. The vaccine was demonstrated to be safe and immunogenic in infants and young children.
showing homotypic neutralizing antibodies (Vesikari et al., 1983). Live oral bovine rotavirus vaccines have been proven to induce good protection in Finland (Vesikari et al., 1983; Vesikari et al., 1984), USA (Clark et al., 1988) and Peru (Lanata et al., 1989) while the RIT4237 vaccine failed to demonstrate protective efficacy in the Gambia and Rwanda in Africa (Hanlon et al., 1987; Lanata et al., 1989) and Arizona in Native Americans (Santosham et al., 1991). Studies involving the RIT4237 vaccine candidate demonstrated variable efficacy and did not always demonstrate protection against subsequent severe infections with human rotavirus strains. Consistent protection was not observed and the vaccine was, therefore, discontinued and withdrawn from trials during the 1980s.

2.12.2.2  Bovine rotavirus vaccine strain WC3

The bovine rotavirus vaccine strain WC3 (Wistar cow) is derived from the VP7 serotype 6 of bovine rotavirus isolated at Wistar Institute, Philadelphia, USA. The vaccine was developed by cell culture, whereby the vaccine was passaged once in African green monkey kidney (AGMK) and passaged three times in CV1 cells, plaque purified twice in MA104 cells and then serial passaged in CV1 cells for up to 12 passages (Clark et al., 1986).

Safety, immunogenicity and efficacy: Efficacy studies of the WC3 vaccine conducted in Pennsylvania and Ohio demonstrated it to be safe, nonreactogenic and immunogenic in infants (Clark et al., 1986; Bernstein et al., 1990). Similarly to RIT4237, efficacy studies showed highly variable rates of protection. In Pennsylvania, WC3 vaccine failed to prevent re-infection with rotavirus but demonstrated protection rates of 76% against any rotavirus diarrhoea and 100% against severe cases during a predominantly G1 season (Clark et al., 1988). In Ohio, infants mounted homotypic neutralizing antibody responses against WC3 but no protection against rotavirus diarrhoea or the development of severe cases were observed during the predominant serotype 1 rotavirus season (Bernstein et al., 1990). In the Central African Republic, the WC3 vaccine failed to demonstrate efficacy against rotavirus diarrhoea that
was predominantly VP7 serotype 1 (George-Courbot et al., 1991). The vaccine demonstrated poor protection and, therefore, development of the vaccine was discontinued.

Rhesus rotavirus vaccine

2.12.2.3 Rhesus rotavirus vaccine strains MMU18006

Rhesus rotavirus vaccine (RRV) strain MMU18006 was isolated from diarrhoeal stool of a rhesus monkey that was passaged nine times in primary or secondary monkey kidney cells and a further seven times in semi-continuous diploid fetal rhesus lung (DBS-FRhL₂) cells (Kapikian et al., 1986). This RRV was characterized as VP7 serotype 3 with VP4 serotype 5B and not related to human strains. The vaccine was developed at the US National Institute of Health.

Safety, immunogenicity and efficacy: Efficacy trials of RRV vaccine were conducted in Maryland, Sweden, Venezuela, New York, Arizona and Finland. Protective efficacy elicited by the vaccine was inconsistent ranging from 0-85% (Vesikari et al., 1986; Losonsky et al., 1986; Gothefors et al., 1989; Rennels et al., 1990; Perez-Schael et al., 1990, Madore et al., 1992). Serum neutralizing antibody responses were predominantly homotypic (Green and Kapikian, 1992). Heterotypic protection against VP7 serotype 1 was, however, also detected in several trials (Rennels et al., 1990; Madore et al., 1992). The vaccine was demonstrated to be safe and immunogenic even though one-third of infants developed low-grade fever for 3-4 days after vaccination (Kapikian et al., 1985; Christy et al., 1986). A $10^5$ pfu dose of RRV was associated with a fever in 64% of infants in Sweden, 79% of infants in Finland and 50% of infants in the US (Vesikari et al., 1986; Losonsky et al., 1986; Gothefors et al., 1989). It was shown that the higher dose of RRV was well tolerated in younger infants. In Venezuela, a greater protective efficacy was demonstrated against severe cases of rotavirus diarrhoea in which the circulating strain serotype 3 (G3) was the same vaccine serotype (Perez-
Schael et al., 1990). The vaccine demonstrated varying protective efficacy and as a result the studies were discontinued.

2.12.2.4 Lamb rotavirus vaccine (LLR)

A lamb rotavirus strain vaccine was developed at Lanzhou, China by ZS Bai in 1997. The vaccine strain includes P[12] G10 specificity and reassortants. The strain was isolated from a local lamb with diarrhoea, and grown in primary calf kidney cells. Phase 3 trials demonstrated that the vaccine was safe and immunogenic in infants aged 6-24 months (Bai, 1997). The vaccine was licensed in China in 2001 (WHO, 2000).

Reassortant animal-human rotavirus strains

A modified Jennerian approach was initiated because of the reduced ability of RIT4237, WC3 and RRV to induce heterotypic protection after vaccination.

2.12.2.5 Human-rhesus rotavirus reassortant vaccines

A human-rhesus rotavirus reassortant vaccine was developed to yield reassortant rhesus rotaviruses expressing important epidemiological VP7 serotypes G1-G4. The isolates of rhesus-human reassortants were RRV strain MMU18006 (G serotype 3) and human rotavirus strains D (G serotype 1), DS-1 (G serotype 2), and ST3 (G serotype 4). The vaccine was developed by cell culture, where the human-RRV reassortants were recovered after co-infection of AGMK cell culture with RRV strains as well as serotype 1 and 2 that was isolated from diarrhoeal stools of hospitalized children and (MMU18006) serotype 3, which was isolated from asymptomatic newborn infants. The culture was then passaged in gnotobiotic calves. The vaccine strains were plaque purified 3 times in AGMK cells and the vaccine was prepared in DBS-FRhl2 cells (Midthun and Kapikian, 1996).
Safety, immunogenicity and efficacy: The vaccine was developed with the idea that the reassortants would stimulate homotypic immunity by inducing a neutralizing antibody response against different serotypes in order to produce a broad immune response (Midthun and Kapikian, 1996). The three efficacy trials of monovalent human-RRV reassortants vaccine (serotype 1 RRV or RRV vaccine) were done in Rochester, Finland and Peru (Lanata et al., 1989; Madore et al., 1992; Vesikari et al., 1992). Following vaccination, 15-20% of children developed a fever compared to placebo recipients. Neutralizing antibody responses to the vaccines were 65% to RRV and 54% to serotype 1 RRV. During a rotavirus season where serotype 1 was predominant, protective efficacy was higher in serotype 1 RRV recipients (77%) compared to RRV recipients, which was 66% against rotavirus diarrhoea.

In the second efficacy trial of monovalent human-RRV reassortant vaccine (serotype 1 x RRV or serotype 2 x RRV or placebo) in Finland, fever was detected at a higher percentage in vaccinees (about 21-25%) compared with the placebo recipients. During a rotavirus season where serotype 1 was predominant, protective efficacy was noted in 67% of serotype 1 x RRV and 66% on serotype 2 x RRV vaccines (Vesikari et al., 1992). The third efficacy trial was conducted in Peru with monovalent human-RRV reassortants vaccine (serotype 1 x RRV, serotype 2 x RRV, RRV vaccine or placebo). Fever was also noted more frequently in RRV recipients. Serological responses (IgG) were detected in 50% of RRV, 50% of serotype 1 x RRV and 70% in serotype 2 x RRV as well as 18% of placebo recipients. Protective efficacy was observed in RRV vaccine recipients at a level of 30% against any rotavirus diarrhoea (Lanata et al., 1995).

After monovalent reassortants were found to be safe and immunogenic, RRV-TV was developed with each of the three reassortants (human rotavirus strains D (G serotype 1), DS-1 (G serotype 2), and ST3 (G serotype 4) and MMU18006 (G serotype 3) providing coverage against the four common VP7
serotypes of rotavirus. A large efficacy trial was completed in both developing and developed countries using RRV-TV and 3 trials were performed in the US. Trials in Finland, Peru, Venezuela, and Brazil were also initiated using a $10^4$ pfu dose and were completed at $10^5$ pfu, i.e. the dose submitted for licensing. The vaccine was administered orally in three doses separated by at least 3 weeks in order to optimize the immune responses (Lanata et al., 1989; Madore et al., 1992; Vesikari et al., 1992).

2.12.2.6 Rhesus-human reassortant (RRV-TV)

The vaccine consists of RRV (with serotype 3) and three reassortants including D x RRV (serotype 1), DS-1 x RRV (serotype 2) and ST3 x RRV (serotype 3). The parent animal strain used was G3P5B rhesus rotavirus.

Safety, immunogenicity and efficacy: In August 1998, the FDA licensed the first rotavirus vaccine, i.e. the tetravalent rhesus-human reassortment vaccine (Rotashield, Wyeth Laboratories, Philadelphia USA). Efficacy trials were conducted in the USA (Rennels et al., 1996), Finland (Joensuu et al., 1997) and Venezuela (Perez-Schael et al., 1997) with a $4 \times 10^5$ pfu dose. The clinical trials were shown to be safe and highly effective. RRV-TV had exhibited good protective efficacy in both studies. The protective efficacy in Finland was 68% and in Venezuela was 48% against any rotavirus disease and 100% and 70%, respectively against severe rotavirus disease (Joensuu et al., 1997; Perez-Schael et al., 1997). In the US, RRV-TV safely prevented half of all mild, as well as 80% of severe episodes, and nearly all hospitalizations associated with rotavirus diarrhoea (Rennels et al., 1996).

The US Advisory Committee on Immunization Practices immediately recommended the vaccine for routine immunizations of all children in the USA. The vaccine was administered to over 600,000 infants in the first 9 months of the programme (Smith et al., 2003), and was given to children
during the routine immunizations programme at 2, 4, and 6 months of age. A booster or catch-up immunization was also provided at any time up to 7 months of age. Later in July 1999, the US CDC suspended the vaccine as it was considered to be associated with an increased risk of developing intussusception, particularly during the first 2 weeks after administration of the first dose (CDC, 1999a; Murphy et al., 2001). After a further 3 months, the ACIP also withdrew its recommendation for the use of the vaccine (CDC, 1999b). According to the CDC National Immunization survey in 19 states, most cases of intussusceptions occurred in children who were older than 90 days at the time of immunization, and who had received a booster. The risk of intussusceptions was estimated at 1 in 30 000–50 000, and there were 15 cases of intussusception in vaccine-treated infants (Murphy et al., 2001).

2.12.2.7 Bovine-human reassortant vaccines

Bovine-human reassortant rotavirus vaccines include a tetravalent WC3 rotavirus reassortant vaccine with genes coding for the VP7 of three major serotypes of rotavirus (G1, G2, and G3), as well as VP7 protein isolated from human rotavirus (W179) and a human VP4 reassortant with P[8] specificity. The vaccine should have induced antibodies broadly reactive to the three common serotypes of rotaviruses sharing P[8] specificity. In an efficacy trial of a two-dose regimen of $10^7$ pfu of the WC3 reassortant vaccine, protection was 67% against all rotavirus diarrhoea and 69% against severe rotavirus diarrhoea (Clark et al., 1996).

2.12.2.8 Hexavalent human rotavirus-bovine rotavirus reassortant vaccine

The second-generation hexavalent human-bovine rotavirus vaccine is a tetravalent human – bovine rotavirus (UK) reassortant with VP7 specificity for serotype G1-G4, G8 and G9. The vaccine was developed in order to cover the
emerging serotype G8 and G9 rotavirus strain in developing countries. Based on the WHO report (WHO, 2000), the median peak incidence of intussusception occurred at 3-8 months. The administration of hexavalent BRV-based vaccine should be in 2 dose schedules, with the first dose administered at 0-4 weeks of age and the second dose administered at 4-8 weeks of age (Kapikian et al., 2005). The vaccine was developed in order to eliminate or significantly reduce the risk of intussusception associated with rotavirus vaccines.

**Multinational rotavirus vaccines licenced worldwide**

Recent rotavirus vaccine developments include two new vaccines: the RotaTeq™ vaccine developed by Merck (Blue Bell, PA, USA) and the Rotarix® vaccine developed by GlaxoSmithKline Biologicals, Rixensart Belgium, which are both live oral vaccines. The widespread use of these two vaccines should have a significant impact on reducing death, hospitalization and medical visits associated with rotavirus diarrhoea globally. Because of uncertainty of possible risk of intussusception, the two vaccines were tested in both developed and developing countries. The vaccines should ideally demonstrate safety and high efficacy against severe rotavirus diarrhoea and should not be associated with increased risk of intussusception.

**2.12.2.9 Attenuated human vaccine (Rotarix®, GlaxoSmithKline)**

Rotarix® is a live human attenuated monovalent P1A[8] G1 rotavirus vaccine from human serotype RIX4414 developed by GlaxoSmithKline Biologicals, Rixensart, Belgium. Rotarix® vaccine was developed from a strain of rotavirus (89-12) isolated from a patient in Cincinnati by Bernstein et al. (1998). This strain 89-12 was first attenuated by passaging 43 times and was then cloned and further passaged in Vero cells and renamed RIX4414. The vaccine was
formulated as a lyophilized powder that needs to be reconstituted with liquid
calcium carbonate buffer before being administered (De Vos et al., 2004).

**Safety, immunogenicity and efficacy:** The phase I trial was evaluated in
adults, children and infants. The vaccine was found to be safe with only a mild
fever of short duration noted. Vaccine efficacy showed 89% against any
rotavirus disease and 100% against severe rotavirus infections. In Latin
America, a phase II trial with two doses of the vaccine provided a protection
rate of >80% against moderate to severe rotavirus diarrhoea (De Vos et al.,
2004). In the middle-income countries such as Mexico, Venezuela, Brazil as
well as Singapore, the vaccine showed an efficacy of 70-85% against any
rotavirus diarrhoea and 85-93% against severe diarrhoea (Salinas et al.,
2005).

A large phase III efficacy trial of Rotarix® to evaluate safety was conducted
with more than 60 000 infants enrolled in 11 Latin American countries and
Finland (Ruiz-Palacios et al., 2006; Linhares et al., 2008). Efficacy was
evaluated in more than 20 000 infants. The vaccine was provided together
with childhood vaccines (except oral polio vaccine, which was given two
weeks after or before administration of the vaccine). The study reported a
reduction in serious adverse events and hospitalization in the vaccine
recipient group. Efficacy of 80-85% was observed against severe rotavirus
diarrhoea and hospitalization (Ruiz-Palacios et al., 2006; Linhares et al.,
2008). Protection of more than 86% was reported not only in serotype G1P[8]
but also G3P[8], G4P[8] and G9P[8] whereas a lower efficacy of 41%  was
observed in G2P[4]. In Finland from 2001-2002, infants received a vaccine
dose of $10^{4.7}$ fluorescent focus units (ffu), and efficacy was found to be 73%
against any episode of rotavirus gastroenteritis.

In Singapore, the vaccine was administered together with routine childhood
vaccines. The trial was conducted using 2464 participants, and the vaccine
was found to be safe with no side effects observed (even at a higher dose of $10^{6.1}$ ffu). Similar results were also observed in Finland where the vaccine was highly immunogenic with protection levels of 97.8-100% depending on the dose given. Interference with other childhood vaccines was not observed. In the United States, infants aged 5-15 weeks were given either placebo or $10^{5.2-6.4}$ ffu vaccines. The vaccine did not show any increase in fever, vomiting and diarrhoea in all the participants (Dennehy et al., 2005).

In six European countries, protection of 87%, 96%, 100% where observed against any rotavirus gastroenteritis, severe disease and hospitalization associated with rotavirus, respectively (Vesikari et al., 2007). Serotype specific protection against G3, G4 and G9 was similar to G1 with more than 95% and an efficacy of 75% against G2 and hospitalization attributable to rotavirus was observed. The vaccine was considered effective against severe rotavirus disease regardless of the strain circulating. Rotarix® was licensed outside the United States, and is currently available in the European Union and other countries including Latin America, Malawi and South Africa. Safety and immunogenicity of the vaccine has been conducted in South Africa in the Madibeng and Ga-Rankuwa districts north of Pretoria. The results from double-blind placebo-controlled phase II trial to evaluate co-administration of RIX4414 and oral poliovirus vaccine show that the vaccines were well tolerated and immunogenic in South African infants (Steele et al., 2008).

2.12.2.10 Pentavalent WC-based bovine-human reassortant (RotaTeq™)

The RotaTeq™ vaccine is a live, oral, attenuated vaccine from bovine-human reassortment strains that contains five reassortant viruses with G1P[7], G2P[7], G3P[7], G4P[7] and G6P[8] combination. The parent animal strain used was G6P7 bovine strain WC3. The vaccine is suspended in a buffered
stabilizer solution that can be directly administered to the infants in three oral doses beginning at six weeks.

**Safety, immunogenicity and efficacy:** Because of uncertainty to the possible risk of intussusception, the pentavalent RotaTeq™ vaccine was tested in a trial involving a minimum of 60 000 study participants. The large cohort was used to identify cases of intussusception, other serious adverse events and within the large group, a smaller group was followed for efficacy and detailed safety. The vaccine and the placebo were administered at three doses given at 6 to 12 weeks of age and the following doses every 4 to 10 weeks thereafter. There were 70 301 participants in the study from 11 countries (Vesikari *et al.*, 2006), which includes the United States, Puerto Rico, Finland, Mexico, Guatemala, Costa Rica, Jamaica, Sweden, Germany, Belgium, Italy and Taiwan. Intussusception was closely monitored during the 42 days post vaccination period. The results of the phase III study in Europe and US have shown high protective efficacy of 98% against severe G1-G4 rotavirus diarrhoea. Hospitalization associated with G1-G4 rotavirus diarrhoea was reduced by 96%, any gastroenteritis was reduced to 59% with medical emergency visits associated with G1-G4 rotavirus reduced by 94% (Vesikari *et al.*, 2006). Efficacy against any severe rotavirus gastroenteritis in the USA was 89.5% and the vaccine was well tolerated when given with other EPI vaccines (Rodriguez *et al.*, 2007b). After 42 days post vaccination, intussusception was observed in six cases among RotaTeq™ recipients, and five cases of intussusception in the placebo group with an adjusted relative risk of 1.6 (95% CI: 0.4, 6.4) (Matson, 2006). RotaTeq™ was subsequently licensed in many countries including the United States, Europe, Latin America and Asia.

### 2.12.2.11 Human neonatal rotavirus strains

The neonatal vaccine strain M37 was isolated in Venezuela, demonstrated to be safe and immunogenic in children (Vesikari *et al.*, 1991; Midthun *et al.*, 1991). The attenuated human neonatal rotavirus strain M37 was developed
based on the observations that neonatal strain produce asymptomatic rotavirus infection and infected children are more protected against reinfection (Bishop et al., 1983). The vaccine provided poor protection in Finland (Vesikari et al., 1991).
**Table 2:1** Live rotavirus vaccine candidates which are currently licenced or under development

<table>
<thead>
<tr>
<th>Company</th>
<th>Vaccine composition</th>
<th>Status of vaccine</th>
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<tr>
<td><strong>Licensed vaccines</strong></td>
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<tr>
<td><strong>LLR</strong></td>
<td>Lanzhou Institute Of Biologicals Products (China)</td>
<td>Monovalent lamb G10P[12]</td>
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<tr>
<td><strong>RotaTeq™</strong></td>
<td>Merck (USA)</td>
<td>Pentavalent human-bovine reassortant G1-G4 WC3, PIA[8] WC3</td>
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<tr>
<td><strong>Rotarix®</strong></td>
<td>GlaxoSmithKline (Belgium)</td>
<td>Human G1P[8]</td>
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<tr>
<td><strong>Early clinical development</strong></td>
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<td>Human-bovine (UK) reassortant G1-G4 UK</td>
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<td>Neonatal Human G3P6]</td>
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<tr>
<td><strong>Pre-clinical development</strong></td>
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<td>Virus-like particle</td>
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<td><strong>DNA</strong></td>
<td>Massachusetts School of Medicine</td>
<td>DNA vaccines</td>
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CHAPTER 3: PROSPECTIVE HOSPITAL-BASED SURVEILLANCE TO ESTIMATE ROTAVIRUS BURDEN IN THE GAUTENG AND NORTH WEST PROVINCES OF SOUTH AFRICA DURING 2003-2005

3.1 Abstract

BACKGROUND: Worldwide, epidemiological studies have shown that rotavirus infection is the most common cause of severe diarrhoea in young children. In Sub-Saharan Africa, the annual mortality rate of gastroenteritis associated with rotavirus infection alone is estimated to account for approximately 145 000 deaths in children less than 5 years of age (Mølbak et al. 2000). Thus, there is an urgent need to promote the importance of rotavirus vaccination as primary public health intervention to reduce the burden of disease of rotavirus diarrhoea. We conducted prospective, hospital-based surveillance for rotavirus diarrhoea among children less than 5 years of age at the tertiary care Dr George Mukhari Hospital and at the secondary care Brits Hospital in the Gauteng and North-West Provinces, respectively. Estimates indicate that these hospitals cater for up to 80% of children less than 5 years admitted for diarrhoea within the catchment study area.

OBJECTIVES: To assess the incidence and burden of rotavirus-associated disease in two South African hospitals. Reliable information on rotavirus infections is essential to identify important features in the epidemiology of the disease in South Africa and to provide baseline and up-to-date estimates of the extent to which severe rotavirus diarrhoea impacts on the South African healthcare system.

METHODS: The study was conducted according to the generic WHO rotavirus hospital-based burden of disease protocol. During 2003 through 2005, the demographic, clinical data and diarrhoeal stool specimens were collected from children under the age of 5 years attending Dr George Mukhari
and Brits Hospitals. The samples were screened for Group A rotavirus using enzyme immunoassay (DAKO, Dakocytoimulation, Denmark).

RESULTS: There were 65,971 paediatric patients treated at the Dr George Mukhari Hospital during the study period. Of these, 4,072 were children less than 5 years of age who presented with acute gastroenteritis or diarrhoea at hospital admissions. Stool specimens were collected from 2,512 of these patients. Stool samples were found to be positive for rotavirus antigens at levels of 17%, 21% and 25% in 2003, 2004 and 2005, respectively. At Brits Hospital, a total of 8,960 children under 5 years of age received treatment. Of these, 1,340 children presented with acute gastroenteritis. A total of 629 diarrhoeal stool specimens were collected. Fourteen percent (23/165), 20% (49/249) and 23% (47/215) of the stool samples were found to be positive for rotavirus in 2003, 2004 and 2005, respectively. A similar peak of rotavirus infection was observed in both hospitals during winter season at prevalence rates between 56% and 59%.

An estimated annual prevalence of 22.8% (95%CI 21.2%, 24.5%) of the children hospitalized with diarrhoea at Dr George Mukhari Hospital were rotavirus-positive, while the corresponding figure for those treated at Brits Hospital was 18.2% (95%CI 14.9%, 22.1%). Among children less than 5 years of age admitted to Dr George Mukhari Hospital, we estimated that 5.5% (95%CI 5.1%, 6.0%) were due to rotavirus infection. Our incidence estimates indicates that one in every 50 to 70 children in the area is likely to be hospitalized with rotavirus diarrhoea between birth and 2 years of age.

CONCLUSION: These figures suggest that diarrhoeal diseases and rotavirus infection, in particular, place a significant strain on the South African health care system and support the general introduction of a rotavirus vaccine.
3.2 Introduction

Diarrhoeal diseases are a major public health problem, with high morbidity, mortality and economic loss for families and societies. Each year, diarrhoeal disease in children under 5 years of age causes over 1 billion diarrhoeal episodes and is responsible for over 2.5 million deaths (Kosek et al., 2003). The greatest burden of diarrhoeal disease occurs in children of developing countries where the incidence of diarrhoea-associated morbidity has been estimated at 6-7 episodes per child per year. This compares to 1-2 episodes experienced by children in developed countries per year (Santosham et al., 1997).

Worldwide, epidemiological studies have shown that rotavirus infection is the most common cause of severe diarrhoea in young children. Group A rotaviruses are most prevalent in human disease, accounting for 95% of infections, and are the leading cause of paediatric hospitalization resulting from severe dehydrating gastroenteritis (Estes and Kapikian, 2007). Annually, rotavirus infection is estimated to cause 111 million episodes of gastroenteritis requiring home care, 25 million clinic visits, 2 million hospitalizations and approximately 600 000 deaths in children under 5 years of age. Most children experience rotavirus gastroenteritis by the age of 5, and 1 in 5 will visit the clinic, 1 in 65 will be hospitalized and approximately 1 in 293 will die (Parashar et al., 2003). In Sub-Saharan Africa, the annual mortality rate of gastroenteritis associated with rotavirus infection alone is estimated to account for approximately 145 000 deaths in children less than 5 years of age (Mølbak et al., 2000).

Rotavirus diarrhoea is not amenable to prevention by improved sanitation or water supply and decreasing the impact on public health is dependent on case management with rehydration therapy as well as on primary prevention by vaccination (Vesikari, 1997). If ongoing trials of rotavirus vaccines already
licensed in industrialized countries and future trials of rotavirus vaccine candidates under development show sufficient efficacy and safety in children in low and middle-income countries with proven high rotavirus disease burden, these countries may consider including rotavirus vaccines into the national immunization programs. It is accordingly important to measure the proportion of children hospitalized with acute diarrhoea that have rotavirus disease as well as the incidence of such severe illness in the community, i.e. one of the two components is a predictor of rotavirus mortality, the other being rotavirus disease burden estimation (Mølbak et al., 2000).

Epidemiology studies conducted in developed countries indicate that annual childhood morbidity associated with severe rotavirus diarrhoea is high. For example, in the United States rotavirus infections are estimated to occur in 2.7 million children under the age of five years, resulting annually in 500 000 physician visits, 160 000 emergency room visits, 50 000 hospitalizations and 20-40 deaths. The cost of medical care has been estimated to be 274 million dollars and more than 1 billion dollars in societal cost (Parashar et al., 1998b; Tucker et al., 1998). Approximately 4 out of 5 children will develop rotavirus gastroenteritis, 1 in 7 will require a medical visit to a clinic or emergency room, 1 in 78 will be hospitalized and 1 in 195 000 will die from rotavirus diarrhoea.

In other developed countries such as Spain, the annual incidence of rotavirus infection requiring hospitalization in children less than 5 years of age was found to be 1-hospital admission per 1000 children and during winter this increased to 2.5/1000. The annual medical care cost is approximately 3.6 million euros (Gil et al., 2003). In Sweden, annually it was estimated that the rate of hospitalization associated with rotavirus gastroenteritis in children less than 4 years of age was 1500-1700 cases, corresponding to 3.7/1000 hospitalizations. The annual cost of medical care is US$1.8-2 million per year (Johansen et al., 1999b). In Italy, the prevalence of rotavirus infection requiring hospitalization is estimated to be 20-40% (Ruggeri and Declich,
1999). In England and Wales, it is estimated that the rate of hospitalization associated with rotavirus infection was 5/1000 children per year (Ryan et al., 1996). In Hungary, it was estimated that rotavirus gastroenteritis causes about 21% of severe diarrhoeal episodes, resulting in 8.4 hospital admissions per 1000 children under the age of 4 years (Szucs et al., 1999). In Poland, it is estimated that annually rotavirus associated hospitalization was 3.1/1000 children under the age of 5 years and 5.2/1000 in children under 2 years of age with the mean hospital stay of 9.5 days (Mrukowicz et al., 1999).

In developing countries the burden of rotavirus infection is significantly higher with a correspondingly high infant mortality rate. Based on estimates in a 1986 study, of 120 million episodes of rotavirus gastroenteritis, 18 million episodes were moderately severe with 35-52% requiring hospital admission and 873 000 deaths in infants and young children less than 5 years (Institute of Medicine, 1986). In Peru, it is estimated that 1 in 1.6 children will experience an episode of rotavirus diarrhoea, 1 in 9.4 will seek medical care, 1 in 19.7 will require hospitalization, and 1 in 375 will die of the disease. This represents 384 000 cases, 64 000 clinic visits, 30 000 hospitalizations and 1600 deaths with an associated medical care cost of US$2.6 million (Ehrenkranz et al., 2001). In Hong Kong, the annual estimated rate of hospitalization for rotavirus infection was 8.8/1000 children less than 5 years of age and 18.4 admissions/1000 children less than 1 year old (Nelson et al., 2005). In Venezuela, it is estimated that each year 118 000 children experience rotavirus infections requiring medical care of which 39 000 children require hospitalization (Salinas et al., 2004). In the Republic of Korea, the annual incidence of rotavirus disease is estimated to 56.9 cases per 1000 children less than 5 years of age (Kim et al., 2005).

In Africa, the African Rotavirus Network surveillance has provided extensive information on the serotype distribution of rotaviruses infecting African children (Steele and Ivanoff, 2003; Peenze et al., 2008) but data on rotavirus
disease burden in Sub-Saharan Africa are scarce (Mølbak et al., 2000, Valentiner-Branth et al. 2003; Binka et al., 2003). However, many studies in Africa (Steele and Ivanoff, 2003; Peenze et al., 2008; Cunliffe et al., 1998): including South Africa (Steele et al., 2003), Ghana (Armah et al., 2001; Binka et al., 2003), Malawi (Cunliffe et al., 1998; Cunliffe et al., 1999), Tunisia (Trabelsi et al., 2000), Cameroon (Esona et al., 2003), Guinea-Bissau (Fischer et al., 2000), Kenya (Kiulia et al., 2006), Côte d'Ivoire (Akoua-Koffi et al., 2007) and Nigeria (Pennap et al., 2000) indicate that rotavirus diarrhoea contributes importantly to childhood diarrhoea on the continent.

While previous studies have determined the proportion of South African children less than 5 years of age admitted to tertiary care hospitals with diarrhoea that have rotavirus disease (Steele et al., 2003), the studies have been too small to precisely estimate the proportions in smaller age categories and no attempts have thus far been made to estimate the incidence of severe disease in the communities served by Dr George Mukhari Hospital and by the secondary care Brits district hospital.

Efficacy studies of the Rotarix® vaccine (GSK, Rixensart, Belgium) were recently completed within communities in the Madibeng and Ga-Rankuwa districts north of Pretoria. It is necessary to have reliable estimates of the incidence of rotavirus infection in South African communities where this vaccine was tested. In order to advise decision-making on future vaccination, we intended with adequate precision, to determine the proportion of children less than 5 years hospitalized with diarrhoea that had rotavirus disease. In order to advise such decisions, we also wished to break down the estimates in age categories and, referring to previous findings (Cunliffe et al., 1998; Steele et al., 2003; Bahl et al., 2005), describe the seasonality of rotavirus diarrhoea (Steele et al., 1986; Cunliffe et al., 1998). Moreover, we wished to determine these proportions in both a tertiary and a secondary care hospital and make rough estimates of the incidence of severe rotavirus diarrhoea in the
communities served by these hospitals. On the basis of reaching all eligible children with acute gastroenteritis treated at the hospital, outpatients children were included. Reliable information on rotavirus infections is essential to identify important features in the epidemiology of the disease and to provide baseline and up-to-date estimates of rotavirus infection in South Africa healthcare system.

3.3 Materials and methods

3.3.1 Study population, hospitals and catchment areas.

The Gauteng and the North-West provinces of South Africa have temperate climatic conditions with four seasons characterized by summer rainfall (mid October to mid-February) and moderately cold and dry winters during May through July. To determine the incidence and the impact of rotavirus-associated gastroenteritis among children less than 5 years of age, on January 1, 2003, a study was initiated to examine stools for rotavirus from children with diarrhoea who were admitted to the Dr George Mukhari Hospital located in Ga-Rankuwa District in Gauteng Province. On July 1, 2003, a similar surveillance study was initiated in the Brits District Hospital in the neighbouring Madibeng district of the North-West Province. Ga-Rankuwa, Soshanguve and Mabopane districts (Gauteng province) and parts of the Madibeng district in the North-West Province constitute the catchment areas of Dr George Mukhari Hospital whilst the Madibeng district is also served by Brits Hospital. This report is based on data collected in the two hospitals until the end of December 2005. Dr George Mukhari Hospital is a tertiary care, 1,600-bed facility that renders a service to about 6 million people and serves as a referral hospital to 43 communities and 4 provinces. In contrast, Brits Hospital is a secondary care facility and is situated at Madibeng district with 60 inpatient beds (Figure 3:1).

In the catchment areas, there were 84 484 children under 5 years of age from the two provinces (StatsSA, 2001). The areas include urban, rural and informal communities with moderate to low socio-economic status. The
The government provides basic services such as water and sewage to ~90% of the population in Dr George Mukhari catchment areas and to ~60% in Madibeng district. Unemployment in the area is slightly above 40% and low education levels of 20% are seen in the Dr George Mukhari catchment areas as opposed to the 35% in the Madibeng district (Municipal Demarcation board SA, 2008).

The study was conducted according to part I of the WHO “Generic protocol for hospital-based surveillance to estimate the burden of rotavirus gastroenteritis in children” (WHO/V&B/02:15).

### 3.3.2 Ethical approval

Ethical approval was obtained from the Research Ethics and Publication Committee of the Medical University of Southern Africa (Medunsa, now University of Limpopo (Medunsa Campus) (MP 46/2005). Verbal informed consent was obtained from parents or other caregivers accompanying children admitted to the 8-bed Brits paediatric ward or treated in the casualty unit and children admitted to the Dr George Mukhari paediatric ward (which caters for children from birth to 12 years of age) for diarrhoea. The reason for not distinguishing between the outpatients and those admitted to the small paediatric ward of Brits Hospital is that, most children with dehydrating diarrhoea are rehydrated in the Brits casualty unit and in the ward. This is in contrast to the outpatient and inpatient department of Dr George Mukhari Hospital.

Cases of diarrhoea were defined as an episode of diarrhoea with three or more watery stools in a 24 hours period and/ or loose or watery bowel movements that exceed the normal daily number of bowel movements with or without vomiting.
The caregiver completed a general as well as a disease-specific questionnaire that were designed to capture vital socio-demographic information including: (1) age, gender and place of residence (formal, informal and rural settlement), (2) illness history- date of the onset of the symptoms, duration of diarrhoea, maximum number of bowel movements during 24 hours period, date of discharge, other symptoms including fever, vomiting and the duration, (3) type of food given to the baby either bottle-fed or breast-fed and (4) any treatment provided by alternative health care providers before inclusion into the study (Appendix A). The clinical surveillance forms were used to determine the severity of clinical dehydration by weight loss of 5-10% of admission weight, frequency of diarrhoea and vomiting, fever, dehydration and type of treatment required. Rusuuka and Vesikari, (1990) numerical scale was used to grade severity of rotavirus diarrhoea. The clinical surveillance form was completed by 5th year medical students (Appendix B).

Based on the knowledge of existing health care utilisation patterns, senior health care officials in the area (Community Paediatrician Dr N Morale and District Family Physician for the North West Province, Dr John Tambo) were asked to estimate what proportion of children less than 5 years of age in the Dr George Mukhari and Brits catchment areas who required treatment for acute diarrhoea in a health facility were likely to be referred or themselves seek treatment in one of these hospitals. Their educated guess was 65%. Moreover, a health utilization survey in Madibeng undertaken in March through November 2004 according to part II of the WHO generic protocol mentioned above (WHO/V&B/02:15) showed that 76% (95% CI 72%, 80%) of children with diarrhoea requiring facility-based treatment sought care at the Dr George Mukhari or Brits Hospital.

Information was obtained on child populations and this was broken down on each of the first 5 years of life in the 4-catchment regions from Statistics South Africa, 2001 census (StatsSA, 2001). Acknowledging that, due to higher
mortality, the number of children in the younger age categories would be slightly underestimated, an equal distribution of children across ages was assumed. In addition, the population in the study catchment area was assumed to experience the average South African population growth of 2%. These population figures were used to derive the denominators to calculate overall and age-specific incidences of severe rotavirus diarrhoea.
Figure 3:1 Map of Madibeng and Ga-Rankuwa district indicating the burden of rotavirus disease studies at the Dr George Mukhari and Brits Hospital
3.3.3 Data collection

The hospital nominal book was used to count the total number of children less than 5 years of age that were admitted during the study period. The following data were collected every month from both sites: total number of paediatric medical visits (both outpatients and inpatients); total number of children less than 5 years of age; total number of hospitalizations; acute gastroenteritis or diarrhoeal visits. The total number of stool specimens collected was equal to the number tested for rotavirus to determine number of rotavirus-positive samples. To assess differences in the clinical characteristics and demographics, patient data from patients who gave stool samples were compared with those that did not donate a stool sample.

3.3.4 Data analysis

Data were entered and analyzed using MS Excel™, MS Access™ and the freely available statistical and graphical programming environment R (www.r-project.org). Graphs were made describing the seasonal pattern of rotavirus diarrhoea in the two hospitals. The proportions of children with diarrhoea at Dr George Mukhari and Brits Hospitals that had rotavirus in their stools was calculated, with the corresponding 95% confidence intervals (95%CI) employing Wilson’s method (Newcombe and Altman, 2000). Generalised linear models from a binomial family with a logarithmic link function were used to compare the proportions of children with rotavirus at the two hospitals and to describe the relationship between the proportion and age. The analysis was done separately in infants and in older children, because there were significant and substantial differences in the relationships between the two age groups.

To derive the numerators in the annual age-specific incidence of severe rotavirus illness, the number of children treated for diarrhoea at Brits Hospital
from January 1, 2004 to December 31, 2005 who had rotavirus-positive stool specimens were counted. In each age category, the number of rotavirus diarrhoea cases treated at Brits Hospital in 2003 was estimated to equal the average of the subsequent two years. The sum of the estimates and the observed number of cases in 2004 and 2005 were added to the number of children admitted for the treatment of diarrhoea at Dr George Mukhari Hospital (from January 1, 2003 to December 31, 2005) who were estimated to be rotavirus positive. The 95%CI of the estimates was calculated based on the observed rather than on the estimated number of rotavirus cases, thereby not increasing the precision of the estimates beyond what the observations could justify. In order to estimate the two outer bounds of the actual number of children in the two hospitals’ catchment areas that were treated in a health facility for rotavirus diarrhoea, the numerators were adjusted by dividing with 0.65, representing the above-mentioned guessimate of 65% coverage of hospitalization anticipated to be captured by the hospitals, and 0.8, which represents the upper 95% confidence limit of the same estimate derived from the health utilization survey. As the denominator, the number of children in each age category estimated to be living in the three Dr George Mukhari catchment areas and in Madibeng during each of year 2003, 2004 and 2005 was used.

### 3.3.5 Collection of stool samples

Diarrhoeal stool samples were collected from children less than 5 years of age presenting at the hospital admission Paediatric ward and Paediatric Outpatient Department. A single stool specimen was obtained within 48 hours upon admission to exclude nosocomial infection. Infants from the neonatal ward were excluded.
3.3.6 Preparation of faecal samples

Faecal suspension of a pea size amount of stool was made with 10 ml of distilled water and vortexed for approximately 2 min to break-up macroscopic debris throughout the specimen. In addition, the raw stool was aliquoted and a pea size of faecal material was sent to Microbiology Department (Medunsa) for bacterial and parasites analysis. All specimens were stored at 4°C until further tested.

3.3.7 Enzyme immunoassay

All faecal suspensions were screened for group A rotavirus using commercially available enzyme immunoassay IDEIA™ Rotavirus test (DAKO, Dakocytomation, Denmark). The test was carried out according to manufacturer’s instruction. The assay utilized a polyclonal antibody in a solid phase sandwich enzyme immunoassay to detect group A rotavirus in faecal suspensions. In brief, 100 μl of faecal suspension and enzyme conjugate antibody were added to the microtitre plates, which were pre-coated with a rotavirus specific polyclonal antibody and incubated at 37°C for 1 hour. Both the negative and positive controls were included. Rotavirus antigens present in faecal suspension adhere to the rotavirus antibody on the solid phase and enzyme conjugate antibody detects to form an antibody-antigen-antibody complex. After a wash, the unbound materials were removed and 100 μl of substrate was added for 10 min incubation. The presence of antibody-antigen complex resulted in a colour change, which was stopped by dilute sulphuric acid solution. The reaction was then measured photometrically by a spectrophotometer at 460 nm. The cut-off value was calculated by adding 0.100 absorbance unit to the negative control. The specificity and sensitivity of the IDEIA™ Rotavirus test is 98.4% and 98.7%, respectively.
3.4 Results

3.4.1 Diarrhoeal disease

Between 2003 and 2005, the disease burden of rotavirus diarrhoea at the tertiary hospital Dr George Mukhari was identified from a total of 65 971 paediatric admissions between ages 0-12 years. Of the total paediatric admissions, 4 072 (1 200 outpatient and 2 872 inpatient) children less than 5 years of age presented for the treatment of acute gastroenteritis or diarrhoea at the Dr George Mukhari Hospital. This translated into 1:16.2 (6%) children presenting at the hospital for treatment. Of those treated, diarrhoeal stool specimens were collected from 2 512 (62%) children (Table 3:1).

The majority of children (~90%) hospitalized for diarrhoeal disease were aged between 0 and 24 months (Table 3:2 and Table 3:3). The data from Brits Hospital shows that from 8 950 paediatric hospital admissions 1:6.6 children were treated for diarrhoeal diseases (Table 3:1).

In order to verify the representative profiles of patients enrolled in the study population, comparisons were made between patients from whom the stool samples were collected and from whom stool sample was not collected. A significant difference in the age distribution between patients who provided stools samples compared to those who did not was observed. Fewer children were between age 0-6 months and more children between ages 18 to 60 months in children were among those who did not provide stool samples (Table 3:4).
<table>
<thead>
<tr>
<th>Year</th>
<th>Total admissions (0-12 years)</th>
<th>CHILDREN UNDER 5 YEARS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outpatient</td>
<td>Inpatient</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dr George Mukhari Hospital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>16 340</td>
<td>3 641</td>
</tr>
<tr>
<td>2004</td>
<td>19 817</td>
<td>3 971</td>
</tr>
<tr>
<td>2005</td>
<td>18 093</td>
<td>4 109</td>
</tr>
<tr>
<td>Total</td>
<td>54 250</td>
<td>11 721</td>
</tr>
<tr>
<td>Brits Hospital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>1 883</td>
<td>-</td>
</tr>
<tr>
<td>2004</td>
<td>3 751</td>
<td>-</td>
</tr>
<tr>
<td>2005</td>
<td>3 316</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>8 950</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3:2 Dr George Mukhari Hospital: Sample size and percentage of rotavirus positive children (1 January 2003 to 31 December 2005)

<table>
<thead>
<tr>
<th>Description</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children* admitted with diarrhoea (from hospital nominal books, includes 38 children aged 60 months or more captured in the database)</td>
<td>4 072</td>
</tr>
<tr>
<td>Proportion</td>
<td>%</td>
</tr>
<tr>
<td>Children* captured in database</td>
<td>3 604</td>
</tr>
<tr>
<td>Children* with specimens</td>
<td>2 512</td>
</tr>
<tr>
<td>Children* with specimens, Rotavirus positive</td>
<td>527</td>
</tr>
<tr>
<td>Under two with specimens</td>
<td>2 181</td>
</tr>
<tr>
<td>Under two with specimens, Rotavirus positive</td>
<td>498</td>
</tr>
</tbody>
</table>

* The hospital dataset includes 38 children aged 60 or more months.

Table 3:3 Numbers of children admitted, specimens examined and children found to be rotavirus positive in Brits Hospital (16 July 2003 to 31 December 2005)

<table>
<thead>
<tr>
<th>Description</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children admitted with diarrhoea (from hospital nominal books, includes 20 patients whose age were not known captured in the database)</td>
<td>1 340</td>
</tr>
<tr>
<td>Proportion</td>
<td>%</td>
</tr>
<tr>
<td>Children* with specimens captured in database</td>
<td>636</td>
</tr>
<tr>
<td>Children* with specimens, Rotavirus positive</td>
<td>108</td>
</tr>
<tr>
<td>Under two with specimens</td>
<td>566</td>
</tr>
<tr>
<td>Under two with specimens, Rotavirus positive</td>
<td>97</td>
</tr>
</tbody>
</table>

*At Brits Hospital, data were collected only from those patients from whom a specimen was obtained and analysed.
<table>
<thead>
<tr>
<th>Question</th>
<th>Dr George Mukhari: patients with specimens</th>
<th>Dr George Mukhari: patients without specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion</td>
<td>%</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3m</td>
<td>207 /1559</td>
<td>13.3</td>
</tr>
<tr>
<td>≥3m to 6m</td>
<td>315 /1559</td>
<td>20.2</td>
</tr>
<tr>
<td>≥6m to 9m</td>
<td>279 /1559</td>
<td>17.9</td>
</tr>
<tr>
<td>≥9m to 12m</td>
<td>192 /1559</td>
<td>12.3</td>
</tr>
<tr>
<td>≥12m to 18m</td>
<td>247 /1559</td>
<td>15.8</td>
</tr>
<tr>
<td>≥18m to 2y</td>
<td>98 /1559</td>
<td>6.3</td>
</tr>
<tr>
<td>≥2y to 5 y</td>
<td>198 /1559</td>
<td>12.7</td>
</tr>
<tr>
<td>≥5y</td>
<td>23 /1559</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>741 /1577</td>
<td>47.0</td>
</tr>
<tr>
<td>Male</td>
<td>836 /1577</td>
<td>53.0</td>
</tr>
<tr>
<td><strong>Admitted to ward</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1155 /1577</td>
<td>73.2</td>
</tr>
<tr>
<td><strong>Housing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional hut</td>
<td>1 /1563</td>
<td>0.1</td>
</tr>
<tr>
<td>Shack</td>
<td>775 /1563</td>
<td>49.6</td>
</tr>
<tr>
<td>Modern</td>
<td>787 /1563</td>
<td>50.4</td>
</tr>
<tr>
<td><strong>Water supply</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>15 /1563</td>
<td>1.0</td>
</tr>
<tr>
<td>Communal tap</td>
<td>806 /1563</td>
<td>51.6</td>
</tr>
<tr>
<td>Indoor tap</td>
<td>742 /1563</td>
<td>47.5</td>
</tr>
<tr>
<td><strong>Toilet facilities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1 /1563</td>
<td>0.1</td>
</tr>
<tr>
<td>Pit</td>
<td>825 /1563</td>
<td>52.8</td>
</tr>
<tr>
<td>Bucket</td>
<td>3 /1563</td>
<td>0.2</td>
</tr>
<tr>
<td>Flush</td>
<td>734 /1563</td>
<td>47.0</td>
</tr>
<tr>
<td><strong>Breastfed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breastfed only</td>
<td>597 /1360</td>
<td>43.9</td>
</tr>
<tr>
<td><strong>Feeding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breastfed only</td>
<td>101 /591</td>
<td>17.1</td>
</tr>
<tr>
<td>Mixed feeding</td>
<td>490 /591</td>
<td>82.9</td>
</tr>
<tr>
<td><strong>Desire for food</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased</td>
<td>52 /1491</td>
<td>3.5</td>
</tr>
<tr>
<td>Decreased</td>
<td>808 /1491</td>
<td>54.2</td>
</tr>
<tr>
<td><strong>Previous management</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Went to clinic</td>
<td>994 /1514</td>
<td>65.7</td>
</tr>
<tr>
<td>Given ORS</td>
<td>921 /1395</td>
<td>66.0</td>
</tr>
</tbody>
</table>
3.4.2 Rotavirus diarrhoea

Rotavirus is associated with severe diarrhoea and requires hospitalization. The virus was associated with 1:4.7 (21%) of stool samples collected (Table 3:2 and Table 3:3). The data indicate that from hospitalized children 1:4 children were treated for diarrhoeal diseases and on average rotavirus antigen was identified at 22.8% of children admitted at Dr George Mukhari Hospital whereas at Brits Hospital 18.2% children had rotavirus diarrhoea.

Yearly prevalence of rotavirus infections was as follows: At Dr George Mukhari Hospital during 2003, 165 (17%) stool samples were found to be positive for rotavirus (19% of the inpatients and 12% of the outpatients). During 2004, 134 (21%) of the collected specimens were found to be rotavirus positive (24% of the inpatients and 14% of the outpatients) and in 2005, 228 (24%) stool samples were rotavirus positive (26% inpatients and 17% outpatients). At Brits Hospital, 14% (23/165), 20% (49/249) and 21% (47/215) of the stool samples were found to be positive for rotavirus from 2003 through 2005, respectively (Table 3:1). The general characteristics of all the children enrolled in the study are listed in Table 3:4. The relevant information was not provided for all the children enrolled, therefore, the total number in each category was not the same. The median duration of hospitalization for children with gastroenteritis associated with rotavirus was 4 days and ranged from (1 to 19 days). This increased to 7 days when children presented with gastroenteritis together with other infections or pre-existing conditions such as malnutrition, HIV/AIDS, bronchopneumonia, meningitis, convulsions, failure to thrive and neonatal sepsis.

3.4.3 Demographic characteristics of rotavirus infection

Rotavirus gastroenteritis was more frequently detected among inpatients, at 81.6%, compared to outpatients, at 18.4%. Comparison of gender proportion and stool
collection at the two hospitals upon admission and comparison of socioeconomic and demographic indicators in patients who gave stool samples and those that did not was conducted. Of these children at Dr George Mukhari Hospital, the majority were boys (53.0%) compared to girls (47.0%). The trend continued at Brits Hospital where slightly more boys than girls were infected with rotavirus. The household environmental conditions were used as an indicator of socioeconomic status. The analysis was characterized as urban, rural formal settlement, or informal settlement, water supply and sanitation. The study showed that rotavirus infection is universal with similar infection rates. There was no significant difference found between rotavirus positive versus rotavirus negative results and the stool collection success or failure according to socioeconomic level (Table 3:4).

3.4.4 Seasonality of rotavirus diarrhoea

According to our data, rotavirus-associated gastroenteritis occurs throughout the year. However, two seasonal peaks of rotavirus incidence were observed around April and August during the cool and dry months of the year, respectively. The peak prevalence of rotavirus infection in hospitalized children was 56% in May 2003 and in June 2004 and 2005 at 59% and 56%, respectively. At Brits Hospital, from the second semester of 2003 to the end of 2005, a similar peak of rotavirus infection in children less than 5 years of age was observed (Figure 3:2). Relatively low prevalence of rotavirus infection was noted during the months of the summer season October through March. Figure 3:2 shows the monthly distribution of rotavirus diarrhoea among children less than 5 years of age admitted to Dr George Mukhari and Brits Hospitals. The prevalence of acute gastroenteritis is shown as white solid bars and number of rotavirus infection in black solid bars.
Figure 3:2 Proportion of rotavirus diarrhoea among children under 5 years of age admitted to Dr George Mukhari hospital (Jan 2003-Dec 2005) and treated for diarrhoea at Brits hospital (Jul 2003-Dec 2005). The number of hospitalizations is shown as bars, distinguishing between cases from which we isolated rotavirus (black) and the other cases (white). The curve indicates the percentage of diarrhoea cases from which we isolated rotavirus.
3.4.5 Proportion of rotavirus diarrhoea in hospitalized children

Among the 11 721 patients admitted to the Dr George Mukhari paediatric ward, 9 886 (84%) were below 5 years of age (Table 3:1). Of these, 2 553 (26%) were from Dr George Mukhari catchment area and were admitted for diarrhoea. Stool specimen were analyzed for rotavirus in 1 870 (73%) of these cases, while stool specimens could not be obtained from the remaining patients because they did not pass a stool before being discharged (394 /2 553) and (289/2 553) children died before a stool sample could be collected. Stool specimens were examined from all the 451 children less than 5 years of age who visited Brits Hospital for diarrhoea during 2004 and 2005.

Among children admitted with diarrhoea to Dr George Mukhari Hospital and from whom a stool specimen was obtained, the median maximal number of bowel movements during any 24 hr period and the number of days with vomiting before admission according to the caretakers were 4 and 1, respectively, identical to the corresponding values for those from whom a specimen was not obtained. On the other hand, the median age of those with stool specimens collected was 8 months against those who failed to give a stool specimen was 11 months. When estimating the number of all children admitted to Dr George Mukhari Hospital who had rotavirus diarrhoea, an age-stratum-specific proportions of stool specimens with rotavirus among those who had given a stool specimen was accordingly applied as well as to those patients who had not given clinical specimens, thereby adjusting for the slightly different age distribution between the two groups of children.

The proportion of children treated for diarrhoea at Brits Hospital and from whom rotavirus was isolated were 44/236 (18.6%; 95%CI 14%, 24%) in 2004 and 38/215 (17.7%; 95%CI 13%, 23%) in 2005 (Table 3:5). The corresponding proportions among children admitted to Dr George Mukhari Hospital were 126 of 652 (19.3%; 95%CI 16%, 22%) in 2003, 115/470 (24.5%; (95%CI 21%, 29%) in 2004; and 195/747 (26.1%;
95%CI 23%, 29%) in 2005. **Table 3:5** also displays the age-specific proportions of children under 5 years of age treated at Brits Hospital and admitted to the Dr George Mukhari Hospital paediatric ward who were identified as having rotavirus diarrhoea, as well as the number of all children admitted with diarrhoea to Dr George Mukhari Hospital that were estimated to have rotavirus diarrhoea, assuming the same age-specific isolation proportions in all diarrhoeal cases. This translates to an annual average age-adjusted proportion of 22.8% (582/2 553) for children admitted to Dr George Mukhari Hospital.

In both hospitals, the proportion of children above 2 years of age who had rotavirus diarrhoea was approximately 10%, while it was close to 23.5% among the 1 year olds. Among all 12-59 month old children, the regression model showed that this proportion decreased by 4% (95%CI 2%, 6%) for every month increase in age. In contrast, among infants, the proportion with rotavirus diarrhoea was much higher at Dr George Mukhari Hospital (27.6%; 95%CI 25.1, 30.2%) as compared to Brits Hospital (19.1%; 95%CI 15.2%, 23.7%). Adjusting for age, the proportion of infants with rotavirus was 40% (95%CI 11%, 81%) higher in Dr George Mukhari than in Brits Hospital, and adjusting for hospital, it increased by 6% (95%CI 3%, 8%) for every month increase in age.

**Table 3:6** shows that three quarters (528/707) of the children who were hospitalized with rotavirus diarrhoea were infants, 90% (633/707) less than 18 months of age and 95% (669/707) less than 2 years of age.

### 3.4.6 Overall and age-specific incidence of severe rotavirus diarrhoea

According to StatsSA, there were 84 484 children under 5 years of age in the four encatchment regions for the two study hospitals, 6 218 in Garankuwa, 19 318 in
Mabopane, 28 882 in Soshanguve, and 30 066 in Madibeng, with approximately equal numbers of children in each 1-year age category.

Based on the above-mentioned assumption that, within each age category, the proportions of children with rotavirus were the same irrespective of whether they had provided a stool specimen that underwent analysis, estimates indicated that 582 cases of severe rotavirus diarrhoea were admitted to Dr George Mukhari Hospital during the study period. Similarly, assuming that at Brits Hospital, the number of cases with diarrhoea and rotavirus illness in 2003 averaged that in the following two years, estimates indicated that 125 children were treated for rotavirus diarrhoea at Brits Hospital during the entire 3-year period. Thus, overall, 707 cases of rotavirus diarrhoea were included in the numerator of the incidence calculations; the age breakdown is given in Table 3:5. Dividing the numerators by the number of children (overall and in each age category) estimated to be living in the three Dr George Mukhari Hospital catchment areas and in Madibeng during each of the years 2003, 2004 and 2005 and dividing by the 80% to 65% coverage of hospitalization anticipated to be captured by the hospitals, an average yearly rotavirus incidence rate ranging from 329 [95%CI 313 to 358] to 404 [95%CI 385 to 441] per 100,000 children under 5 years of age was obtained. The age-specific incidence rate estimates are given in Table 3:6.

The incidence was relatively low during the first 3 months of life, highest in the last 9 months of infancy, decreasing sharply thereafter (Table 3:6). The incidence estimates indicates that one in 45 [100 000/(441*5)] to 64 (100 000/(313*5)] children under 5 years of age in this population is likely to be hospitalized with rotavirus diarrhoea between birth and 5 years of age. Likewise, one in 50 [100 000/(1 003*2)] to 70 [100 000/(711*2)] children is likely to be hospitalized with rotavirus diarrhoea between birth and 2 years of age.
Table 3:5 Age-stratified proportion of children hospitalized at Dr George Mukhari and treated at Brits Hospital for diarrhoea with rotavirus-positive stool specimens.

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Hospital: Dr George Mukhari hospital (January 1, 2003, - December 31, 2005)</th>
<th>Brits hospital (January 1, 2004 - December 31, 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion rotavirus + of children with diarrhoea who were tested</td>
<td>Estimated number of children with rotavirus diarrhoea</td>
</tr>
<tr>
<td></td>
<td>% rotavirus +</td>
<td>95% CI</td>
</tr>
<tr>
<td>0-2</td>
<td>29 / 218</td>
<td>13.3</td>
</tr>
<tr>
<td>3-5</td>
<td>111 / 366</td>
<td>30.3</td>
</tr>
<tr>
<td>6-8</td>
<td>119 / 372</td>
<td>32.0</td>
</tr>
<tr>
<td>9-11</td>
<td>76 / 259</td>
<td>29.3</td>
</tr>
<tr>
<td>0-11</td>
<td>335 / 1 215</td>
<td>27.6</td>
</tr>
<tr>
<td>12-17</td>
<td>65 / 318</td>
<td>20.4</td>
</tr>
<tr>
<td>18-23</td>
<td>16 / 125</td>
<td>12.8</td>
</tr>
<tr>
<td>12-23</td>
<td>81 / 443</td>
<td>18.3</td>
</tr>
<tr>
<td>0-23</td>
<td>416 / 1 658</td>
<td>25.1</td>
</tr>
<tr>
<td>24-59</td>
<td>20 / 212</td>
<td>9.4</td>
</tr>
<tr>
<td>All</td>
<td>436 / 1 870</td>
<td>23.3</td>
</tr>
</tbody>
</table>
Table 3:6 Estimated age-specific incidence of rotavirus diarrhoea requiring hospitalization among 84 484 children in North West and Gauteng Provinces of South Africa.

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Estimated no. of children with rotavirus diarrhoea&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Estimated population at risk</th>
<th>Incidence per 100 000 referral = 80%&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Incidence per 100 000 referral = 65%&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no</td>
<td>95% CI</td>
<td>2001</td>
<td>2003 – 2005</td>
</tr>
<tr>
<td>0-2</td>
<td>42</td>
<td>31</td>
<td>56</td>
<td>4 614</td>
</tr>
<tr>
<td>3-5</td>
<td>182</td>
<td>161</td>
<td>205</td>
<td>4 614</td>
</tr>
<tr>
<td>6-8</td>
<td>175</td>
<td>153</td>
<td>199</td>
<td>4 614</td>
</tr>
<tr>
<td>9-11</td>
<td>129</td>
<td>111</td>
<td>150</td>
<td>4 614</td>
</tr>
<tr>
<td>0-11</td>
<td>528</td>
<td>489</td>
<td>567</td>
<td>18 455</td>
</tr>
<tr>
<td>12-17</td>
<td>105</td>
<td>87</td>
<td>125</td>
<td>8 132</td>
</tr>
<tr>
<td>18-23</td>
<td>36</td>
<td>27</td>
<td>50</td>
<td>8 132</td>
</tr>
<tr>
<td>12-23</td>
<td>141</td>
<td>121</td>
<td>167</td>
<td>16 264</td>
</tr>
<tr>
<td>0-23</td>
<td>669</td>
<td>629</td>
<td>721</td>
<td>34 719</td>
</tr>
<tr>
<td>24-59</td>
<td>38</td>
<td>26</td>
<td>54</td>
<td>49 765</td>
</tr>
<tr>
<td>All</td>
<td>707</td>
<td>673</td>
<td>771</td>
<td>84 484</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sum of estimated number of children given in Table 3:3.

<sup>b</sup>No. of cases/100 000 children/year. Corrected for 65% or 80% coverage of all hospitalizations anticipated at Dr George Mukhari and Brits Hospital.
3.4.7 Clinical symptoms

Fever and vomiting were symptoms commonly associated with rotavirus diarrhoea and vomiting was reported more frequently in the rotavirus positive patients 63% than those without rotavirus infection. Low grade fever did not show any significant difference between children infected with rotavirus and the children whom were not infected. Rotavirus infection was commonly associated with bronchopneumonia (70%), following the seasonal influenza during winter season. The mean hospital admission for rotavirus infections in children less than 5 years of age was reported to be 4 days, ranging from (1-7 days) (Data not shown). The difference between clinical history of diarrhoea from patients from whom the stool samples were collected and not collected, fever and vomiting were seen more in children from whom the stool samples were collected, which indicates that patients whose samples were collected had moderate to severe infection.

3.4.8 Treatment

During the study period, high proportions of children (64%) with acute gastroenteritis were treated with oral rehydration therapy at home prior to hospital admissions. Majority of children infected with rotavirus (74%) were treated with antibiotics at the hospital.

3.5 Discussion

The burden of diarrhoeal disease in developing communities has been estimated to be around five times higher than developed countries (Murray et al., 1996). In South African children under the age of 5 years, diarrhoea was the leading cause of death in black and coloured children (Westaway and Viljoen, 2000). Notwithstanding, prior to data generated in this study, there was not any information about rotavirus-associated burden of disease in South Africa. This is the first study to estimate population-based
incidence of hospitalization for rotavirus diarrhoea in Africa. The high incidence of severe rotavirus diarrhoea reflects the substantial public health impact of this single and potentially preventable disease.

While studies in the United States provided data on costs of diarrhoeal hospitalization and outpatient visits (Zimmerman et al., 2001), the few burden of disease trials in sub-Saharan Africa indicate different sets of issues when compared to developed countries. Some of these issues include: insufficient research on diarrhoea, childhood diseases and HIV relative to developed countries; the interaction between nutrition and diarrhoeal diseases and the logistics of introducing vaccines against childhood diarrhoeal diseases (Isaakidis et al., 2002). In countries such as Guinea Bissau, Malawi and Burkina Faso, malaria, diarrhoea and lower respiratory infections are the most common diseases among infants. These acute diseases emphasise the need for active and early management of infected infants (Mølbak et al., 2000; Vaahtera et al., 2000; Würthwein et al., 2001).

This prospective hospital-based study of rotavirus disease burden provides information on the importance of rotavirus gastroenteritis as a cause of morbidity and mortality in South African children aged between 0 and 60 months. The study was designed according to the generic WHO protocol, required for advocacy regarding the testing and introduction of rotavirus vaccines.

The data clearly demonstrates the high burden of rotavirus-associated disease in South Africa. During 2003 through 2005, the average annual rate of rotavirus infection was 17%, 21%, and 25%, respectively, indicating that a substantial number of South African children who are treated for diarrhoea are infected with rotavirus. Interestingly, South African incidence estimates of severe rotavirus diarrhoea among children less than 5
years of age are similar to incidence estimates obtained in a North Indian study (Bahl et al., 2005). In both studies, the rotavirus disease burden was highest among infants between 2 months and 18 months of age, declining substantially thereafter. Rotavirus seems to have considerably lower pathogenicity in children older than 2 years of age (Fischer et al., 2002). The South African analysis indicates that rotavirus causes one in 50 to one in 70 children to be hospitalized during their first two years of life. Rotavirus diarrhoea accordingly contributes importantly to the disease burden in young children, and emphasizes the need for vaccination at an early age once rotavirus vaccines are incorporated into child health promotion programs.

Out of 9,886 children under 5 years of age that were admitted and treated at Dr George Mukhari Hospital, 2,553 (26%) were admitted for diarrhoea. Among these, it was estimated that 22.8% (582/2,553) had rotavirus diarrhoea. These results are in line with hospital-based studies from many other countries (Cunliffe et al., 1998). Assuming that rotavirus was the only cause of diarrhoea in children less than 2 years of age (Fischer et al., 2002), 549 (582-33) or 5.5% (95%CI 5.1%, 6.0%) of all admissions in children less than 5 years at Dr George Mukhari Hospital would be due to rotavirus infections. This illustrates that deploying an effective rotavirus vaccine with high coverage could substantially reduce not only the disease burden in the community but also the load on the paediatric ward of hospitals and thereby health facility costs. A separate economic analysis has been conducted to examine the high financial burden of rotavirus diarrhoea at Dr George Mukhari Hospital, highlighting the benefits that rotavirus immunization could have on the health system in South Africa (MacIntyre and De Villiers, J Infect Dis Accepted).

A seasonal pattern of rotavirus infection was observed in the children treated at Dr George Mukhari Hospital and at Brits Hospital corresponding to the cooler and drier months of the year. These results correlate with the seasonal trend of rotavirus infection
reported in previous studies in South Africa (Steele et al., 1986). These figures support the fact that majority of children with rotavirus gastroenteritis were hospitalized during the winter season. During the winter peaks, the proportion of rotavirus diarrhoea could exceed 50%, while during the summer months, it was less than 5%. These findings may have implications for immunization (WHO, 2007) and case management strategies.

The protective effect of breast-feeding on infectious disease risk is well documented. Lack of breast-feeding, severe malnutrition, inappropriate use of oral rehydration solutions and scarce health care facilities, were previously reported as the major risk factors for severe dehydration in children with diarrhoea (Faruque et al., 1992). In the present study, the majority of the children (over 58%) less than one year in age were breast-fed. Breastfeeding was either exclusive or the child was breastfed together with infant formula or solids. No significant association between breast-feeding practices and the risk of rotavirus gastroenteritis was found and it appears that protective maternal antibodies had no significant effect on the prevalence of rotavirus infection. This outcome differs from the study done by Walther et al. (1984) where formula feeding, in the absence of breast-feeding, was associated with rotavirus infection.

Adequate water supplies, sanitation facilities, access to health care facilities, education and knowledge of rehydration therapy are the main factors thought to prevent diarrhoeal disease and improve child welfare. The present study does not indicate a difference in rotavirus infection with respect to geographical area, whether urban, rural and/or informal settlement. These results seem to reinforce the idea that rotavirus infection is global and that the infection rate is similar in both developing and developed countries. Nevertheless, the major difference remains the high mortality rate associated with diarrhoeal disease in developing countries. This might be due to a number of factors such as poor healthcare facilities, malnutrition, poor access to rehydration therapy and co-infections such as HIV, M. tuberculosis and malaria. The importance of rotavirus-
associated gastroenteritis in sub-Saharan Africa and the recent promising interim analysis from a phase III efficacy study of rotavirus vaccine in South Africa (Madhi et al., 2008), indicate that rotavirus immunization will have a large impact on the health of African infants and young children.

It is known that the majority of deaths due to rotavirus infection occur as a result of the primary infection. Poor nutritional status predisposes a greater diarrhoeal frequency due to impaired host defences, decreased gastric acidity, or nutritional deficiencies (Black and Lanata, 1995). Although diarrhoeal disease is a significant cause of childhood morbidity and mortality, especially among malnourished children, the present study showed that 34% of children were malnourished.

The rate of hospital admissions for gastroenteritis in children less than 5 years of age in the study population was 7%. This estimate rate is relatively low. This may be due to improvements in the health care services available to the communities involved in this study or to improved treatment and management of diarrhoeal diseases, such as the use of appropriate oral rehydration solution (ORS) at home. In many regions of the world the usage of oral rehydration therapy (ORT) has declined (Ram et al., 2008). Contrarily, in this study most of the caretakers or mothers were aware of the proper usage of ORS (64%) and it appears that ORT may have improved many of the most severe cases of gastroenteritis. A similar study conducted during the late 1990s at the Ga-Rankuwa Hospital indicated that about 67% of mothers offer their children ORS if they have diarrhoea (Mawela and De Villiers, 1999), emphasizing the need for effective implementation of preventive measures against rotavirus disease.

The peak prevalence rates of rotavirus infection observed was 56-59% between 2003 and 2005, and the greatest incidence of hospitalizations to treat gastroenteritis occurred
during the dry and cooler months of the year. The mortality rate of diarrhoeal diseases at Dr George Mukhari Hospital was 7% in 2003 (Dr George Mukhari Hospital, unpublished data). However, it was difficult to determine the mortality rate of rotavirus gastroenteritis because most of the children died before the stool samples were collected. Furthermore, not all of the children who were infected with rotavirus were followed to determine whether they survived after leaving the hospital. Therefore, a community-based survey of rotavirus infection could be conducted in future and linked to the hospital-based data to determine more precise estimates of rotavirus mortality.

South Africa has a unique hospital referral system whereby the clinics are the primary care level of admission and provide good treatment management and only exceptional or severe cases are referred to secondary and tertiary health care level hospitals.

At Dr George Mukhari Hospital, the proportion of diarrhoea at the outpatients ward was found to be less significant due to the fact that the outpatient ward caters for clinics for different chronic diseases, for example, Neonatal, Asthma, Oncology, General and HIV clinic. The total admissions in the outpatients ward also includes follow up or review cases.

Although the average annual collection rate of stool samples was relatively low (59%), the study population is representative of all paediatric patients in the study area. The low collection rate may be ascribed to: (1) limited staff, collecting the stool specimens mainly during weekdays, and during the weekends only when the children were still admitted, (2) hospital strikes, (3) patients been discharged before producing stool samples or diarrhoea has stopped, (4) mistrust and suspicions about what the stool samples might be used for, due to cultural beliefs.
This study had several limitations. First, the proportion of rotavirus cases in children admitted to Dr George Mukhari Hospital and from whom a sample was not obtained may not be the same as the proportion of rotavirus cases in children who did provide a stool specimen. However, similar stool frequency and days with vomiting and the fact that the age imbalance between the two groups was adjusted for, makes this an unlikely source of serious bias. Second, the corrective factor of 65% (public health expert guesstimate) to 80% (upper 95%CI of the proportion obtained in our Health Utilization Survey) may have been inaccurate. On the other hand, the large prospective survey where most children of a relatively well-described population visited a secondary and a tertiary care referral hospital over up to a three-year period and in whom rotavirus was identified using standardized protocols is likely to generate data adequate for disease burden estimations in this and in similar African populations.

In conclusion, our findings indicate that the deployment of a safe and effective rotavirus vaccine, the only known preventive measure against rotavirus infection, should be a public health priority in South Africa. The data presented here can contribute to cost-effectiveness studies of rotavirus vaccination programs once they are rolled out in South Africa.
4 CHAPTER 4: MOLECULAR EPIDEMIOLOGY OF ROTAVIRUS STRAINS RECOVERED IN GA-RANKUWA BETWEEN 2003 TO 2005 DURING SOUTH AFRICAN ROTAVIRUS VACCINE TRIALS

4.1 Abstract

BACKGROUND: Rotavirus infection remains a significant clinical problem throughout the world and is the most common cause of severe dehydrating gastroenteritis in infants and young children. Severity as well as incidence of rotavirus disease can be reduced through the implementation of an effective vaccine programme. The aim of this study was to characterize rotavirus strains circulating within South African communities during the time the monovalent rotavirus vaccine candidate (Rotarix®, G1P[8]) was being tested.

OBJECTIVES: Firstly, to describe in detail the molecular epidemiology of rotavirus infections in South Africa during consecutive rotavirus seasons, through genotyping of rotavirus-positive samples collected throughout each year. Secondly, to monitor any changes in the prevalence of common rotavirus strains circulating and lastly, to identify uncommon strains not represented in the vaccine that may be important after introduction of a rotavirus vaccine in South Africa.

METHODS: Between 2003 and 2005, 527 rotavirus-positive stool specimens were collected from children less than 5 years of age who were treated for diarrhoea at Dr George Mukhari Hospital. Polyacrylamide gel electrophoresis (PAGE) was performed on extracted rotavirus double stranded (dsRNA). Rotavirus P and G typing was carried out utilizing semi-nested RT-PCR amplification of the VP7 and VP4 genes.
RESULTS: During 2003, a short electropherotype was noted in 62% of the rotavirus strains detected. However, in 2004 and 2005, these strains were largely replaced by rotaviruses showing a long electropherotype at levels of 96% and 98%, respectively. Predominant rotavirus strains detected included G2P[4]/G2P[6] in 2003, G1P[8]/G1P[6] in 2004 and G3P[8]/G3P[6] in 2005. The P[6] genotype were detected in 30% of specimens over the three year time span. In addition, unusual rotavirus strains G12P[6] and G8 were also detected at low frequency.

CONCLUSION: These results reflect the diversity of rotavirus strains circulating within the communities served by the Dr George Mukhari Hospital. While annual variation in the circulating rotavirus strains may have implications for the efficacy of a monovalent vaccine candidate, strain replacements by alternate or unusual rotaviruses were not observed in this setting. Further monitoring is required to determine the impact of rotavirus vaccine.
4.2 Introduction

Rotavirus infection remains a significant clinical problem throughout the world and is the most common cause of severe dehydrating gastroenteritis in infants and young children. Recently, it was estimated that rotavirus disease is responsible for approximately 527 000 deaths in children less than 5 years of age, primarily in developing countries (WHO, 2007). In sub-Saharan Africa alone is estimated to account for approximately 230 000 deaths in children less than 5 years old (Proceedings of 4th Africa Rotavirus symposium, Mauritius 2008).

The genetic and antigenic diversity of the outer capsid proteins, VP7 and VP4, allow the classification of rotaviruses into G and P types, respectively. Both VP4 and VP7 are important targets for vaccine development because these proteins independently elicit protective neutralizing antibodies (Estes and Cohen, 1989; Hoshino and Kapikian, 2000). Epidemiological studies of rotavirus infections have shown that there is great diversity of rotavirus strains circulating throughout the world. Currently there are 23 G genotypes and 28 P genotypes identified in humans and animals (Estes, 2001; McNeal et al., 2005; Rahman et al., 2005a; Khamrin et al., 2007a; Matthijnssens et al., 2008; Solberg et al., 2009; Ursu et al., 2009). Five human group A rotavirus strains which are frequently detected globally include strains with serotype G1, G2, G3, G4 and G9 specificity in association with P[8] and P[4] genotypes (Hoshino and Kapikian, 2000; Estes and Kapikian, 2007). However, in developing countries alternative strains may predominate that may include P[6] genotypes and G8 serotypes (Cunliffe et al., 1999; Armah et al., 2001; Adah et al., 1997, Steele et al., 2003; Desselberger et al., 2001).

The introduction of new live oral rotavirus vaccines has pioneered the prospect of substantial reductions in child morbidity and mortality globally. The two new rotavirus vaccines licensed for use are RotaTeq™, developed by Merck (Blue Bell, PA, USA) and
Rotarix® developed by GlaxoSmithKline Biologicals, (Rixensart, Belgium). RotaTeq™, based on homotypic immunity, consists of bovine-human mono-reassortant strains with G1P[7], G2P[7], G3P[7], G4P[7] and G6P[8] specificity (Heaton et al., 2005). The large RotaTeq™ vaccine trials of 70 301 participants from 11 developed countries demonstrated high efficacy against severe rotavirus diarrhoea and no association with an increased risk of intussusception (Vesikari et al., 2006).

Rotarix® is a live human attenuated monovalent P1A[8]G1 rotavirus vaccine (De Vos et al., 2004) based on heterotypic immunity. Results from phase III Rotarix® efficacy studies performed in Latin America and Finland demonstrated high efficacy against severe rotavirus diarrhoea associated with G1P[8], G3P[8], G4P[8] and G9P[8] strains and no association with an increased risk of intussusception (Ruiz-Palacios et al., 2006). However, the vaccine appears to be less effective against G2P[4] rotavirus strains of any disease severity but was effective against severe G2 rotavirus infections (Vesikari et al., 2007).

The World Health Organization (WHO) recommended that safety and immunogenicity of rotavirus vaccines should be demonstrated in developing countries in Africa and Asia. Thus, Rotarix® was evaluated in the Madibeng and Ga-Rankuwa districts in South Africa from 2001 onwards to determine: (a) non-interference of the rotavirus vaccine with oral polio vaccine (OPV) (Steele et al., 2008); (b) the appropriate dosage and regimen for the rotavirus vaccine administration; (c) rotavirus vaccine safety in children with HIV and; (d) the efficacy of the rotavirus vaccine in an African setting. The studies included: (a) two phase II immunogenicity trials (2 versus 3 doses) where OPV was co-administered at 6 and 10 weeks and at 6,10 and 14 weeks of age (2001-2004); (b) a phase III efficacy study (2005-2008); and (c) a safety and immunogenicity trial in HIV infected infants (2005-2008). The results from co-administration of OPV with Rotarix®
demonstrated that the rotavirus vaccine does not interfere with OPV, is well-tolerated and immunogenic in South African infants (Steele et al., 2008).

The ability of the currently available vaccines to provide protection against unusual or uncommon rotavirus strains circulating in developing countries cannot be predicted. Epidemiological studies of rotavirus infections have shown that there is great diversity of rotavirus strains circulating throughout the world. Therefore, the effectiveness of rotavirus vaccines against diverse strains should be monitored over the long-term period. The aim of this study was to describe the genetic diversity of rotavirus strains circulating within communities during the same time period the Rotarix® vaccine was being tested. In addition, rotavirus strain surveillance studies should document rotavirus serotypes in circulation before and after the implementation of rotavirus vaccination programs in order to monitor any changes in the prevalence of commonly circulating rotavirus strains.

4.3 Materials and methods

4.3.1 Stool collection

Between 2003 and 2005, stool samples were collected from children less than 5 years of age treated for gastroenteritis at both the paediatric inpatient and outpatient departments at Dr George Mukhari Hospital, North of Pretoria, South Africa. Infants in the neonatal ward were excluded from the study. A single stool specimen was obtained within 48 hours of admission to exclude nosocomial infections. Informed consent was obtained verbally from the parent or caretaker accompanying the child. The research protocol received approval from the Ethics Committee of University of Limpopo (Medunsa Campus). One gram of faecal material was sent to the Microbiology Department (University of Limpopo) for bacterial and parasitic enteropathogen analysis. A 10% faecal suspension of pea-sized stool in 10 ml of distilled water was made and stored at 4°C until analysed.
4.3.2 Detection of bacteria and parasites

Stool samples were also examined for bacterial enterotoxigenic *E. Coli* (ETEC), enteric pathogenic *E. Coli* (EPEC), enteroinvasive *E. Coli* (EIEC), *Campylobacter, Shigella, Salmonella, Clostridium difficile, Yersinia* and parasites, which included *Cryptosporidium parvum, Microsporidium, Entamoebas,* and *Giardia lamblia* using standard techniques like wet preparation, staining and culture (Stanier *et al.*, 1977).

4.3.3 Detection of rotavirus group A

All stool samples collected were analysed for the presence of rotavirus antigens using the commercially available enzyme immunoassay IDEIA™ Rotavirus test (DAKO, Dakocytomation, Denmark), according to the manufacturer’s instructions (refer to section 3.3.7).

4.3.4 Electrophoretic migration pattern of dsRNA

All rotavirus positive faecal samples were analysed by PAGE to characterized rotavirus strains. Briefly, RNA was extracted from the rotavirus-positive 10% faecal suspensions using 1/10 volume 1M sodium acetate containing 1% sodium dodecyl sulphate (SDS) (pH 5.0) and incubated at 37°C for 15 min as previously described by Steele and Alexander (1987). An equal volume of 1:1 phenol-chloroform was added and incubated at 56°C to disrupt the virus particles and release the viral dsRNA genome. After centrifugation at 13 500 x g, the upper aqueous phase containing the dsRNA was precipitated in ice-cold absolute ethanol and 3M sodium acetate (pH 5.0) overnight at –20°C. Following centrifugation at 13 500 x g for 15 min the dsRNA pellet was resuspended in PAGE dye containing glycerol and bromophenol blue (0.001%). The extracted RNA was resolved on 10% polyacrylamide gels overlaid with a 3% stacking gel, using a discontinuous Tris-glycine buffer system at 100 Volt (V) for 18 hours at
ambient temperatures. RNA segments were visualized by silver staining as described by Herring et al. (1982).

### 4.3.5 RT-PCR RNA extraction

The viral dsRNA was extracted from rotavirus-positive 10% faecal suspensions using Tri-Reagents-LS (Molecular Research Centre Ohio, USA) according to the manufacturer's instructions. In brief, 500 µl of Trizol was added to 200 µl stool supernatant and briefly vortexed and incubated at ambient temperature for 3 min and 100 µl chloroform was then added. After centrifugation at 13 500 x g for 15 min, the upper aqueous phase was precipitated in 700 µl ice-cold isopropyl alcohol and incubated at -20°C overnight. This was followed by centrifugation at 13 500 x g for 15 min and dsRNA pellet was air dried and resuspended in 15 µl of diethylpyrocarbonate (DEPC) treated water and used for RT-PCR. Samples that failed to generate PCR products were extracted by QIAamp viral RNA extraction method (QIAGEN, Hilden, Germany). This was employed according to the manufacturer's instructions. In brief, 140 µl of stool sample was added to 560 µl lysis buffer containing AVL with 1µg/ µl carrier RNA and 560 µl of ethanol to improve the binding of viral RNA to the QIAamp membrane. After lysis and 1 min centrifugation, the dsRNA was bound to the membrane of a QIAamp spin column with collection tube. The impurities were removed with 500 µl of Buffer AW1 and AW2, each wash followed by centrifugation at 6 000 x g for 1 min and 13 500 x g for 3 min, respectively. The viral RNA was eluted from spin the column by the addition of 60 µl buffer AVE, followed by incubation at ambient temperature for 1 min and centrifugation at 6 000 x g for 1 min. The viral RNA was then stored at –20°C until further analysis.
4.3.6 RT-PCR

The dsRNA extracted was used as the template for reverse transcriptase (RT) to synthesize cDNA copies from both strands. Briefly, a 10 μl reaction mixture containing 8 μl of the viral RNA and 10 pmol of each of the oligonucleotide primers pairs, sBeg/End 9 or 9con1/EndA, were used to reverse transcribe the VP7 gene of 1062 bp and 903 bp, respectively (Gouvea et al., 1990; Das et al., 1994; Gault et al., 1999). The non-degenerate primer set Con2/Con3 or degenerate primer set VP4F/VP4R primers were used to amplify a fragment of the VP4 of 876 bp and 663 bp, respectively (Gentsch et al., 1992; Simmonds et al., 2008) (Table 4:1). A negative control consisting of a previously confirmed negative rotavirus stool sample was also included. The reaction mixture was then incubated at 95°C for 5 min to denature dsRNA, followed by brief centrifugation and immediately cooled on ice. The RT reaction mixture was bought to 13.2 μl containing 0.25 μl 10 mM of each deoxynucleoside triphosphates (dNTPs) (Bioline, UK), 5 U of Reverse transcriptase, avian myeloblastosis (AMV) (Roche Boehringer), 1 x AMV buffer (5x 250 mM Tris-HCl (pH 8.5), 40 mM MgCl₂, 150 mM KCl; supplied with enzyme) (Roche Boehringer) and incubated at 42°C for 25-30 min.

4.3.7 PCR amplification

RT-PCR amplification was performed in a total volume of 40 μl reaction mixture containing 1 μl 10 mM of each dNTPs, 1 x NH₄ Reaction Buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8); supplied with enzyme], 1.5 μl 50 mM MgCl₂, 0.3 μl 5 U/μl BIOTAQ™ DNA polymerase (Bioline, UK) and sterile Baxter water. The PCR master mix was added to RT reaction mixture. The reaction tubes were then placed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystem). The amplification cycle was performed as follows; initial denaturation cycle at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 42°C for 30 seconds, and extension at 72°C for 1 min. A final extension step of 72°C for 7 minutes was included and tubes held at 4°C until the thermocycler was terminated manually. The amplification
products were analysed and visualised by electrophoresis on 1% agarose gel (Bioline, UK) after staining with ethidium bromide (0.6 µg/ml). Electrophoresis was carried out in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.9) at 90 V at ambient temperature for 45 min. The molecular size marker (Bioline Inc, USA) was used to verify the size of the amplicons.

4.3.8 Rotavirus genotyping

The G and P typing was carried out utilizing a nested multiplex PCR and G-specific and P-specific primers described by Gouvea et al. (1990), Gentsch et al. (1992), Das et al. (1994), Cunliffe et al. (1999), Mphahlele et al. (1999), Iturriza-Gómara et al. (2000b), Iturriza-Gómara et al. (2004b), Banerjee et al. (2007) and Simmonds et al. (2008). In brief, 0.5-4 µl of the amplicons obtained from the first round PCR served as a template for genotyping. Genotyping amplification was carried out in a 40 µl reaction mixture containing template DNA, 1 µl 10 µM of each dNTPs, 1 x NH4 Reaction Buffer [supplied with enzyme], 1.5 µl 50 mM MgCl₂, 0.3 µl 5 µ/µl BIOTAQ™ DNA polymerase (Bioline, UK), sterile Baxter water and 10 pmol/µl of each genotype specific oligonucleotide primers. For G-typing, oligonucleotide primers sets used were G1-G4, G8, G9 and G12 together with 3'-anchor primer RVG or EndA described by Gouvea et al. (1990), Gault et al. (1999), Iturriza-Gómara et al. (2000b), Iturriza-Gómara et al. (2004b) and Banerjee et al. (2007) or oligonucleotide primers described by Das et al. (1994) and Cunliffe et al. (1999), which includes G1-G4 and G8-G9 with 5'-anchor primer 9con1 (Figure 4:1). For P-typing, the oligonucleotide primers used were P[4], P[6], P[8],P[9], P[10], P[11] and P[14] with con3 or VP4F described by Gentsch et al. (1992), Mphahlele et al. (1999), Iturriza-Gómara et al. (2000b), Iturriza-Gómara et al. (2004b) and Simmonds et al. (2008) (Table 4:1 and Figure 4:1).
Figure 4:1 Agarose gel showing the G and P genotyping of human group A rotavirus VP7 and VP4 genes by semi-nested PCR for G and typing. 100bp ladder, G9 (1) G1 (2); G2 (3); G3 (4); G8 (5); G6 (6); G10 (7), RT-VP7 (8); G5 and; RT-VP4 (9); P[4] (10) P[6] (11); P[8] (12) and 100 bp ladder (13)

The reaction tubes were then placed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystem). The PCR was performed at 94°C for 2 min followed by 30 cycles of 94°C for 1 min; 42°C for 1 min; 72°C for 1 min, and a final extension at 72°C for 7 min, before being stored at 4°C. The amplicons were analysed and visualised by electrophoresis on 2% agarose gel as described above.

Non-typeable G and P genotypes were further analysed using animal G and P primers described by Gouvea et al. (1994a) and Gouvea et al. (1994b), respectively. The oligonucleotide primers used were G5, G6, G8, G10 and G11 for G-typing and for P-typing P[6], P[7], P[5], P[1] and P[11] (Table 4:1) and the same PCR amplification program and analysis of amplicons described above were employed. Those G or P amplicons that could not be identified utilizing the current human or animal primers available were subjected to cloning and sequencing.
Table 4:1 The oligonucleotide primers utilized for the amplification and genotyping of the VP7 and VP4 genes of rotavirus strains including primer sequences, primer positions on the respective genes, expected amplicon size and the reference article

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Nt Position</th>
<th>Amplicon (size)</th>
<th>References</th>
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<tr>
<td><strong>First Round PCR VP7 and VP4</strong></td>
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<tr>
<td>sBeg9</td>
<td>GGCTTTAAAAGAGAGAAATTTTC</td>
<td>1-21</td>
<td>1062 bp</td>
<td>Gouvea et al. (1990)</td>
</tr>
<tr>
<td>End9</td>
<td>GGTTCACATCATACAATTCTAAATCTAAG</td>
<td>1062-1036</td>
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<td>Gouvea et al. (1990)</td>
</tr>
<tr>
<td>sBeg9</td>
<td>GGCTTTAAAAGAGAGAAATTTTC</td>
<td>1-21</td>
<td>944 bp</td>
<td>Gouvea et al. (1990)</td>
</tr>
<tr>
<td>EndA</td>
<td>ATAGTATAAAATACTTGGCCACCACCA</td>
<td>922-944</td>
<td></td>
<td>Gault et al. (1999)</td>
</tr>
<tr>
<td>Con3</td>
<td>TGGCTTCGCCATTTATAGACA</td>
<td>11-32</td>
<td>876 bp</td>
<td>Gentsch et al. (1992)</td>
</tr>
<tr>
<td>Con2</td>
<td>ATTCGGACCATTTTATAACC</td>
<td>868-887</td>
<td></td>
<td>Gentsch et al. (1992)</td>
</tr>
<tr>
<td>VP4 F</td>
<td>TATGCTCCAGTNAATTGG</td>
<td>132-149</td>
<td>663 bp</td>
<td>Simmonds et al. (2008)</td>
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<td>VP4 R</td>
<td>ATTCATTTTCTTTCCATAATG</td>
<td>775-795</td>
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<td>Simmonds et al. (2008)</td>
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<td><strong>Second Round PCR</strong></td>
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<td><strong>A : VP7 Genotyping</strong></td>
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<tr>
<td>9Con1</td>
<td>TAGCTCCTTTTAATGTATGG</td>
<td>37-56</td>
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<td>9T-1</td>
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<td>9T-3P</td>
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<td>484-503</td>
<td>466 bp</td>
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<td>9T-4</td>
<td>GGGTCGATGGAAAATTCT</td>
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<td>403 bp</td>
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<td>9T-9B</td>
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<td>110 bp</td>
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<td>MW-8</td>
<td>TCTTCAAATATGTGCTAGTG</td>
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<td>651 bp</td>
<td>Cunliffe et al. (1999)</td>
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<tr>
<td><strong>B : VP7 Genotyping</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVG9</td>
<td>GGTCACTCATACATAATTCT</td>
<td>1062-1044</td>
<td></td>
<td>Gouvea et al. (1990)</td>
</tr>
<tr>
<td>aAT8</td>
<td>GTCACACCATTGTAAATTCG</td>
<td>178-198</td>
<td>885 bp</td>
<td>Gouvea et al. (1990)</td>
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<tr>
<td>aBT1</td>
<td>CAAGTACTCAATCAATGATCG</td>
<td>314-335</td>
<td>749 bp</td>
<td>Gouvea et al. (1990)</td>
</tr>
<tr>
<td>aCT2</td>
<td>CAATGATATTAACACATTTTCGTG</td>
<td>411-435</td>
<td>652 bp</td>
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<tr>
<td>aDT4</td>
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<td>Primer</td>
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<td>Amplicon (size)</td>
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<td>-----------------</td>
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</tr>
<tr>
<td>mG9</td>
<td>CTTGATGTGACTAYAAATAC</td>
<td>757-776</td>
<td>305 bp</td>
<td>Iturriza- Gómara et al. (2004b)</td>
</tr>
<tr>
<td>mG3</td>
<td>ACGAACTCAACACGAGAGG</td>
<td>250-269</td>
<td>812 bp</td>
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<tr>
<td>mG10</td>
<td>ATGTCACTACARATACTGG</td>
<td>666-687</td>
<td>396 bp</td>
<td>Iturriza- Gómara et al. (2004b)</td>
</tr>
<tr>
<td>G12</td>
<td>CCGATGGACGTAACGGTGA</td>
<td>548-567</td>
<td>512 bp</td>
<td>Banerjee et al. (2007)</td>
</tr>
<tr>
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**C : VP7 Genotyping**

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<tr>
<td>EndA</td>
<td>ATAGTATAAAAATCTTGGACCACCA</td>
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<td>Gault et al. (1999)</td>
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<td>aAT8</td>
<td>GTCACTCACTTTGGAAATTCG</td>
<td>178-198</td>
<td>767 bp</td>
<td>Gouvea et al. (1990)</td>
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<tr>
<td>aBT1</td>
<td>CAAGTACTCAATCAATGATGG</td>
<td>314-335</td>
<td>631 bp</td>
<td>Gouvea et al. (1990)</td>
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<td>aCT2</td>
<td>CAATGATATTTAACACATTTTTCTG</td>
<td>411-435</td>
<td>534 bp</td>
<td>Gouvea et al. (1990)</td>
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<tr>
<td>aDT4</td>
<td>CTTTCTGTTGAGGGTTTG</td>
<td>480-498</td>
<td>465 bp</td>
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<tr>
<td>mG9</td>
<td>CTTGATGTGACTAYAAATAC</td>
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<td>188 bp</td>
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<td>mG3</td>
<td>ACGAACTCAACACGAGAGG</td>
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<td>692 bp</td>
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<tr>
<td>mG10</td>
<td>ATGTCACTACARATACTGG</td>
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<td>276 bp</td>
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<tr>
<td>G12</td>
<td>CCGATGGACGTAACGGTGA</td>
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<td>394 bp</td>
<td>Banerjee et al. (2007)</td>
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**Animal primers**

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<td>sBeg</td>
<td>GGCTTTAAAAGAGAGAATTTC</td>
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<td>aFT5</td>
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<td>779-760</td>
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<td>aDT6</td>
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<td>499-481</td>
<td>500 bp</td>
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<td>aHT8</td>
<td>GTGCCTCATTCCGGAACCG</td>
<td>273-256</td>
<td>274 bp</td>
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<td>aET10</td>
<td>GAAGTCGCAACGGGTGAA</td>
<td>714-697</td>
<td>715 bp</td>
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<td>aBT11</td>
<td>GCAACTCAGATTGCTGATGAC</td>
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<td>337 bp</td>
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<td>Amplicon (size)</td>
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<td>--------</td>
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<td>-----------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Second Round PCR</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A: VP4 Genotyping</td>
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<td></td>
</tr>
<tr>
<td><strong>Con3</strong></td>
<td>TGGCTTCGCCATTTATAGACA</td>
<td>11-32</td>
<td></td>
<td>Gentsch <em>et al.</em> (1992)</td>
</tr>
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<td>1T-1 or dP[8]</td>
<td>TCTACTGGATAACGTGC</td>
<td>339-356</td>
<td>345 bp</td>
<td>Gentsch <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td>TCTACTGGRTTRACNTGC</td>
<td>339-356</td>
<td>345 bp</td>
<td>Iturriza- Gómara <em>et al.</em> (2000b)</td>
</tr>
<tr>
<td>2T-1</td>
<td>CTATTGTAGAGGGTAGAGTC</td>
<td>474-494</td>
<td>483 bp</td>
<td>Gentsch <em>et al.</em> (1992)</td>
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<tr>
<td>3T-1</td>
<td>TGAGACTGCAATTGGAC</td>
<td>385-402</td>
<td>391 bp</td>
<td>Gentsch <em>et al.</em> (1992)</td>
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<td>4T-1</td>
<td>ATCATAGTTAGTGTCGG</td>
<td>575-594</td>
<td>543 bp</td>
<td>Gentsch <em>et al.</em> (1992)</td>
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<td>mP11</td>
<td>GTAACATCCAGAATGTG</td>
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<td>312 bp</td>
<td>Iturriza- Gómara <em>et al.</em> (2004b)</td>
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<td>4943</td>
<td>GGTGTAGTTCCCTGCGTA</td>
<td>271-291</td>
<td>280 bp</td>
<td>Mphahlele <em>et al.</em> (1999)</td>
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<tr>
<td><strong>B: VP4 Genotyping</strong></td>
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<tr>
<td>VP4 F</td>
<td>TATGCTCCAGTNAATTGG</td>
<td>132-149</td>
<td></td>
<td>Simmonds <em>et al.</em> (2008)</td>
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<td>1T-1 or dP[8]</td>
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<td>TCTACTGGRTTRACNTGC</td>
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<td>474-494</td>
<td>351 bp</td>
<td>Gentsch <em>et al.</em> (1992)</td>
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<td>385-402</td>
<td>135 bp</td>
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<td>575-594</td>
<td>462 bp</td>
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<td>mP11</td>
<td>GTAACATCCAGAATGTG</td>
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<td>180 bp</td>
<td>Iturriza- Gómara <em>et al.</em> (2004b)</td>
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<td>4943</td>
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<td>414 bp</td>
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Table 4.1 (Continued)

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<th>Amplicon (size)</th>
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<td>C: VP4 Genotyping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con2</td>
<td>ATTTCGGACCATTTATAACC</td>
<td>868-887</td>
<td></td>
<td>Gentsch et al. (1992)</td>
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<td>pB223</td>
<td>GGAACGTATTTCTAATCCGGTG</td>
<td>574-594</td>
<td>314 bp</td>
<td>Gouvea et al. (1994a)</td>
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<td>pGott</td>
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<td>465-487</td>
<td>423 bp</td>
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<td>pOSU</td>
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<td>389-412</td>
<td>502 bp</td>
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<td>pUK</td>
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<td>336-354</td>
<td>555 bp</td>
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<td>pNCDV</td>
<td>CGAACGCGGCGGTGGTAGTGG</td>
<td>269-289</td>
<td>622 bp</td>
<td>Gouvea et al. (1994a)</td>
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</tbody>
</table>

Y=C or T  
R=A or G  
N=A, G, C or T
4.3.9 Molecular cloning

The VP7 genes of selected rotavirus strains were cloned and sequenced to confirm the RT-PCR G typing results. The samples that were selected represented various genotypes detected during different years. Rotavirus PCR products (10 µl) of the VP7 gene (1062 bp) were excised from a 2% low melting agarose gel (NuSieve® GTG®, FMC BioProducts USA, or Sigma-Aldrich, Germany) and purified with the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. In brief, 300 µl of buffer QC was added to solubilize PCR fragment excised from agarose gel and provide appropriate condition for binding of DNA to the silica membrane. The solution was incubated at 50°C for 10 min with brief vortexing. A total volume of 100 µl of isopropanol was added. The mixtures were subsequently applied to QIAquick spin column with 2 ml collection tube and centrifugation at 6 000 x g for 1 min allowing nucleic acid to bind to the column. Impurities were washed away with 750 µl of Buffer PE and DNA was eluted in 50 µl of buffer EB after 1 min centrifugation at 6 000 x g.

The PCR products were cloned into pGEM-T® Easy Vector according to manufacturer's instructions. The ligation reaction contains 5 µl of 2x rapid ligation buffer, 1 µl 50 ng/ µl of pGEM-T® Easy Vector, 1 µl of T4 DNA ligase, 1-3 µl of PCR product and deionised water with total volume of 10 µl. The reaction was incubated at 4°C overnight. The second step was transformation into pGEM-T® Easy Vector clones. For transformation, after centrifugation 2 µl of each ligation reaction was transferred to sterile eppendorf tube and placed on ice. Fifty microlitre (50 µl) of JM 109 competent cells (Promega, Madison WI, USA) and 950 µl of SOC medium was added to each tube and incubated for 90 min in a 37°C orbital shaker at 150 rpm. These transformations were plated onto nutrient agar containing ampicillin, isopropyl β-D- thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and incubated for 16-24 hours at 37°C. Two white colonies from each sample carrying plasmid were inoculated into 5 ml Luria-Bertani (LB) broth with ampicillin and incubated in a 37°C orbital shaker (Forma Scientific, Inc., Ohio, USA) at 200 rpm for 12-16 hours.
4.3.10 Isolation of recombinant plasmid

4.3.10.1 Extractions using Qiagen QIAprep spin Miniprep Kit

The procedure consists of three basic steps according to manufacturer's instructions:

1. Alkaline lysis method; after centrifugation of incubated tubes at 2 400 x g in a Roto-Unill centrifuge (Zeiss West Germany), the supernatant was discarded and the cells resuspended with 250 µl of buffer PI and 250 µl of buffer P2 (lysis buffer) and mixed 4-6 times.

2. Adsorption of DNA onto QIAprep membrane; a total amount of 350 µl of neutralization buffer (buffer N3) was added and centrifuged for 10 min at 17 900 x g to neutralize and adjust high salt binding condition and to pellet the cell debris. The supernatant of the solutions were then placed on QIAprep spin column in a 2 ml collection tube. After centrifugation for 1 minute at 6000 x g, the flow through was discarde.

3. Washing and elution of plasmid DNA; impurities were washed away with 500 µl of buffer PB and 750 µl of buffer PE. The QIAprep spin column was placed on a clean 1.5 ml microcentrifuge tubes; DNA was eluted in 50 µl of buffer EB after been centrifuged for 1 minute at 6000 x g.

4.3.10.2 Confirmation of correct insert

The restriction enzyme Not I (Promega, Madison WI, USA) was used to digest recombinant plasmids to ensure that the insert of the correct size had been cloned. The restriction digestion mixture contains 1 µl of clone (DNA), 2 µl of restriction enzyme 10 x buffer (60 mM Tris-HCl, pH 7.9, 1.5M NaCl, 60 mM MgCl₂, 10 mM DTT; supplied with enzyme), 0.2 µl Acetylated BSA (10 µg/µl; supplied with enzyme), 0.5 µl of Not I (10 u/µl) and 16.3 µl sterile Baxter water. After incubation at 37°C for 60 min, the samples were electrophoresed at 90 V on a 2% agarose gel with ethidium bromide (0.6 µg/ml) and visualized under ultraviolet light.
4.3.11 Sequencing

The purified recombinant plasmids were sent to the University of Stellenbosch, South Africa for direct sequencing using an ABI 3100 automated sequencer. The nucleotide sequences were determined for both strands using M13 forward and reverse primers. The amino acid sequences of the VP7 genes were deduced and amino acid and nucleotide sequences were compared with the corresponding sequences of representative strains of human, porcine and bovine group A rotavirus VP7 genes available from the EMBL/GenBank data libraries under the following accession numbers (Table 4:2). The DNA sequences for the VP7 genes of the South African G1, G2, G3, G8, G9 and G12 strains were submitted directly to Genbank and assigned the following accession numbers (Table 4:2).

4.3.11.1 Sequence alignment

For accurate alignment, the consensus sequences of the VP7 gene were aligned and analysed manually with Chromaslite (www.technelysium.com.au) and BioEdit (www.mbio.ncsu.edu/bioEdit/bioedit.html) software packages. The following reference rotavirus strains available in the National Center for Biological Information (NCBI) databases and accession numbers were used (Table 4:2). Multiple sequence alignment was implemented by Mafft software packages (www.align.bmr.kyushu-u.ac.jp/mafft/online/server/).
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G12 Genotype
4.3.12 Phylogenetic analysis

The genetic relatedness among the VP7 genes of South African rotavirus strains were determined by constructing a phylogenetic tree utilizing the neighbour joining, clustering methods, seqboot program, maximum parsimony analysis and maximum likelihood distance (Phylip version 3.6 and MEGA version 4.1 softwares). Distance calculations were made using the Kimura two-parameter model on MEGA version 4.1 software (Kumar et al., 2001). Genetic distances within genotypes were calculated using replicate data sets of 1000. Each method has its own strengths and weaknesses, but most importantly, they give similar results that give confidence to the final tree. The neighbour joining method gives a lot of different parameters to confirm the reliability of the phylogeny obtained whereas maximum likelihood gives a phylogeny with a small number of informative sites while mean genetic distances was measured by the Kimura-2 parameter model available in MEGA version 4.1 software. The phylogenetic group evidence shows the bootstrap values greater than 50 were considered statistically significant. The graphic representation of phylogenetic tree was displayed using the Treeview program, version 6.6.

4.4 Results

4.4.1 Samples

During the three years of surveillance, a total of 2 512 diarrhoeal stool samples were collected from children aged less than 5 years. Of these, 527 were positive for group A rotaviruses. Rotavirus prevalence (86.8%) was significantly greater in children less than 2 years of age. A marked seasonal trend of rotavirus infection was noted with bimodal peaks during the cooler, dry months of the year (Seheri et al., J Infect Dis Accepted).
4.4.2 Detection of rotavirus, bacterial and parasitic enteropathogens

A total of 1,092 diarrhoeal stool samples were tested for rotavirus, bacterial and parasites. Rotavirus was the most frequent enteropathogen detected at 316 (29%), followed by *Escherichia coli* 53 (4.8%), *Shigella flexneri* and *Shigella sonnei* combined at 25 (2.2%) and *Cryptosporidium parvum* 21 (1.9%) were also detected. A specific pathogen was not diagnosed in the majority of cases 677 (62%) (Figure 4:2). Rotavirus infection was associated in 10 cases of dual infections with enteropathogenic *E. coli*, *Cryptosporidium parvum*, *Shigella flexneri*, *Entamoeba*, *Salmonella species* or *Campylobacter jejuni*.

![Figure 4:2 Percentage of diarrhoeal episodes associated with specific enteropathogens from 1,092 stools sample at Dr George Mukhari Hospital](image)

4.4.3 Polyacrylamide gel electrophoresis (PAGE)

A wide variety of rotavirus electropherotypes were detected during the study period from 471/527 (89%) stool samples. A total of 56/527 (11%) of the stool samples failed to demonstrate the dsRNA electropherotypes (negatives). On the other hand, only 9/471
(2%) of the results showed mixed long and short electropherotypes and 12/471 stool samples showed faint positive (not clear) results. Rotavirus strains varied annually and the variations in electrophoretic migration pattern were particularly noticeable in segments 5 and 6, in segments 7, 8, 9 and in segments 10 and 11 and not in segments 1 to 4. In 2003, the long electropherotype that was noted exclusively in 2002 was largely replaced by strains displaying a short RNA migration pattern (62%). In total, ten different electropherotypes (S1-S5 and L1-L5) were observed during 2003. In 2004 and 2005, rotavirus strains with long electropherotypes once again predominated with six (S1-S2, L1 and L4-L6) and seven electrophoretic (S4, L1-L2 and L4-L7) patterns detected, respectively. Variations in electrophoretic migration pattern are shown in Figure 4:3 and 4:4).

The predominant rotavirus electropherotypes noted at Dr George Mukhari Hospital were as follows: in 2002 (unpublished data), a long pattern designated L4 predominated, and continued to predominate in 2003 together with the short pattern, designated as S1. In 2004 and 2005, the long patterns designated as L4 and L5 predominated, respectively. It was noted that, even though appearing at different prevalence rates throughout the study period, the L4 strain exhibited a consistent pattern appearance compared to other strains (Table 4:3).
Table 4.3. Rotavirus electropherotypes detected at the Dr George Mukhari Hospital in stool samples collected from children under five years of age between 2003 and 2005

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<td></td>
<td>134</td>
<td></td>
<td>228</td>
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</tr>
</tbody>
</table>

L designates: Long electrophoretic pattern
S designates: Short electrophoretic pattern
**Figure 4:3** Electrophoretic migration patterns of rotavirus dsRNA isolated at Dr George Mukhari Hospital during 2003
4.4.4 G and P rotavirus genotypes

The G and P types of rotavirus strains collected between 2003 and 2005 from children with acute gastroenteritis are summarized in Figure 4:5, Figure 4:6 and Table 4:4. Rotavirus dsRNA was extracted from 457/527 (86.7%) stool samples and the samples were subjected to RT-PCR and genotyping. The most common human G- and P- genotype combinations detected were G2P[4] and G2P[6] (47%) in 2003, G1P[8] and G1P[6] (56%) in 2004 and G3P[8] and G3P[6] (69%) in 2005. Mixed infections of G- and P- genotype were observed and approximately 2% of the rotavirus-positive samples could not be genotyped (Table 4:4).
In 2003, the serotype G2P[4]/G2P[6] were detected in 67 (47%) specimens with G1P[8]/G1P[6] and G9P[6]/G9P[8] noted in 33% and 10% of specimens, respectively. The majority of the strains (56%) detected during the 2004 rotavirus season were G1P[8] and G1P[6]. Serotype G3P[8] (13%), G3P[6] (9%), G12P[6] (7%), G2P[4] (2%) and G8P[6] (2%) were also detected during this time. In the 2005 rotavirus season, the G1 strains predominating in 2004 were replaced by serotype G3P[8] (51%) and G3P[6] (17%) strains. In addition, G1P[8], G12P[6], G1P[6] and G8P[6] strains were also detected at relatively low levels of 6%, 5%, 4%, and 3%, respectively.

During the study period the globally important genotypes G1, G2, G3 and G9 were associated with P[4], P[6] and P[8] and G4 was not detected. Overall the most predominant strains in 2003, 2004 and 2005 were mainly G2 (50%), G1 (57%) and G3 (70%), respectively. Of note were the rotavirus strains carrying unusual G12 and G8 specificities that were also detected at a low frequency. The results have also shown that G1 and G3 strains were strongly associated with the long electrophoretic pattern whereas G2 strains were associated with the short electrophoretic pattern (data not shown). Serotype G8 and G9 types were associated with both short and long electrophoretic patterns while all G12 genotypes exhibited the single long electrophoretic pattern (data not shown).

Figure 4: Distributions of G-types of rotavirus strains detected at Dr George Mukhari Hospital between 2003 and 2005
The human P[8] genotype was found to be present throughout the study period, and was predominant in 2004 (61%) and 2005 (60%) (Figure 4:6). The P[4] genotype was predominant in 2003 (46%) coinciding with the circulation of increased numbers of G2 strains. The P[6] genotype was also detected at an incidence of 30% during the study period.

![Figure 4:6 Distribution of P-types rotavirus strains detected at Dr George Mukhari Hospital from 2003-2005](image)

Overall, the global common G/P combinations of rotavirus strains identified during the study period were G1P[8] (22.3%), G2P[4] (14.2%), G3P[8] (24.5%) and G9P[8] at (1%). The relative prevalence of the common G/P combinations varied over time from year to year and season to season (Table 4:4).

Uncommon rotavirus and potential reassortant strains detected during the study period were G1P[6], G1P[4], G2P[6], G3P[6], G3P[4] G8P[6], G8P[4], G12P[6], G9P[4] and G9P[8]. The P[6] and G8 rotavirus strains have been frequently detected in Africa.
## Table 4.4: Rotavirus strains circulating at Dr George Mukhari Hospital during 2003-2005

<table>
<thead>
<tr>
<th></th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>Total (%)</th>
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<td><strong>Common G/P combinations</strong></td>
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<tr>
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<td>6</td>
<td>8</td>
<td>20 (4.4)</td>
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<td><strong>Combined Total</strong></td>
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<td>124</td>
<td>190</td>
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</table>
Mixed infections of the G and P genotypes and non-typeables strains were also observed. Varied mixed infections observed consisted of a single P genotype with two or three G genotypes or two P genotypes with one or two G genotypes. The P type mixed infections observed was G2 associated with P[4] and P[6] or G2 with P[4] and P[8] with P[4] predominating with a short RNA electropherotype pattern. These results suggest coinfection of an individual with DS-1 and Wa strains. The other mixed infection observed was G1 with P[8] and P[6], or P[8] and P[6] with predominant P[8] or P[6] with long RNA electrophoretic pattern. These mixed infections, demonstrate the co-circulation of different rotavirus genotypes with the long electrophoretic pattern infecting the same host cell. The overall prevalence of mixed infections was less than (5%) 22/457. Twenty specimens appeared to be non typeable as to G or P types (Table 4:4). The samples were subjected to various parameters including different primers, and dsRNA extraction methods. Unfortunately, the VP7 or VP4 genes PCR products were not successfully amplified by RT-PCR.

4.4.5 Sequencing results

The results from sequence alignment and phylogenetic analysis of the VP7 genes of 21 rotavirus strains detected in South Africa showed nucleotide and deduced amino acid sequence homology and identity of 97-99% when compared with corresponding international G1, G2, G3, G8, G9 and G12 strains (data not shown). These results confirmed the genotyping results obtained by semi-nested PCR.

4.4.6 Phylogenetic analysis

The evolutionary distances between South African isolates and the international rotavirus strains were investigated using pairwise comparison from multiple sequence alignments. After using Parsimony, maximum likelihood, neighbor joining and MEGA methods the overall tree topologies of VP7 gene analyses were similar. The differences
observed were that the genotypes were distributed differently but with the same branch
topologies. The number in the internal node represents the bootstrap value as
percentage of all trees (Figure 4:7-4:12).

The phylogenetic analysis of the G1 rotavirus strains identified 7 distinct genetic
lineages, designated lineage I-VII. The genetic lineage II was further divided into four
sublineages IIa-IIId, comprising human, porcine and bovine rotaviruses (Figure 4:7).
The South African G1 strains fell into two different lineages, with one strain collected in
2004 in lineage I and three strains collected in 2003 and 2004 in lineage II. The G1
strains, SA648DGM/03, SA1199DGM/04 and SA2107DGM/04, exhibited 97% and 99%
identity to Japanese (JP-7265 [EF079066]) and Italian G1 strains (PA5/03 [DQ377595]),
respectively, and clustered in lineage II. Remarkably, an additional G1 strain
(SA4799DGM/04) exhibited 99% identity to G1 strains from India (ISO-4/02
[AY098670]), Thailand (1604-Thai [DQ512981] and CMH036/04 [EF199716]) and were
found in lineage I.

The phylogenetic analysis of the South African G2 rotavirus strain and the international
representative strains clustered into seven distinct clusters I-VII (Figure 4:8). The single
G2 rotavirus strains (SA2124DGM/03) analysed was found in cluster V and formed a
single branch with the strain (GH1803 [AY261353]) detected during 1999 in Ghana. The
South African G2 strain showed the highest VP7 sequence identity (99%) to the strains
from West Africa, including Ghana (GH1803 [AY261353]), Burkina Faso (BF3704/99
[AY261356]) and Cote d’Ivoire (CI1735/99 [AY261354]). The South African G2 strain
was almost identical (98%) to G2 strains previously circulating in the same area during
1994-1998 (906SB/98 [AY261347]).
Figure 4:7  Unrooted phylogenetic consensus tree of VP7 gene nucleotide sequences showing the phylogenetic classification of rotavirus genotypes G1 and international reference strains (Table 4:2). The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05.
Figure 4.8 Unrooted phylogenetic analyses of VP7 deduced nucleotide sequences of serotype G2 and international reference strains. The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05.
Phylogenetic dendrograms of the G3 rotavirus strains of humans, porcine, bovine, equine and simian revealed four lineages I-IV (Figure 4:9). Within lineage III three sublineages designated S1-S2 and S4 were also identified. The two G3 rotavirus strains (CC425/USA [AJ311738] and CMH120/04 [DQ923797] Thailand) belonged to lineage II-sublineage S3 formed a lineage or a cluster below lineage IV. From our data sets the nucleotide sequences of South African G3 strains (SA1198DGM, SA1401DGM, SA1306DGM, SA1380DGM, SA1304DGM and SA188DGM) recovered in 2004 and 2005 were closely related to G3 strains from China, Russia and Thailand with 99% identity. The South African G3 strains belonged to the lineage II-sublineage S4 with a G3 strain from China (L478 [EU708580] collected between 2006 and 2008, a G3 strain from Vietnam (VN374 [DQ995489]) collected between 2001 and 2003 and a G3 strain from Spain (115/G/ Madrid [DQ440616]) collected in 2004 (Figure 4:9). The close genetic relationship of three G3 strains (SA1198DGM, SA1306DGM and SA1304DGM) isolated during the rotavirus season 2004 revealed a distinct single branch in sublineage S4.

The phylogenetic dendrogram of the G9 rotavirus strains classified into six distinct clusters, i.e. cluster I-VI (Figure 4:10). The South African G9 rotavirus strains were grouped together into cluster III with some of the strains from African countries and Bangladesh. The South African G9 strains clustered in lineage III, strain DGM2144/03 clustered with strain (KY6923/02 [DQ822604]) from Kenya while strains DGM2171/03 and DGM2125/03 formed a separate sublineage (albeit with a low bootstrap value) and were more closely related to G9 strains from Cameroon (3710CM [AY816184], Malawi (MW69 [AJ250545]), South Africa (GR10924/99 [FJ183360]) and Bangladesh (Bangla 39/96 [EF694183]). The degree of nucleotide identity among three South African G9 strains was 99% to strains from India and Thailand (S23 [AJ491187] and 97CM113 [AY866505]) and also displayed 99% homology to a Southern African G9 strain detected during a previous rotavirus season in Botswana (BS1414/02 [DQ822599]).
Figure 4:9 Unrooted phylogenetic analyses of VP7 deduced nucleotide sequences of serotype G3 and international reference strains. The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05.
Figure 4:10 Unrooted phylogenetic analyses of VP7 deduced nucleotide sequences of serotype G9 and international reference strains. The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05.
As indicated in Figure 4.11, the G8 rotavirus strains formed three clusters I-III with other international reference strains. The South African G8 strain clustered with all human African G8 strains cluster IV, detected during 1999 in South Africa, Malawi, Kenya, Nigeria and Guinea Bissau. At the nucleotide level, the South African G8 strains were closely related to a G8 strain identified in the Republic of Congo (DRC86/04 [DQ005120]) with 99% identity. Within the cluster IV, the South African G8 strain was closely related to strain Si/KY1646/99 ([FJ386444]) detected during 1999 from Kenya with nucleotide homology and identity of 98%.

Phylogenetic analysis of the VP7 nucleotide sequences of genotype G12 strains identified four lineages I-IV (Figure 4.12). The seven South African G12 rotavirus strains sequenced formed an independent cluster in the lineage III by showing a novel sublineage or monophyletic branch. All the G12 strains detected were closely related to Eastern India G12 strains with 99% identity at the nucleotide level (ISO23 [DQ099753]).
Figure 4:11 Unrooted phylogenetic analyses of VP7 deduced nucleotide sequences of serotype G8 and international reference strains. The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05
Figure 4:12 Unrooted phylogenetic analyses of VP7 deduced nucleotide sequences of serotype G12 and international reference strains. The tree was generated using neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values. The scale bar indicates an evolutionary distance of 0.05.
4.5 Discussion

This study complements both the ongoing rotavirus-associated burden of disease study at Dr George Mukhari Hospital and the Rotarix® trials that have been conducted in South Africa since 2001. Results from these studies emphasize the immense impact that rotavirus infection has on South African children and the South African healthcare system. In hospitalised children at Dr George Mukhari Hospital, a marked seasonal pattern of rotavirus infection has been observed during the cooler and drier months of the year with a peak prevalence of between 56% and 59% (Seheri et al., J Infect Dis Accepted).

Rotavirus strain surveillance is necessary prior to, and during, the introduction of rotavirus vaccines in Africa. Our study provides knowledge on, or confirms previous findings of, rotavirus strains co-circulating in South Africa. The current study indicates variations within the common rotavirus strain serotypes and genotypes over a 3-year period and also the variety shown within these rotavirus strains as indicated by the presence of uncommon genotypes and mixed rotavirus infections within communities. This study complements and adds to previous molecular epidemiological studies performed in the Ga-Rankuwa area, South Africa (Steele et al., 1995; 1999; 2003).

Rotavirus circulating in Ga Rankuwa, South Africa during 1988/1989

In the 1980’s, strain surveillance in Ga-Rankuwa, South Africa showed serotype G1 rotaviruses were most commonly detected (44%), followed by strains with the G4 (24%) and G2 (23%) serotypes. During the same time period, the serotype G4 were also fairly common in the adjoining areas of Pretoria and Johannesburg (Steele et al., 1995). Serotype G3 strains also appeared to be more common in Johannesburg than in Ga-Rankuwa (Pager et al., 1998). The P typing of the rotavirus strains showed that P[8]
viruses were observed in approximately two-thirds of cases, followed by P[4] strains (22%) and P[6] strains (10%) (Steele et al., 1995).

Serotype G8 rotavirus strains, previously thought to be more common in bovines and were detected in humans in the mid-1980s in Ga-Rankuwa (Steele et al., 1995). Mixed infection with more than one serotype of rotavirus was detected in approximately 2% of cases and indicated the potential for rotavirus reassortment in nature (Steele et al., 1995; 1999).

**Rotavirus circulating in Ga-Rankuwa during 1998-1999**

Specimens collected during the 1998/1999 seasons in Ga-Rankuwa identified the four globally predominant rotavirus strains, namely serotype G1, G2, G3 and G4. Compared to the strains circulating a decade prior, serotype G1 was still predominant (67%). The G1 genotype was divided mainly amongst strains with a G1P[8] genotype, and, to a lesser extent, the G1P[6] strains. Serotype G2 was detected at comparable levels (20%) and G3 serotypes were more commonly seen compared to 10 years previously (Steele et al., 2003).

**Rotavirus circulating in Ga-Rankuwa from 2003 to 2005**

The distribution of group A rotavirus genotypes has been shown to vary from one season to the next and between geographical areas. Between 2003 and 2005 all of the globally important genotypes G1, G2, G3 and G9 associated with P[4] and P[8] were detected with the exception of G4 rotavirus strains. Overall, between 2003 and 2005, serotype G3 and G1 rotaviruses were detected at an average prevalence of 36% and 33%, respectively, followed by strains with the G2 serotype (18%). The balance of rotavirus infections was caused by serotype G12 (5%), G9 (5%) and G8 (4%). As expected, the annual rotavirus strain profile varied considerably. In 2003, rotavirus
strains with the G2 serotype were the most prevalent (50%), followed by strains with the G1 serotype (37%). Also noted were strains with the G9 serotype (11%) while strains with the G8 serotype appeared to circulate at low levels and G3 and G4 strains were absent. By 2004, rotavirus strains with the G1 serotype were re-established as the most predominant serotype (60%). The prevalence of strains with the G3 serotype showed a significant increase (to 26%) and appeared as the second-most common strains closely followed by strains with the G12 serotype (7%). The prevalence of G2 strains had dropped (to 3%) and now appeared at similar low levels as G8 strains (3%). By 2005, the rotavirus strain profile showed a massive predominance of strains with the G3 serotype (70%) followed by strains with the G1 serotype (12%). The prevalence of the G12 strains were 6% and G8 serotype remained at 6% while G9 strains were observed in 4% of cases. During 2005, strains with the G2 serotype were absent.

Compared to the strains circulating a decade prior, during the 1998/1999 seasons serotype G1 predominated (67%) divided between G1P[8] and to a lesser extent to the G1P[6] strains. Serotype G2s were detected at comparable levels (20%), whilst serotype G3s were more frequently detected than 10 years previously (Steele and Ivanoff, 2003). However, the G4 strains were detected at a very low frequency. G4 rotavirus strains have not been observed in South Africa and the rest of Africa for nearly 20 years (Steele and Ivanoff, 2003; Mwenda et al., J Infect Dis Accepted; African Rotavirus Network Workshop unpublished data).

At the molecular level, the genetic characteristics of rotaviruses can be followed by noting variation not only in rotavirus genotypes but also in rotavirus electrophoretic patterns over a period of time. Thus, the present study not only showed relatively gross changes as to “short” and “long” electrophoretic patterns associated with fluctuations in the genotypes of circulating rotavirus strains but also more subtle changes within different electrophoretic patterns. The results indicate that the same genotype can
exhibit different electropherotypes and, conversely, the same electropherotype can be associated with different genotypes. Previously unpublished data (noted in 2002) indicated a relatively conserved rotavirus genome (only two different long patterns) and this expanded to ten patterns (including significant variety in short patterns) in 2003. To emphasize the molecular and evolutionary adaptability of rotaviruses, again, this molecular variation bottle-necked in 2004 when only six patterns were noted before expanding to an additional seven patterns in 2005. The results have shown that mostly G1 and G3 were strongly associated with the long electrophoretic pattern whereas G2 was associated with the short electrophoretic pattern. The G2P[6] strains exhibiting short RNA electropherotype patterns were observed. Similar findings were reported in West Africa (Armah et al., 2001). Also observed at low frequency were G8 and G9 types associated with both short and long electrophoretic patterns. All G12 genotypes exhibited the long electrophoretic pattern.

Serotype G1 strains are considered the most common strain in circulation globally. For the past 20 years in South Africa, the G1 rotavirus strains have been the most commonly detected. The prevalence of G1 strains during the study period showed a dramatic increase during 2004 and were less common during 2005. Comparing the nucleotide sequence among South African G1 strains, the strains revealed a similarity of 99% and these strains appeared to be closely related to the G1 strains from Japan and Italy (EF079066-7265/JP and DQ377595-PA103/02). The phylogenetic analysis of the VP7 sequence of G1 from different countries revealed seven genetic lineages I-VIII (Berois et al., 2003; Arista et al., 2006). Analysis of South African G1 strains (two from the 2003 season and two from the 2004 season) revealed three that clustered in the G1 genetic lineage II, sublineage IIb (Phan et al., 2007b) and were closely related to the Italian strain PA5/03. A single strain detected in 2004 clustered in G1 genetic lineage I, sublineage Ia and was closely related to G1 strains from Thailand and India (Phan et al., 2007b). The results suggest intratypic diversity within the G1 genotypes circulating in South Africa and indicate that monotypes within one serotype emerge from a pool of related strains rather than through continuous point mutations (Palombo et al., 1993).
Continuous monitoring of G1 molecular epidemiology over time will aid in identifying multiple G1 lineages circulating within a population and any changes in these lineages from one season to the next.

There have been concerns raised about strain replacement in response to vaccine pressure. In particular, studies from Brazil documented an increase in serotype G2P[4] strains in areas where the monovalent G1P[8] vaccine was being utilized (Gurgel et al., 2007; Nakagomi et al., 2008). Rotavirus vaccine trials were conducted in South Africa between November 2001 and March 2002 and between October 2002 and March 2003 (Steele et al., in press) within the same area where this study was conducted. While the number of children vaccinated with the monovalent vaccine was limited and may not have been sufficient to drive vaccine pressure, the predominant G1P[8] rotavirus strains circulating in 2002 (unpublished data), were largely replaced by G2P[4] strains in 2003 which were in turn, replaced by G1P[8] strains in 2004. By 2005, the G3P[8] and G3P[6] strains were the most significant strains isolated from children treated at the hospital. This underlines the extent to which the incidence and distribution of group A rotavirus genotypes may vary during a rotavirus season and from one season to the next. These results may have implications as to the efficacy of a monovalent rotavirus vaccine that has been tested within the Madibeng and Ga-Rankuwa communities. Regular three to four year peaks occur with serotype G2 strains in the Ga-Rankuwa area. Thus, the high prevalence of G2 strains in 2003 may reflect the normal cyclical pattern observed in South Africa from 1984 to 1998 (Page and Steele, 2004b) and the significance of G2 or any other serotype emergence after vaccine introduction needs to be carefully monitored and analyzed.

The phylogenetic analysis of G2 rotavirus strain detected in South Africa revealed a close relationship to strains isolated in West Africa (Ghana, Burkina Faso and Cote d'Ivoire) during 1999 (Page and Steele, 2004a). The G2 strain analysed demonstrated 98% homology and identity with the G2 strains previously detected in the same area during 1994-1998 (906SB/98 [AY261347]). These results may suggest that G2
rotaviruses might have originated from South to West Africa or West to South Africa due to migration and travelling, although further analysis will be required to prove this hypothesis. Further studies describing the phylogenetic relationships of strains are required to investigate the transmission of genotypes from one country to another.

Similarly to G2 strains, G3 have also demonstrated cyclic patterns of emergence and disappearance and in South Africa were responsible for the majority of rotavirus infections during 2005. Phylogenetic analysis of South African serotype G3 strains from 2004 and 2005 revealed clustering with G3 strains from China, Vietnam and Spain in lineage III sublineage IIIId. Furthermore, the phylogenetic analysis also revealed three strains (SA1198DGM, SA1306DGM and SA1304DGM) grouped together in a single branch in sublineage IIIId indicating the close genetic relationship between those strains. Phan et al. (2007a) and Wang et al. (2009) have suggested that G3 strains within lineage III sublineage IIIId are part of a “new G3 variant” and detection and predominance in South Africa supports the emergence of this variant in Africa. The relationship between rotavirus strains from Africa and those from Asia and Europe were seen in the phylogenetic analyses of both G1 and G3 strains. Further monitoring of strains from Asia and Europe into Africa should be conducted to investigate how the movement of people and/or other reservoirs affects the epidemiology of rotavirus stains within African populations.

Early studies indicated the P[6] genotype to be associated with asymptomatic, less virulent, neonatal infection (Gorziglia et al., 1988). However, the results from the Dr George Mukhari Hospital are consistent with previously published papers that reported a high prevalence of the P[6] genotype with symptomatic infection and in association with wide variety of G genotypes (Santos et al., 1994; Timenetsky et al., 1994; Steele et al., 1995; Cunliffe et al., 1999; Steele et al., 2003). Strains with the P[6] genotype were in the minority in 1988/1989 and were most frequently associated with G4 strains. However, by 1998/1999 season, P[6] strains were regularly detected with both G1 and G3 specificity. Between 2003 to 2005, the prevalence of P[6] was still increasing and
P[6] was not only associated with G3 and G1 serotypes but also to a lesser extent with G9, G8 and G12 serotypes. These results seem to indicate that P[6] strains are common in newly introduced G types and emerge during interspecies transmission by genetic reassortment between human and animal rotaviruses (Martella et al., 2006; Nguyen et al., 2007). The epidemiological significance of the P[6] VP4 protein on rotavirus transmission and reassortment dynamics, therefore, requires additional investigation and further studies involving full genome sequencing.

Sequence analysis revealed that G8 strains associated with the short electropherotype was closely related to strains from the Republic of Congo (DQ005120- DRC86) and Malawi (AJ278254- MW23) with homology of 99% and 96%, respectively (data not shown). Rotavirus G8 strains have been detected from humans and animals including cattle, pigs and horses (Gouvea et al., 1994b; Isa et al., 1996; Adah et al., 2003). The South African G8 strain demonstrated nucleotide homology and identity of 97% with the experimentally infected vervet monkey G8 strain (Si/KY1646/99 [FJ386444]) detected during the 1999 from Kenya. The results generated in this study are consistent with various studies in Africa demonstrating high prevalence of G8 rotavirus strains. Therefore, the high frequency of G8 strains in Africa implies a continuous circulation of this strain in African populations, probably in part due to the close contact and living conditions between humans and animals (Adah et al., 1997; Cunliffe et al., 1999; Steele et al., 1999; Fischer et al., 2000; Armah et al., 2001; Adah et al., 2003). These might imply potential interspecies rotavirus transmission. Therefore, any rotavirus vaccine destined for introduction in Africa will have to demonstrate protection against serotype G8 strains.

In South Africa, the first G9P[6] strain was isolated from a child in the Pretoria area in October 1997 (Page et al., J Infect Dis Accepted). Subsequently, additional G9 strains were detected during the 1998 and 1999 rotavirus seasons. Serotype G9 strains were
also detected in the latter part of 1998 from the neonatal ward of the Ga-Rankuwa Hospital when G9P[6] was predominant in the ward. These G9P[6] strains persisted in the ward during 1999, despite the introduction of G1P[8] and G1P[6] strains into the ward in early 1999. Despite being identified periodically in South Africa, G9 strains have never been identified as the predominant circulating serotype within the general population like G9 predominant seasons seen in Ghana, Mauritius and Kenya (Page et al., J Infect Dis Accepted). Phylogenetic analysis of the 2003 South African G9 strains revealed two sublineage clusters. The first related to G9 strains detected in strains from Bangladesh, Malawi, Cameroon and South Africa during the 1998/1999 seasons and the second related to Kenya and Botswana detected in 1999 and 2002, respectively but forming a distinct subgroup. These results suggest that G9 strains continue to circulate at low levels within the South African population and are introduced from a pool of related strains and from neighbouring countries.

The emergence of unusual rotavirus strains G12P[6] with a long electropherotype were observed for the first time in South Africa in late 2004 and 2005. The G12 serotype has been reported all over the world including Philippines, Thailand, Japan, Korea, United Kingdom, Belgium, Argentina, Nepal, India, Bangladesh and Brazil (Taniguchi et al., 1990; Griffin et al., 2002b; Pongsuwanna et al., 2002; Das et al., 2003; Shinozaki et al., 2004; Castello et al., 2006; Samajdar et al., 2006; Banerjee et al., 2007b; Rahman et al., 2007). The G12 rotavirus strains identified were identical to strain IS023, DQ099753 from Eastern India (99% homology). Recent strain surveillance studies have demonstrated the G12 serotype in at least three locations in South Africa and indicated that these strains appear to be a recent introduction into South Africa (Page et al., 2009). Interestingly, the phylogenetic analysis of the VP7 nucleotide sequences of the South African G12 strains were clustered into single branch in the lineage III. The data indicates that the introduction of the G12 rotaviruses in the country appeared genetically the same by showing a novel sublineage with bootstrap proportion of 62% to other international reference strains. Unfortunately, the direct evidence to support the origin of these G12 strains in the country and changing pattern of genotypes remained
undetermined. Based on these observations, it raises the possibility that G12 rotavirus strains may be next important genotype to emerge and current vaccine candidate will have to demonstrate efficacy against these strains.

At present, rotavirus vaccines, RotaTeq™ and Rotarix ® have been designed to protect against the globally most common rotavirus strains, G1-G4. The WHO recommended that safety and immunogenicity of these vaccines should be demonstrated in both developed and developing countries before receiving global recommendation from this organisation. To date, the two rotavirus vaccines have been licensed in 89 and 105 countries, respectively, and 17 countries have introduced routine rotavirus vaccination programs. There are a number of challenges facing these vaccines in poor countries as was previously documented with rotavirus vaccine RIT4237 and WC3 in Africa (Hanlon et al., 1987; George-Courbot et al., 1991). Immune challenges included high maternal antibody titres and breastfeeding at the time of vaccination, malnutrition or the presence of interfering microorganism in the gut. Each of these factors could inhibit the immune response to the vaccine. In Africa there is marked diversity of circulating, unusual rotavirus strains (African Rotavirus Network Workshop, unpublished data) and these findings are further supported by strain surveillance from the Ga-Rankuwa area. Thus, alternative VP7 or VP4 antigens including G8 or P[6] or the removal of the G4 antigen from the current formulation could be considered for vaccine development for Africa. The increasing number of reports from different countries about emergence of novel G and P types raises concerns for the effectiveness of rotavirus vaccine candidates. The rotavirus strains of the G1P[6], G2P[6], G3P[6], G8P[6] and G12P[6] were responsible for a substantial percentage (30%) of infections. Among these, 2% strains exhibited the G8 serotype with both short and long electropherotype migration patterns.

A low level of untypeable G and P rotavirus strains were noted during the study period (~3%). This may have been partly due to standard genotyping primers not being able to
anneal to, and so not detect, uncommon or unusual strains. This raises concern as to the significance of these omissions in epidemiology studies of rotavirus strains. These results emphasize the need to constantly monitor and improve the effectiveness of current genotyping methods and to respond to changes associated with genetic drift and shift of the viral strains.

Developing countries may show higher frequency of mixed rotavirus infections. Mixed rotavirus infections were detected frequently during 2003 at 4-5% within the Ga-Rankuwa study population, together with predominant strains. Interestingly, unusual combination of rotavirus strains identified were G2 with P[4] as well as P[8] or G2 with P[4] and P[6] or G1 with P[8] and P[6] with a predominant strain. Most of these mixed rotavirus infections occurred within a single rotavirus season, had identical long or short electrophoretic pattern, which suggested that they might belong to the same genogroup. The segmented nature of the rotavirus genome allows for genetic reassortment during mixed infections leading to these atypical strains and populations within developing countries with constant exposure to enteric pathogens may provide fertile ground for these events to occur. It is well documented that natural reassortants exist as progeny from co-circulating strains (Iturriza-Gómar et al., 2001). The emergence of novel rotaviruses in the region needs to be taken into consideration where vaccine efficacy is concerned. It is, thus, important to continue with such studies to monitor the rotavirus strains associated with severe gastroenteritis in a hospital setting after the introduction of a rotavirus vaccine.

Conclusion

The results presented confirm a marked diversity of rotavirus strains in circulation in South Africa that has been documented by various studies (Steele et al., 1999; Page and Steele, 2004b) with the high prevalence of types G1-G3, G8 and G9 associated with P[8], P[6] and P[4]. Also very significant was the presence of unusual strains such
as G12, not previously found in the South Africa. Furthermore, the present study noted mixed rotavirus infections and untypeable strains. The study supports the need for continued national rotavirus serotype surveillance and identification of changes in emerging rotavirus strains within South Africa.
CHAPTER 5: USE OF RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS TO DETERMINE HUMAN GROUP A ROTAVIRUS SUBGROUPS.

5.1 ABSTRACT

BACKGROUND: The VP6 monoclonal antibodies (MAbs) described by Greenberg et al. (1983) have been used successfully in epidemiological studies to determine rotavirus subgroups. However, genetic and antigenic diversity of rotaviruses appears to be more extensive than previously anticipated and currently the sub-grouping ELISA is considered unreliable due to limited availability of MAbs specific for all rotavirus variants. Accumulation of point mutations through genetic drift of the rotavirus genome, and consequent change in capsid epitopes may contribute to serological assay failure. The aim of the current study was to determine genetic variability of rotavirus strains in the Ga-Rankuwa area by developing a restriction fragment length polymorphism (RFLP) method that may be widely used as an assay for VP6 subgrouping.

OBJECTIVES: (1) To determine rotavirus VP6 antigenic specificity utilizing subgroup-specific MAbs, (2) To evaluate the use of restriction enzymes in the designation of Group A subgroup specificity (3) To assess genetic diversity by RT-PCR amplification and sequencing of VP6 gene (379 bp) and comparing local sequences with reference strains from the Genbank, (4) To devise a simple RT-PCR RFLP analysis method to rapidly detect VP6 subgroups.

METHODS: To examine genetic variation in the VP6 gene between different strains of rotaviruses, this study compared the serological method (n=186) to RT-PCR - restriction fragment length polymorphism (RFLP) assays of rotavirus VP6 amplicons (n=72) using restriction endonucleases AcI, Ddel and Rsal, in separate reaction mixtures. The genetic variations detected by RFLP were compared to variation detected by sequence
analysis of the VP6 gene (379 bp) fragment. Twenty-nine representative strains were characterized by sequencing and phylogenetic analysis.

**RESULTS:** Serological subgrouping of the specimens’ yielded subgroup I (44%), subgroup II (50%) and subgroup I + II (6%). Endonuclease restriction produced a total of 11 restriction profiles designated A to K [Acil (A-F), Ddel (G-I) and Rsal (J-K)]. Examination of deduced amino acid (aa) and nucleotide sequences revealed high sequence homology (97-99%) with corresponding international reference strains. The comparison of the deduced aa sequences showed a wide range of variable regions. The phylogenetic analysis revealed four genetic lineages comprising subgroup I, subgroup I + II, subgroup non-I non-II and subgroup II.

**CONCLUSION:** Analysis with Rsal clearly differentiated the subgroups without showing multiple restriction sites and partial digests. The deduced amino acid sequences indicated eight variable regions across the subgroup I and subgroup II strains. Notably, those regions appeared to correlate well with rotavirus subgroup I and subgroups II delineation, suggesting these regions are functional recognition sites for subgroup epitopes. Comparison of the sequences of VP6 amplicons with reference strains and phylogenetic analysis revealed four genetic lineages describing subgroup I 26% (7/29), subgroup II 66% (19/29) and subgroup non-I non-II 7% (2/29) and subgroup I + II 3% (1/29) specimens. The RFLP method allowed a rapid comparison of rotavirus subgroups and investigation of the evolution of rotavirus VP6 genes.
5.2 Introduction

The product of the VP6 gene is the major component of the structural inner capsid layer. This is expressed at high levels, is highly antigenic and is the main target of a variety of diagnostic assays used for rotavirus detection. During the infection process, VP6 plays important structural, immunological and morphogenic roles, interacting with the inner layer protein VP2 and the two outer capsid proteins VP7 and VP4. It is a trimeric protein that is stabilized by noncovalent bonds. Although VP6 is the most immunogenic rotavirus protein, in vitro studies have indicated that antibodies against VP6 do not inhibit rotavirus replication and they do not have neutralizing activity either in vivo or in vitro when fed orally. That is, VP6 antibodies may not neutralize virus in the intestinal lumen or tissue cultures (Estes and Cohen, 1989).

Rotaviruses are classified into three important antigenic and genetic specificities based on group, subgroup and serotypes (Estes and Kapikian, 2007). Among all groups, VP6 shares a group antigen that is common among all viruses of a given group and these group-specific epitopes have been mapped between aa residues 48 and 75 (Kohli et al., 1992). Rotaviruses are classified into seven distinct antigenic groups (A-G). Epidemiological studies have shown that groups A, B, C infect both humans and animals whereas rotavirus groups D, E, F and G are found mainly in animals to date (Estes, 2001). The VP6 protein of groups A, B and C are hydrophobic, each contains a single hydrophilic region between aa 90 and 110, contain a high percentage of β structure and a low percentage of α helix structure.

Group A rotaviruses are responsible for significant morbidity and mortality in humans. They are classified into subgroups depending on the presence or absence of two conformational epitopes called subgroup I and subgroup II. Rotaviruses may be classified as subgroup I, subgroup II, subgroup non-I non-II or subgroup I + II (Estes and Cohen, 1989). Monoclonal antibodies (MAbs) directed against VP6 and against
specific subgroup I (detected by MAb 255/60) or subgroup II (detected by MAb 631/9) epitopes were described by Greenberg et al. (1983). Other MAbs directed against VP6 have been developed by Beards et al. (1984); Liprandi et al. (1990) and Lopez et al. (1994) to differentiate subgroup I and II rotavirus strains.

Assays using the VP6 monoclonal antibodies described by Greenberg et al. (1983) have been used successfully in early epidemiological studies to determine the rotavirus subgroup and were applicable to large-scale epidemiological studies. Mapping the subgroup epitopes of rotavirus protein VP6 using subgroup specific MAbs 255/60 and 631/9 (described by Lopez et al. 1994) showed that a single aa mutation at position 172 (Met to Ala) or 305 (Asn to Ala) was sufficient to change the subgroup specificity of the human rotavirus Wa VP6 from subgroup II to subgroup I + II. Furthermore, Baozhang et al. (1997) showed that amino acid substitution at position 305, 315 and a region position 296-299 or 301 are capable of changing rotavirus strain EW VP6 from subgroup non-I non-II to subgroup I or subgroup II. At present the sub-grouping ELISA is considered unreliable due to;

1) Limited availability of MAbs specific for all rotavirus variants (Iturriza-Gómara et al. (2002b).
2) The existence of VP6 mutations that may promote cross-reactions in ELISA’s.
3) Constant accumulation of point mutations through genetic drift that may result in serological assay failure.

As genetic and antigenic diversity of rotaviruses may be more extensive than previously anticipated, genetic variants have the potential to provide antigenically distinct strains with similar serotypes (O’Halloran et al., 2002). This prompted the gradual replacement of VP6 subgrouping using monoclonal antibodies with molecular typing methods, including hybridisation techniques, standard and real time PCR. Molecular typing of VP6 subgroup has been used in large-scale epidemiological studies (Iturriza-Gómara et al.,
The report by Iturriza-Gómara et al. (2002a) described a restriction fragment length polymorphism (RFLP) method for VP6 subgrouping. The assay distinguished all four subgroups of rotaviruses by digesting partial length (379 bp) amplicons of the VP6 gene using a single restriction enzyme (AciI). The RFLP analysis revealed four restriction profiles, with each subgroup showing a unique pattern of fragments when separated by agarose gel electrophoresis. The subgroup I strains showed the original VP6 fragment (379 bp) with no cut while the subgroup I + II demonstrated a cut in one place to yield two fragments of 285 bp and 94 bp. The restriction profile represented by subgroup non-I non-II showed two cuts to yield three fragments of 167, 119 and 94 bp whereas the subgroup II showed three cut to yield four fragments of 202, 83, 62 and 32 bp.

To complement the Iturriza-Gómara study, the current project selected VP6 amplicons (379 bp) from human group A rotavirus strains detected in South Africa and analyzed these amplicons by RFLP profiling using restriction endonucleases AciI, DdeI and RsaI. Through this approach, the study aimed to identify genetic variability of rotavirus strains by enhancing the current RFLP method, thereby, developing a simple and rapid technique that may be widely used in VP6 subgroup typing assay.

Within this study, the VP6 subgroup of South African rotavirus strains was first determined utilizing VP6-specific monoclonal antibodies. Representative subgroups were selected and the strains were subjected to RT-PCR amplification of the VP6 gene and RFLP analysis. The partial length VP6 amplicons were digested with endonuclease AciI (recognition site 5’C↓CGC 3’/ 3’GGC↓G 5’), DdeI (recognition site 5’ C↓TNA G 3’/ 3’ G ANT↓C 5’) and restriction endonuclease RsaI (recognition site 5’ GT↓AG 3’/ 3’ CA↓TG 5’). The restriction fragment length polymorphism cleavage sites and genetic diversity of VP6 gene were also evaluated by sequencing the VP6 fragment. A favourable comparison of these methods may allow the use of RFLP analysis as a
useful adjunct for rotavirus subgrouping. It might also allow insight into genetic variability of circulating rotaviruses and could be used to monitor changes in rotavirus epidemiology during a specific time period.

5.3 Materials and methods

5.3.1 Rotavirus samples

Stool samples were collected from January 2003 through December 2005 from children less than 5 years of age treated for acute gastroenteritis at Dr George Mukhari Hospital. Group A rotavirus antigen was detected from diarrhoeal stool samples using commercially available enzyme immunoassay IDEIA™ Rotavirus test (DakoCytomation, Denmark), according to the manufacturer’s instructions (refer to section 3.3.7).

5.3.2 VP6 subgroup ELISA

VP6 monoclonal antibodies described by Greenberg et al. (1983) and Beards et al. (1984) were used to determine VP6 subgroup and group specificity, respectively. Briefly, 96-well NUNC (Roskilde, Denmark) flat bottom microplates were coated with 100 µl of a 1:75 dilution of coating antibody of rabbit anti-human rotavirus #0903, (DAKO, Cambridgeshire, UK) in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. On the second day, the plates were washed 4-6 times with phosphate–buffered saline containing Tween 20 (PBS/T) (pH 7.2). After washing, 100 µl of PBS/T with ethylenediaminetetra-acetic acid (EDTA) and 50 µl of 10% stool suspension were added into three separate wells on the coated plates and incubated overnight at 4°C. Previously subgrouped rotavirus positive as well as rotavirus negative human stool samples were added as controls. On the third day, the plates were again washed 4-6 times with PBS/T. Three separate tubes containing rotavirus group antigen (Beards et al., 1984), subgroup I (SGI) and subgroup II (SGII; Greenberg et al., 1983) MAbs diluted 1:5000 in PBS/T (pH 7.2) containing bovine serum albumin (BSA;
PBS/T/BSA) were added to consecutive sample wells and incubated at 37°C for 3 hours. The plates were washed 4-6 times with PBS/T. After washing, 100 µl horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Zymed, San Francisco, USA) diluted 1:2000 in PBS/T/BSA was added and incubated at 37 °C for two hours. Lastly, the plates were washed 4-6 times with PBS/T, 100 µl of HRP-substrate hydrogen peroxidase plus 3, 3', 5, 5'-tetramethylbenzidine was added and incubated for 10 minutes in the dark followed by addition of 5% sulphuric acids to stop the reaction. Optical density (OD) readings were obtained using a spectrophotometer set at 450 nm. The results were interpreted as follows:

- **Rotavirus Group A antigen**: OD > 1.0
- **Subgroup I**: OD SGI: SGII > 1.7
- **Subgroup II**: OD SGI: SGII > 2.0
- **Subgroup I+ II**: OD SGI=SGII
- **Subgroup non-I non-II**: OD SGI & SGII ≤ negative control

### 5.3.3 Genomic extraction

Rotavirus dsRNA was extracted from 10% faecal suspensions using Tri-Reagents-LS (Molecular Research Centre Ohio, US) or the QIAamp viral RNA extraction method (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions (refer to section 4.3.5).

### 5.3.4 VP6 amplification by RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify the 379 bp VP6 amplicons using type specific primers (VP6F: 5' GACGGVGCRACATACGTG 3’ and VP6R: 5’ GTCCAATTCATNCCTGCTGG 3’) as described by Iturriza-Gómez et al. (2002b). The same RT-PCR and amplification protocols previously described were utilized (refer to sections 4.3.6 and 4.3.7).
5.3.5 Selection of restriction endonuclease

The computer analysis program Webcutter 2.0 ([http://user.unimi.it/~camelot/tods/cut2.html](http://user.unimi.it/~camelot/tods/cut2.html)) was used for selection of restriction endonucleases. The local VP6 sequences and the international reference strains were imported into the Webcutter program in FASTA format and internal program tools search for selected restriction sites and calculate the restriction fragment size. Restriction enzymes were selected based on the ability to distinguish four different subgroups. In addition to *Acil* utilized by Iturriza-Gòmarra *et al.*, (2002b), restriction endonuclease *Rsal* and *Ddel* were included to assess the VP6 subgroups in the South African rotavirus strains.

5.3.6 Restriction mapping

The partial length VP6 amplicons were digested with endonuclease *Acil* (New England BioLabs), according to manufacturer’s instructions. The volume of amplified PCR products used (8 to 15 µl) depended on amplicon concentration. The amplicons were then digested in a total volume of 26.5 µl containing 15 U of *Acil*, 10x NE Buffer 3 [100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 200 µg/ml BSA, 50% glycerol; supplied with the enzyme] and deionised water and incubated for 16 hours at 37°C. Positive and negative controls were also included. The restriction reaction was then deactivated at 65°C for 20 min and the products were electrophoresed through 3% low melting temperature agarose gel (NuSieve® GTG®, FMC BioProducts USA or Sigma-Aldrich, Germany) containing ethidium bromide (0.6 µg/ml) in 0.5 x TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8.0) at 90 V for 1 hour and visualized under ultraviolet light. A 50 bp molecular weight DNA ladder (Promega, Madison WI, USA) was used to verify the size of the restriction fragments. The comparisons of the restriction profiles were conducted within strains displaying the same subgroups by monoclonal antibodies.
Restriction endonuclease *RsaI* and *DdeI* were also employed to digest the partial length VP6 amplicons. The same protocol as described above was used for restriction endonuclease analysis using 1 U of *RsaI* or *DdeI* (Promega, Madison WI, USA) according to manufacturer’s instructions. In brief, digestion was performed in a total volume of 20 µl containing a template, 10 U/µl *DdeI* restriction enzyme, 1x restriction enzyme buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 0.01% Triton x 100, 50% glycerol [supplied with the enzyme]), 0.2 µl Acetylated BSA (supplied with the enzyme) and deionized water. Restriction enzymes digest with 10 U/µl *RsaI* was also completed in a final volume of 20 µl and contained template, 1x restriction enzyme buffer (10 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol [supplied with enzyme]), 0.2 µl Acetylated BSA (supplied with the enzyme) and deionized water. After 4 hours incubation at 37°C, fragments were electrophoresed at 90 V for 1 hour, using 3% low melting temperature agarose gel (NuSieve® GTG®, FMC BioProducts USA, or Sigma-Aldrich, Germany) with ethidium bromide (0.6 µg/ml) staining in 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) and visualized under ultra violet light. A 50 bp molecular marker (Promega, Madison WI, USA) was used to verify the size of the respective restriction fragments. Similarly to *AciI* restriction, comparisons of the restriction profiles were conducted within strains displaying the same subgroups by monoclonal antibodies.

### 5.3.7 Molecular cloning

A fragment of the VP6 genes of 29 selected rotavirus strains were cloned and sequenced. Briefly, rotavirus PCR products of the VP6 gene (379 bp) were excised from a 2% agarose gel and purified with the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. PCR products were cloned into pGEM-T® Easy Vector (Promega, Madison WI, USA). Restriction with enzyme *Not I* (Promega, Madison WI, USA) was used to digest recombinant plasmids
to ensure that the insert of the correct size had been cloned as previously described (refer to section 4.3.9 and 4.3.10).

5.3.8 Sequencing and phylogenetic analysis

The VP6 insert in purified recombinant plasmids was sequenced using the ABI 3100 automated sequencer at the University of Stellenbosch using VP6 forward and reverse primers or M13 forward and reverse primers. The nucleotide and deduced amino acid sequences of the VP6 amplicons were compared with the VP6 sequences of representative subgroup strains of human rotavirus group A. These reference sequences are available from the PubMed EMBL/GenBank data libraries under the following accession numbers: EF426120- SGII (US0468), EF426140- SGI (US8908), D00325- SGI (1076), D00324- Non-SGI and II (H-2), U36474- Non-SGI and II (EW), D00323- SGI and II (F1-F14) and U36240-SGII (E210). Consensus sequences of the VP6 gene were aligned and analysed (refer to section 4.3.11). Phylogenetic analysis was performed according to the protocol described in the previous chapter (refer to section 4.3.12).

5.4 Results

5.4.1 VP6 subgroup ELISA

One hundred and eighty-six stool specimens from children with acute rotavirus gastroenteritis were tested by VP6 subgroup ELISA using MAbs described by Greenberg et al. (1983) and Beards et al. (1984). Of these rotavirus positive specimens, 50% (93/186) showed subgroup II specificity and 43.5% (81/186) showed subgroup I specificity, detection of both subgroup I + II specificity were found in 6% (12/186) of the specimens and subgroup non-I non-II strains were not identified. The method was able to distinguish between the VP6 subgroups, even though background optical density
readings obtained were very high. The assay was repeated and the results remained unchanged.

5.4.2 Restriction profiles

Seventy-two (72) representative subgroup strains, selected based on common genotypes circulating during the study period were subjected to molecular identification method using the RT-PCR RFLP analysis. The VP6 amplicons were digested by restriction endonuclease Acil to differentiate four rotavirus subgroups as described by Iturriza-Gómara et al. (2002a), and produced six different restriction profiles (designated as A-F). The results of the digestion by Acil indicated the same pattern of cDNA fragments anticipated by computer analysis program. The results were also confirmed by gel electrophoresis of the actual fragments generated by restriction enzyme digestion. The most common restriction profiles detected were profile C (subgroup II) and A (subgroup I) found in 58% and 24% of the strains, respectively. Remarkably, restriction profiles B, D, E and F shared the restriction recognition digest at position 274 to yield restriction fragments 119 bp.

Restriction endonucleases Ddel and Rsal consistently produced restriction profiles G-K. Analysis with restriction enzyme Ddel showed three different restriction profiles designated as G, H and I (Table 5:1). The restriction enzyme Rsal produced profiles useful for the identification of rotavirus subgroups by producing two restriction profiles labelled as J and K.

5.4.3 VP6 subgroup ELISA versus restriction mapping

To verify the results, the rotavirus VP6 subgroups determined by VP6-specific monoclonal antibodies were compared to RT-PCR RFLP analysis with restriction
endonucleases *AciI, Ddel and Rsal*. A minor discord results were found in two samples typed as subgroup I + II by VP6 monoclonal antibodies and the restriction endonucleases which revealed subgroup II. None of the South African strains were identified with subgroup non-I non-II specificity or produced a subgroup non-I non-II restriction profile. The results were in agreement with the results obtained with VP6 monoclonal antibodies.

5.4.4 Subgroup I

The results obtained by RT-PCR RFLP analysis using restriction endonucleases *AciI* matched those obtained with monoclonal antibodies for subgroup I. Profile A, showed an unrestricted 379 bp VP6 amplicon (Figure 5:1 and Table 5:1) whereas profile B, showed incomplete digestion of the 379 bp, yielding two fragments of 379 bp and 119 bp (Figure 5:1 and Table 5:1). Profiles A and B appeared to be associated with subgroup I strains representing genotypes G2P[4], G2P[6] and G9P[6] strains with short electrophoretic pattern. Restriction profile A and B represented 37% (26/72) of the tested strains as subgroup I. A significant positive correlation was observed between the results for subgroup I strains obtained utilizing VP6-specific monoclonal antibodies and restriction mapping using *AciI*.

The subgroup I-specific incomplete digestion, subgroup I + II and subgroup non-I non-II digestion described by Iturriza-Gómara was also observed in restriction profile E (379, 285, 167 and 119 bp), whilst restriction profile F yielded fragments of 379, 167, 119 and 94 bp, indicative of subgroup-I and subgroup non-I non-II specificity (Table 5:1).

Restriction enzymes *Ddel and Rsal* generated three restriction profiles H, I and K representing subgroup I rotavirus strains. Two profiles (H and I) were noted after
digestion with \textit{DdeI}. Profile H yielded fragments of 342, 132 bp and 95 bp. Profile H might reflect complete digestion of the amplicon as profile I showed the same sized fragments as profile H with the addition of unrestricted amplicon (379 bp). The amplicons digested by \textit{Rsa I} showed one cut to yield fragments of 101 bp and 250 bp (restriction profile K) (Table 5:1 and Figure 5:2 and 5:3). A brief analysis shows that the South African G2 rotavirus strains were associated with these three restriction profiles (H, I and K). Unfortunately, reaction with \textit{Dde I} resulted in small restriction fragments (restriction profile H) that were difficult to see on the 2% agarose gel. The restriction profile produced by restriction enzyme \textit{Rsa I} was useful to identify rotavirus subgroups and the results could be read easily on 2% agarose gels.

\textbf{5.4.5 Subgroup II}

In contrast to subgroup I, most VP6 amplicons using restriction endonuclease \textit{AciI} from strains G1, G3, G12 and G9 strains with long electrophoretic pattern yielded restriction fragments of 202, 83, 62 and 32 bp and were named restriction profile C (Figure 5:1). It was noted that restriction of VP6 amplicons from certain of these genotypes showed further partial digestion to yield two extra fragments of 285 and 119 bp; the latter profile was named profile D (Figure 5:1). Significantly, digestion of these VP6 amplicons with restriction endonuclease \textit{AciI} indicated that 60\% (43/72) were subgroup II representing restriction profile C and D.

The restriction profiles generated by restriction endonucleases \textit{DdeI} and \textit{Rsal} produced two profiles G and J, respectively. The restriction profiles were in agreement with the results of VP6 monoclonal antibodies indicating subgroup II rotavirus strains. In brief, after digestion with \textit{Dde I} the samples from representative strains of G1, G3, G9 and G12 with long RNA electropherotypes showed one cut at 27 bp to yield a fragment of 350 bp (restriction profile G). Restriction endonuclease \textit{Rsal} showed one cut at 313 bp
to yield fragments of 66 bp and 313 bp. This restriction profile was named restriction profile J.

Furthermore, two samples (2/12) which were positive for subgroup I + II after utilizing VP6 specific monoclonal antibodies were later found to be subgroup II with restriction enzymes. Restriction profiles of the South African strains indicated that none of the strains presented with the subgroup I + II cut to yield two fragments of 285 bp and 94 bp. Instead samples were identified as restriction profile C representing subgroup II.
Table 5:1 Characteristics of rotavirus subgroups analyzed in this study with ELISA, RT-PCR, restriction enzymes, restriction profiles and sequence fragments recognised by restriction enzymes *Aci*, *DdeI* and *RsaI*

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Profile</th>
<th>Figure</th>
<th>Restriction Fragments</th>
<th>No of samples</th>
<th>ELISA result</th>
<th>Sample sequenced</th>
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<tr>
<td><em>Aci</em></td>
<td>A</td>
<td>5:2</td>
<td>379 bp</td>
<td>17/72 (24%)</td>
<td>SGI</td>
<td>SA480DGM</td>
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<td></td>
<td>B</td>
<td>5:2</td>
<td>379, 119 bp</td>
<td>9/72 (13%)</td>
<td>SGI</td>
<td>ND</td>
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<td></td>
<td>C</td>
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<td>42/72 (58%)</td>
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<tr>
<td></td>
<td>D</td>
<td>5:2</td>
<td>202, 83, 62, 32, 285, 119 bp</td>
<td>1/72 (1%)</td>
<td>SGIi</td>
<td>DGM 1186</td>
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<td>E</td>
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<td>379, 285, 167, 119 bp</td>
<td>1/72 (1%)</td>
<td>SGI</td>
<td>DGM 518</td>
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<td>F</td>
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<td>379, 167, 119, 94 bp</td>
<td>2/72 (3%)</td>
<td>SGI</td>
<td>ND</td>
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<tr>
<td><em>DdeI</em></td>
<td>G</td>
<td>5:3</td>
<td>350 bp</td>
<td>11/29 (38%)</td>
<td>SGIi</td>
<td>SA107DGM</td>
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<td></td>
<td>H</td>
<td>ND</td>
<td>342, 132, 95 bp</td>
<td>1/29 (3%)</td>
<td>SGI</td>
<td>ND</td>
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<td></td>
<td>I</td>
<td>ND</td>
<td>342, 132, 95, 379 bp</td>
<td>1/29 (3%)</td>
<td>SGI</td>
<td>ND</td>
</tr>
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5.4.6 Sequence analyses

The sequences of the VP6 amplicons were aligned and compared with VP6 sequences of human and animal rotaviruses available in NCBI Genbank. The VP6 amplicon from G2 strains showed a homology of 98-100% when compared with the reference G2P[4] strain representing subgroup I strain 98TW762 (DQ898122) from Taiwan and G10P[11] strain N155 (ABY64685) from India. The VP6 amplicons from the G1 strains showed a similar homology with the reference G1P[8], subgroup II strain from the United States US8616 (EF426138) as well as the G9P[8] strain from Rio de Brazil rj7335/03 (DQ498159). Likewise, the VP6 G3 sequences showed the same homology to the G3P[8] subgroup II US reference strain US0468 (EF426120) detected between 1996 and 2002 and G12P[6] reference strain Matlab13-03 (DQ146675) detected during 2003. The G12 VP6 amplicons showed 98%-100% homology with G12P[6] subgroup II when
compared to the US reference strains US56597 (ACJ66753) and South Indian strain CRI10795 (ABN42875).

5.4.7 Sequence analysis and RFLP genetic analysis

The sequence analysis of the partial length VP6 gene confirmed the location and the recognition sites of the restriction enzymes Aci I, Dde I and Rsa I (data not shown). The typing results obtained by PCR-RFLP were confirmed by sequence analysis.

5.4.8 Phylogenetic analyses

The topologies of neighbour joining, maximum likelihood distance and consensus tree were similar. The partial sequences of VP6 gene were used to construct a phylogenetic tree. The phylogenetic tree revealed four main lineages with published human, animal and South African strains. The lineage I represented by subgroup I strains, lineage II represented by subgroup non-I non-II strains, while lineage III showed subgroup I+II specificity and lineage IV consisted of subgroup II rotaviruses. The phylogenetic analysis revealed similarities between the results obtained from VP6 MAb and the South African subgroup I and subgroup II rotavirus strains clustered in lineage I and lineage IV, respectively. None of the South African strains previously identified as subgroup I + II using monoclonal antibodies clustered with lineage III strains but grouped with strains of lineage IV.

Subgroup II strains was further subdivided into five sub-lineages (designated as sub-lineage I-V) and there was no correlation between genotypes and VP6 sub-lineages across a wide variety of electropherotypes (Figure 5:4). The reference strain E210 was distantly related to other subgroup II strains, forming sublineage I. The South African representative strains clustered with reference strains in each lineage except in lineage
II and III. Lineage II was defined as subgroup non-I non-II (EW strain), while lineage III represents equine rotavirus F1-14 strain (subgroup I + II). Identical sequences were also observed among South African strains with subgroup I and subgroup II specificity. Unfortunately, there were insufficient references VP6 sequence data available to allow comparison with further rotavirus subgroups.

5.4.9 Deduced amino acids

The comparative amino acids analysis of the VP6 gene is given in Figure 5:5 and Figure 5:6. The VP6 aa sequences analysis demonstrated homologies of 99-100% when compared to the published rotavirus reference strains. The partially deduced amino acid sequences of the VP6 gene identify at least eight variable regions within subgroup I and subgroup II to published rotavirus reference strains. These residues appeared to be highly conserved in positions common to all subgroup I at aa positions (2, 7, 60, 65, 70, 94, 97 and 103) when compared with subgroup II. Multiple alignment of the deduced amino acid sequences revealed aa substitutions at these sites, phe - tyr, val - ile, asn - ala, gln - asn, gln - glu -, asn - ser, met - leu and ala - ser for subgroup I, respectively (Figure 5:5). While comparing South African subgroup I with subgroup I reference strains, an aa substitution at positions 36 (ile-val) and at 58 (thr-ala) was noted (Figure 5:6).

Subgroup II aa substitutions at positions 58 (ala-ser) and 7 (leu-ile) were present in all South African G1P[8] and G12P[6] strains, respectively, when compared with reference strains, regardless of year of isolation. Random substitutions at aa positions 36 (val-ile), 50 (met-ile) and 98 (pro-gln) were found in subgroup II strains, but were not significantly associated with specific genotypes (Figure 5:5).
Figure 5:1 Partial length VP6 amplicons cleaved by three restriction endonucleases *AciI*, *Ddel* and *Rsal*. The sizes of the individual fragments generated by enzymes are determined by comparison with the position of fragment of known molecular weight marker.
Figure 5.2: Partial length VP6 amplicons cleaved by two restriction endonucleases \textit{Ddel} and \textit{Rsal}. The sizes of the individual fragments generated by enzymes are determined by comparison with the position of fragment of known molecular weight.
**Figure 5:3** Partial length VP6 amplicons cleaved by restriction endonuclease *RsaI*. The sizes of the individual fragments generated by enzymes are determined by comparison with the position of fragment of known molecular weight marker.
Figure 5:4 Unrooted phylogenetic analyses of the VP6 sequences of South African strains and international reference strains (subgroup I, subgroup II, subgroup I and II, subgroup non-I non-II) strains. The tree was constructed using the MEGA 3 neighbor-joining method. Numbers at the nodes indicates the percentages of bootstrap values based on 1000 replicates. The scale bar indicates an evolutionary distance of 0.05.
Figure 5.5 Comparisons of the partial deduced amino acid sequences of South African rotavirus VP6 genes with the VP6 sequences of reference strains. Identical amino acids in each isolate are represented by dots and different amino acids are indicated by one-letter code.
**Figure 5:6** Alignment of the VP6 deduced amino acid sequences of South Africa G2 strains compared with the VP6 sequences of international reference strains. Conserved amino acid residues are indicated by dots and different amino acids are indicated by one-letter code.
5.5 Discussion

Previously, monoclonal antibodies have been used successfully as an epidemiological tool to determine animal and human rotavirus subgroups. Studies have been conducted in different geographical locations to determine subgroup specificities of rotavirus strains, and have shown association between subgroup and RNA electropherotypes (Kalica et al., 1981; White et al., 1984; Steele and Alexander, 1988). However, recent data have indicated that some rotavirus strains failed to react or cross-react with subgroup-specific MAbs. This might be due to the existence of monotypes that cannot be recognised by MAbs, because of point mutations, antigenic shift, genome rearrangement (Coulson et al., 1985; Tian et al., 1993; Desselberger, 1996a; Ramig, 1997) or may be due to the existence of new subgroups. Iturriza-Gómara et al. (2002a) reported the use of AciI digestion in RFLP analysis of VP6 amplicons to differentiate rotavirus subgroups and the current study extends this by comparing two additional restriction endonucleases (Ddel and RsaI) with AciI RFLP analysis.

The technique consists of RT-PCR amplification of the 379 bp VP6 and subsequent digestion of the amplicons with restriction endonucleases to obtain restriction profiles. The restriction endonucleases were selected based on the following; enzymes producing too many small fragments were avoided and the availability of the enzyme in the country and cost were also taken into account. A total of 72 samples were successfully amplified and digested to produce 11 restriction profiles for diversity analysis. The restriction profiles produced were highly variable and unique to different rotavirus subgroups. Comparison was made between the molecular technique (RT-PCR RFLP analysis) and serological assays to investigate the correct assignment of rotavirus subgroup. In addition, representative VP6 amplicons were selected for sequencing and phylogenetic analysis to confirm the RFLP results and comparisons were made with international reference strain VP6 sequences. The results provided insights into genetic variability within rotavirus subgroups that influence subgroup-specific epitopes.
The serological assay used in the current study was the VP6 subgroup monoclonal antibodies 255/60 and 631/9 directed against the presence or absence of two conformational epitopes called subgroup I and subgroup II, respectively (Greenberg et al. 1983). The monoclonal antibodies were successfully used to identify South African rotavirus subgroups as follows: 50% specified subgroup II (93/186), 44% specified subgroup I (81/186), 6% specified both subgroup I and II (12/186) while subgroup non-I non-II were not identified.

South African rotavirus subgroups determined by VP6 subgroup monoclonal antibodies were further analysed by RT-PCR RFLP analysis. The partial-length of the VP6 gene was first reverse transcribed then followed by PCR amplification and digested with *Acil, Ddel* and *Rsal* in separate reactions. The technique was found to be specific as compared to the commonly used monoclonal antibodies against VP6 subgroup, since the results were confirmed by sequencing. Restriction of the VP6 amplicons with endonuclease *Acil* produced six profiles (A – F) that were confirmed through computer analysis program Webcutter 2.0.

While profile A and C compared favourably to previous results reported by Iturriza-Gómez et al. (2002a) when VP6 amplicons were digested with *Acil*, four additional profiles were noted in the South African strains. Profile B, D, E and F all demonstrated the subgroup non-I non-II 119 bp fragment while profiles E and F also demonstrated the subgroup non-I non-II 167 bp fragment. Profiles D and E also showed a subgroup I + II fragment (285 bp). Only SA1186DGM/04 subgroup II specificity (profile D) and SA518DGM/03 subgroup I specificity (profile E) were sequenced. Interestingly, sequence analysis of cloned VP6 fragments (SA1186DGM/04 and SA518DGM/03) did not demonstrate any nucleotide or amino acid sequence variations that would result in the profiles (D and E). The variation observed was only noted by RFLP analysis and electrophoresis in a 2% agarose gel. Further investigation is recommended to look at the possibilities of point mutations in the VP6 genes or mixed infections by sequencing various clones of the VP6 genes of strains SA1186DGM/04 and SA518DGM/03.
The variation between two studies was probably due to the fact that strains analysed were from different geographic region with the possibility of point mutation that might lead to the inclusion or removal of endonucleases recognition sites of the PCR amplicons. Due to the potential of point mutations, further studies are required. The sequence and computer analysis generated by Webcutter 2.0, of the two studies were not verified. Restriction profile C (subgroup II) was the most frequently detected (58%), followed by restriction profile A (subgroup I) (24%) and lastly restriction profile B (subgroup I) (13%). Conserved restriction site was identified at position 274 (119 bp) was present among subgroup II and subgroup I rotavirus strains.

On statistical average the *Rsa I* recognition site 5’ GT↓AG 3’/ 3’ CA↓TG 5’) occurs two times in a 376 bp of the subgroup I rotavirus strains producing three digestion fragments whereas the subgroup II recognition site occurs only once to produce two digestion fragments. Using restriction endonuclease *DdeI*, three distinct restriction profiles were identified.

The examination of restriction profiles obtained revealed that South African subgroup I rotavirus strains exhibiting short electropherotypes were associated with restriction profile A, B, H, I and K, after digestion with three restriction endonucleases. Comparative sequence analysis of G2 strains showed that four South African strains had almost identical sequences on the other hand exhibiting different restriction profile (A and B). Inability to distinguish the sequence analysis variations between the restriction profiles was probably due to cloning and sequencing of only one clone from each specimen and the possibility of point mutations and of mixed infections cannot be ruled out from the current analysis.

The variation in restriction of the VP6 amplicon within rotavirus genotypes reflects genetic variability within that rotavirus subgroup. Restriction profiles A and B (subgroup I) variations, showing no cut and incomplete digests, respectively, were
also considered insignificant. The strains results were identified by monoclonal antibodies as subgroup I. The results could indicate the existence of an unknown subgroup or mutations in the VP6 gene that can potentially result in different fragment patterns, but unfortunately the mixed subgroup results (subgroup I and subgroup non-I non-II) could not confirmed by monoclonal antibodies. Based on electrophoretic pattern, it appears that the genotype G2 can be accurately predicted as subgroup I while other genotypes were more difficult to forecast. All the G2P[4] and G9P[6] strains exhibiting short electropherotypes were classified as subgroup I using monoclonal antibodies and belonged to the restriction profiles A and B. Comparisons of these two studies show that profile B was not previously recognised by restriction endonuclease described by Iturriza-Gómar et al. (2002a). Nevertheless this study was able to demonstrate the presence of an additional subgroup I restriction profile.

The genetic analysis of subgroup II revealed that restriction profiles C, D, E, and F were associated the G1, G3, G9 and G12 exhibiting the long electropherotype. Incomplete digestion was also observed in restriction profile E and F. Incomplete digests were probably due to an enzyme that does not recognize every target site in every cDNA molecule, and sometimes fails to cleave some of its target sites. Additional studies will, however, be required to investigate the VP6 sequences of the four strains displaying these altered subgroup profiles (D, E and F). The RFLP analysis with restriction enzymes Ddel and Rsal (restriction profiles J and K) were able to characterise the subgroup II rotavirus strains correctly and sequence analysis confirmed the results. However, the sequencing and restriction site analysis by Acil showed that the 119 bp fragment shown in both subgroup I and subgroup II were similar only in length. This underscores the unsuitability of using Acil as a differentiating endonuclease enzyme and highlights the need to develop RFLP enzyme such as Rsal to differentiate rotavirus subgroups.

When the deduced aa sequences of the VP6 proteins were aligned against the reference strains to examine the antigenic regions of VP6 subgroup, it was possible
to detect nucleotide and amino acid substitutions. It became evident that single aa substitutions might be responsible for not only identifying different rotavirus subgroups but, also, that point mutations at these sites might result in epitope changes contributing toward MAb failure. A comparison of aa sequences deduced from nucleotide sequences reflected conservation within the VP6 gene – there was complete agreement across genotypes G1, G3, G9 and G12 with long electropherotypes (representing subgroup II). Likewise, homology was noted when comparing the VP6 from G2 strains with the short electropherotype (representing subgroup I). Only one amino acid change was observed in subgroup II strains and two were observed in subgroup I strains when compared to subgroup reference strains. The eight variable sites observed across the subgroups could be those positions that describe the subgroup epitopes. This suggests that those positions might have important antigenic characteristics.

Thus, restriction mapping can be used as a genetic marker to examine rotavirus subgroups. The results obtained by RFLP mostly correlated with the VP6 genogrouping results obtained by Iturriza-Gómara et al. (2002a), except that in the current study four extra RFLP patterns were observed including partial digestion and identification of fragments from subgroup I + II and subgroup non-I non-II profiles. A total of 18% (profiles B, D, E and F) South African strains demonstrated genetic variability in the VP6 gene, such that the RFLP method described by Iturriza-Gómara et al. (2002a) could not be used to assign a subgroup. In addition, more genetic variation (9/72) was noted in the subgroup I-specific strains identified with MAbs than in the subgroup II strains (1/72) detected in the same manner (Table 5:1). Additional studies will be required to investigate this phenomenon.

None of the South African strains were identified as subgroup non-I non-II or both subgroup I + II by RFLP analysis. In contrast to VP6 subgroup monoclonal antibodies, the typing by PCR-RFLP analysis failed to detect subgroup I + II in selected samples and the results were confirmed by sequencing as subgroup II. Iturriza-Gomara and colleagues (2002b) also reported the existence of rotavirus
strains that react with both subgroup-specific MAbs but revealed subgroup II specificity when sequenced. However, the VP6 sequences from the South Africa subgroup I + II specimens were cloned and only one clone sequenced, therefore, the presence of subgroup I + II-specificity or another subgroup I-specific VP6 gene cannot be discounted.

This method used is considered to be convenient for strain differentiation and classification. The test is highly sensitive, specific, and simple to perform and may be regarded as a rapid diagnostic test for group A rotavirus subgroup. This method might be useful for further rotaviruses characterization. Since determination of rotavirus subgroups using monoclonal antibodies has been reported to be difficult, RT-PCR RFLP analysis may become an essential technique to characterize rotavirus subgroups. By developing an RFLP analysis method that can be widely used as diagnostic assays for VP6 subgroup, the Webcutter computer program was applied for selecting restriction endonuclease most suitable for subgroups specific cleavage. This is a simple method for rapidly detecting rotavirus subgroups that could be widely used as a VP6 subgroup assay in many laboratories. Implementing RT-PCR and subsequent RFLP analysis with restriction endonuclease Rsal, Acil and Ddel enabled us to differentiate between different rotavirus subgroups. The use of restriction endonuclease Rsal has allowed simple profiles, easily recognised size patterns for rotavirus subgroups as compared to Acil and Ddel. Hence, digestion with Rsal was the most convenient and easy to read on the agarose gels, without showing various restriction sites and partial digests. Moreover, we cannot exclude the possibility that the use of this restriction endonuclease is limited to the partial length VP6 amplicon (379 bp) and further studies will be required if the entire VP6 gene is utilized.

The phylogenetic analysis revealed four lineages after inclusion of international reference strains; subgroup I, subgroup II, subgroup I + II and subgroup non-I non-II. The two main lineages subgroup I and II were further subdivided into several sublineages depending on genotype and electrophoretic pattern. In general, the
topology of the phylogenetic tree is in agreement with the literature, that single point aa mutations at position 172 (Met to Ala) or 305 (Asn to Ala) was sufficient to change the subgroup specificity (Baozhang et al., 1997).

The sequence analysis detected more variation within the VP6 fragment, compared to RFLP analysis. Nevertheless, the VP6 subgroups determined by sequence analysis, RFLP and monoclonal antibodies were mostly in agreement except few cases where subgroup I + II could not be determined. The subgroup I + II determined by monoclonal antibodies indicated high probability of false positivity caused by cross reaction of subgroup I and subgroup II monoclonal antibodies as was previously described by Iturriza-Gómar et al. (2002b).

In conclusion, the study suggests that RT-PCR RFLP analysis could probably replace the VP6 subgroup monoclonal antibody assays. The RFLP analysis proved to have more potential to accurately detect different rotavirus subgroups and it is also essential to confirm this assay with genomic sequencing to determine the precise genetic mutation. Restriction endonuclease RsaI produced the most accurate RFLP results to rotavirus subgroups.
CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 Rotavirus infection

Rotavirus infection is the most common cause of severe diarrhoea in infants and young children worldwide, despite socio-economic status or environmental conditions. Rotavirus infection causes an estimated 527,000 deaths in children less than 5 years of age each year with the highest childhood mortality rate of approximately 85% in developing countries especially Africa and Asia (WHO, 2007). Approximately 40% all diarrhoeal hospitalization are due to rotavirus gastroenteritis (CDC, 2008). Worldwide, the burden of rotavirus is significantly high. Since 2001, Regional Rotavirus Surveillance network in many countries including the USA, UK, Europe, Asia, Australia, China and Africa under the leadership of WHO and PATH, have gathered information on burden of rotavirus disease. Numerous studies have examined burden of rotavirus disease and its economic impact in different areas of the world. In response to the burden of rotavirus-associated disease, numerous rotavirus vaccines have been developed as a principle strategy to reduce mortality and morbidity associated with rotavirus. Thus, our study provides comprehensive estimates and incidence of rotavirus disease requiring hospital admission, and identifies common strains circulating in children less than 5 years of age during a three-year period from 2003-2005. We collected data and stool samples from Dr George Mukhari and Brits Hospitals in the North West of Pretoria. These data will contribute to baseline up-to-date information on rotavirus infection within the South African health care service and make the general public, policy makers and the paediatricians aware of the impact that rotavirus infections have in the country. This has been the first analysis of the burden of rotavirus disease in South Africa.
6.2 Burden of rotavirus disease

The incidence and burden of rotavirus gastroenteritis remains significantly high among children under 5 years of age in many countries. The current study confirms that rotavirus is the most common cause of severe diarrhoea. Certainly, the data clearly demonstrated that rotavirus infection is responsible for around 22.8% of all annual diarrhoeal hospital admissions in children less than 5 years of age. During the study period from 2003-2005, disease burden of rotavirus infection in South Africa was substantial, and the annual detection of rotavirus infection in the medical facilities under study was found to be 17%, 21% and 25%, respectively. This South African hospital-based study of rotavirus has indicated an average of 20-25% of all hospitalization for diarrhoeal illnesses are rotavirus positive. Our results are consistent with findings that had been reported by several authors (Ryan et al. 1996; Johansen et al. 1999b; Ruggeri and Declich, 1999; Szucs et al. 1999; Mrukowicz et al. 1999; Gil et al. 2003) all of which have motivated efforts to develop rotavirus vaccines. Hence, our study provided new and important insight into disease burden and genetic diversity of rotaviruses circulating as well as clinical severity of rotavirus disease and to a certain extent it provided a baseline for the translation of the disease burden of rotavirus infection onto the South African economy.

6.3 Incidence of rotavirus infection

The annual rate of rotavirus-associated hospitalization at Dr George Mukhari Hospital was 22.8% (95%CI 21.2%, 24.5%) and 18.2% (95%CI 14.9%, 22.1%) at Brits Hospital in children less than 5 years of age. Among children admitted to Dr George Mukhari Hospital, we estimated that 5.5% (95%CI 5.1%, 6.0%) of all admissions were due to rotavirus infection. We estimated that one in every 50 to 70 children less than 24 months of age in these areas were likely to be hospitalized for rotavirus gastroenteritis during 2003 and 2005. Remarkably, the study might underestimate the true incidence of hospitalization due to rotavirus disease in the area. This is because of health
care referral system in South Africa, where children are treated at the primary health care facilities before they can be referred to the tertiary or secondary health care facilities.

Our data can be extrapolated carefully for national or regional information to obtain an accurate estimate of rotavirus burden of disease. Since this is the one of the few reports of rotavirus burden disease study in Africa, this should assist policy makers in decision-making about rotavirus diarrhoea. The estimated annual rotavirus mortality in the African region is significantly high. Recent reports showed the four African countries (Nigeria, DRC Congo, Ethiopia and Angola) are among the top ten countries with the highest childhood mortality attributed to rotavirus (Parashar et al., 2009). Therefore, this information is focussed particularly on developing countries, especially in Africa and Asia and has important implications regarding the impact of rotavirus disease on health care facilities, as well as on monitoring the introduction, roll out and impact of rotavirus vaccines.

The data emphasizes the potential use of effective vaccines to reduce the incidence of severe rotavirus diarrhoeal cases that require hospitalization and, in contrast, the potential reduction in the economic burden associated with rotavirus disease. This, considering that diarrhoeal diseases in children can potentially have a significant impact on family members of infected children and society at large - increased medical expenditure, loss of productivity of family members and hospital overcrowding.

6.4 Relevance of the study to the country

This study together with cost effectiveness study and the Rotarix® clinical trials at Madibeng and Ga-Rankuwa area have generated invaluable evidence and also raised awareness of burden of rotavirus disease and the potential
impact of rotavirus vaccination in the country. As a result, these have provided an excellent reference framework to the Department of Health to prioritize the proposed introduction of a rotavirus vaccine in the Expanded Programme on Immunisation-SA (EPI-SA) to prevent childhood morbidity and mortality in South Africa. To date (August 2009-December 2009), in South Africa more than 659 000 doses of Rotarix® had been distributed in eight of the nine provinces. A factor contributing to slow uptake of the vaccine is mainly logistic problems at the provincial level.

The introduction of rotavirus vaccine in the country is in line with WHO’s Strategic Advisory group of experts (SAGE) recommendations that rotavirus vaccine should be included in all national childhood vaccination programmes including developing countries (WHO, 2009). Thus, the two new rotavirus vaccines were licensed in several countries in the America, European, Eastern Mediterranean, Australia and in South Africa. SAGE also recommended the broadening of age range of Rotarix® or Rotateq™ schedule with the first dose be administered at 6 weeks to 15 weeks of age and the last dose before 32 weeks. These due to the fact that in many developing countries where there are high rates of childhood mortality not all children receive immunization according to the recommended schedule. Therefore, this strategy might potentially increase vaccine coverage even in developing countries (WHO, 2009). The implementation of rotavirus vaccine programmes could have major impact on preventing morbidity and mortality associated with rotavirus infection globally.

The clinical trials conducted at the Madibeng and Ga-Rankuwa area included: two phase II immunogenicity trials (2 versus 3 doses) where oral poliovirus vaccine (OPV) was co-administration at 6 and 10 weeks and at 6,10 and 14 weeks of age (2001-2004); a phase III efficacy study (2005-2008); and a safety and immunogenicity trial in HIV-infected infants (2005-2008). The results from co-administration of OPV with Rotarix® trial demonstrated that the
vaccine is well tolerated and immunogenic in South African infants (Steele et al., 2008). The clinical trial suggests that the HIV infected infants could also receive rotavirus vaccine (Steele et al., 2009). Collectively, together with burden of disease study, the studies have generated the needed information about the public health benefits of rotavirus vaccination in developing countries. Subsequently the WHO SAGE further extended the recommendation for use of rotavirus vaccines to all regions of the world.

6.5 Hospital admission

Hospital admissions of diarrhoeal diseases represent a small percentage of the general population. The proportion of children being treated for diarrhoea at Dr George Mukhari Hospital was found to be 7% for all children less than 5 years of age at both outpatients and inpatients wards. There are a number of factors to be considered in this regard, including: (1) the proportion is relatively low because of the hospital referral system in South Africa where patients should be assessed and treated at primary health care facilities before being referred to secondary or tertiary health care facilities. Thus, the majority of diarrhoeal cases should be treated at clinics and in private practice; (2) the patients involved in this study were from outpatients (OPD) and inpatients wards. At OPD, children are treated for different conditions because of different clinics including Asthmatic, Endocrine, Cancer, HIV, and General clinics, etc. Therefore, many children admitted at the hospital are mainly treated for chronic infections. The study shows that rotavirus infections account for an average of 20-25% of all hospitalization of diarrhoeal illnesses. However, the rate of 56 to 59% was observed during the rotavirus season. This is consistent with the results of a study performed by Conway et al. (1990) which showed that a large proportion of diarrhoeal diseases were attributable to rotavirus group A and accounts for a greater number of hospital admissions. These incur large economic cost in the health care facilities. The costs associated with diarrhoeal disease include direct medical cost (hospital facility and professional, medication and non drug order and diagnostic tests),
direct non-medical cost (travel and other out of pocket cost) and indirect costs (time lost from productive work by the carer).

Rotavirus characterization has provided insight into and underscored the importance of emerging new rotavirus strains. This information will make the public health community even more aware of the importance of candidate rotavirus vaccines. This demonstrates the vital role in assessing the effectiveness of the vaccines by identifying circulating serotypes.

6.6 Age distribution of rotavirus infection

The study indicates that rotavirus-associated diarrhoea in South Africa occurs at an early age (from 0–18 months). However, we observed the peak incidence of rotavirus infection in children aged 3-18 months and the incidence decreases rapidly after 24 months of age. In developed countries the situation is different where children aged 6-23 months are more vulnerable to rotavirus infection (Rodriguez et al., 1977; Barnes et al., 1998) and more than 80% of children developed rotavirus infection during the first three years of life. In developing countries, symptomatic rotavirus infection starts earlier in life in 0-5 months old infants (Glass et al., 1996). The study shows a significant decrease in rotavirus prevalence with aging. As indicated by other studies, the difference in age supports the evidence that vaccination at an early stage will potentially decrease severity of infection and reduce high prevalence of rotavirus infection treated at hospitals (Joensuu et al., 1997).

6.7 Seasonality of rotavirus infection

In many countries during the winter months, there is a higher proportion of rotavirus infections with more than 50% of all diarrhoeal hospital admissions in infants and young children. Likewise, rotavirus infection in South Africa occurs
throughout the year with marked seasonal changes. We observed a peaked incidence during the winter months with low prevalence during summer months. The peak incidence rates of rotavirus infections observed were 56%, 59% and 56% from 2003-2005, respectively, among children less than 5 years of age who were treated for severe diarrhoea. Seasonal distribution of rotavirus data observed is similar to other studies in developed countries with temperate climatic conditions (Koopmans and Brown, 1999; MMWR Recomm Rep, 1999; Rivest et al., 2004; Bresee et al., 2004; Kim et al., 2005; Nakagomi et al., 2005; Gleizes et al., 2006). In the in tropical regions of Africa developing countries increased cases of rotavirus infection are usually experienced during the drier months of the year and a low rate of rotavirus infection during the wet season (Cunliffe et al., 1998, AfrRSN unpublished data).

6.8 Clinical history

Fever and vomiting were commonly associated with rotavirus diarrhoea and vomiting was reported significantly more frequently by caregivers of rotavirus positive patients 63% than those without rotavirus infection. Fever did not show any significant difference between children infected with rotavirus or not. Various studies have shown similar results, where vomiting was more associated with rotavirus diarrhoea (Rodriguez et al., 1977; Pazdiora et al., 1990; Staat et al., 2002). Rotavirus infection is more often associated with severe infection or dehydration compared to other common causes of diarrhoea (Rodriguez et al., 1977) and the most common clinical finding in this study was that rotavirus diarrhoea was associated with bronchopneumonia (about 70%). Similar results have been reported by Pazdiora et al. (1990). The mean hospital stay associated with rotavirus infections was 4 days.

The difference between clinical history of diarrhoea from patients from whom the stool samples were collected and not collected, fever and vomiting were
seen more in children from whom the stool samples were collected, which indicates that patients whose samples were collected had moderate to severe infection.

6.9 Gastroenteritis mortality

The current study provides data on rotavirus gastroenteritis as the major etiological agents of severe diarrhoeal disease in hospitalized infants and young children. In developing countries, diarrhoeal diseases are one of the leading causes of illness and deaths in infants and young children with at least an estimate of 1.4 million deaths annually (Murray et al., 2001). Nevertheless, at Dr George Mukhari Hospital annually mortality associated with diarrhoeal diseases was found to be 7% (Dr George Mukhari Hospital, unpublished data). Although there was a high frequency of rotavirus gastroenteritis at the hospital, unfortunately we could not determine rotavirus mortality rates as most children who had severe diarrhoea with dehydration died before stools could be obtained for study.

6.10 Demographic data

The overall proportion of rotavirus infection was comparable between males and females. More male children (53%) experienced diarrhoeal diseases compared to female children (47%) and were more susceptible to rotavirus infection. This suggests that boys become more frequently sick than girls. The reason for the gender difference was not determined, but the results obtained are consistent with the findings that had been reported previously by Staat et al. (2002), Nguyen et al. (2004) and Kim et al. (2005).

Diarrhoeal disease is the leading cause of death in black and coloured South African children under the age of 5 years (Westaway and Viljoen, 2000). The
The majority of the South African population (75%) lives in rural areas (McKerrow, 2002). In most rural areas, many households are relatively far from health care facilities resulting in reduced use of clinics and hospitals. Informal settlements have increased as many people have moved to the cities to seek job opportunities and this may lead to overcrowding within households and also to unhygienic practices. In recent years, studies have shown that household environment factors are associated with child mortality in urban and rural areas of many developing countries (Cairncross, 1990; Harpham and Stephens, 1991; Macassa et al., 2004). In the informal settlement and rural areas, the household environmental risk factors such as water shortage or contamination, overcrowding, malnutrition due to high food cost and poverty might exacerbate the increased incidence of diarrhoeal diseases. Different results were observed with rotavirus infection. Our data demonstrated that infection rate of rotavirus is similar in rural, urban (formal settlements) and informal settlements. This suggests that household environmental variables including improvement in sanitation and water supply have not been found to reduce the incidence of rotavirus infection. Socio-economic and demographic factors were not considered as risk factors for children to acquire rotavirus disease.

The reports by Faruque et al. (1992) and Gianino et al. (2002) have shown that breast-fed children are less affected by rotavirus infection. Over 58% of children less than one year of age in our study population were breast-fed, but breastfeeding did not appear to have significant effect on prevention of rotavirus gastroenteritis. Breastfeeding and maternal antibodies seem to provide incomplete protection-part of this may be due to the large and diverse infective dose that children in developing countries probably receive when exposed to their first viral gastroenteritis infections.

Effective interventions are needed to reduce childhood mortality. As a result the use of oral rehydration therapy to treat acute gastroenteritis has been
considered as a major public health advance in developing countries. Data indicate a high prevalence (64%) of rotavirus infection and usage of oral rehydration solution for treatment of gastroenteritis at home prior to hospital admissions. This is similar to other studies conducted in South Africa where high proportions of children were treated with oral rehydration therapy during diarrhoeal episodes (Mawela and De Villiers, 1999; Dippenaar et al., 2005). This result may indicate that improved knowledge and maternal education on the usage of oral rehydration solution has resulted in fewer cases of moderate to severe dehydration potentially requiring hospital admissions, which can also be a determinant of childhood mortality. Other factors, including improved living conditions and health care facilities in South Africa might also have a significant impact of rotavirus mortality.

Routine treatment management of acute gastroenteritis is rehydration regardless of the cause. Rotavirus infection has never been regarded as a priority for laboratory diagnosis, since there was no specific treatment. Our study was able to identify incorrect routine treatment of diarrhoeal disease at the hospital where 74% of children with rotavirus infection were given antibiotics. This problem could lead to increasing resistance to antibiotics. It also highlights increasing difficulties in controlling infectious disease associated with decreasing effectiveness of antimicrobial drugs. These results should make paediatricians and policy makers aware of the importance of rotavirus infection in the hospital and the need for vaccination. The study strongly recommends that health care personnel be trained to adhere to management protocol to avoid unnecessary antibiotic prescription.

In developing countries, mortality related to malnutrition is estimated at 50% in children less than 5 years of age (Yigael, 2004). Even though diarrhoeal disease is a significant cause of childhood morbidity and mortality, especially among malnourished children, the present study showed that 34% of children were malnourished. According to Black and Lanata (1995), poor nutritional
status predisposes towards a greater diarrhoeal frequency due to impaired host defences, decreased gastric acidity, or nutritional deficiencies.

### 6.11 Diarrhoeal pathogens

Most diarrhoeal illnesses in children less than 5 years of age were mild to severe cases. Diarrhoea can be caused by a wide diversity of bacterial and viral pathogens. The study was able to describe the distribution of enteropathogens associated with severe diarrhoea at the hospital. Of these pathogens, rotavirus was the leading cause of diarrhoea in 29% of cases, *Escherichia coli* were the most frequent bacterial enteric pathogens isolated (4.8%) followed by *Shigella flexneri* and *sonnei* at 2.2%, and lastly *Cryptosporidium parvum* at 1.9%. Thus, the majority of diarrhoeal incidences were not diagnosed. In contrast to these results, reviews of epidemiological studies in developing countries have shown that a median of 23% of ETEC was responsible for episodes of diarrhoea and 6.3% for rotavirus. However, Bern *et al.*, (1994) reported different results with inpatient studies showing rotavirus responsible for 29% and ETEC 9.3%. As a large proportion of causative agents of diarrhoeal illnesses could not be determined in the present study, the influence of co-infection of rotavirus and bacterial pathogens could not be determined as to its influence on clinical symptoms.

### 6.12 Serological techniques

To determine the incidence and burden of rotavirus disease in children less than 5 years of age, diarrhoeal stool samples were screened for group A rotavirus using enzyme immunoassay IDEIA Rotavirus test. The test is widely used to screen clinical specimens. The application of this assay has significantly increased the recognition and the importance of group A rotavirus in children less than 5 years of age, worldwide.
The study was also able to describe the frequency of subgroup I and subgroup II rotaviruses isolated in Dr George Mukhari Hospital using VP6 specific MAbs developed by Greenberg et al. (1983) and Beards et al. (1984). Early epidemiological studies using MAbs have been successful in the identification of animal and human rotavirus subgroups. Recently, the application to VP6 subgrouping using monoclonal antibodies has been ineffective (Alam et al., 1999; Iturriza-Gómez et al. 2002b). Thus, the study compares serological VP6 subgrouping assays and molecular characterization of the VP6 subgroups. VP6 subgrouping using MAbs was determined in 186 stool specimens from children with acute rotavirus gastroenteritis. Of these, 50% of the rotavirus isolates were found to be positive for subgroup II whereas 44% were found to be subgroup I, and both subgroup I and II were found to be positive in the balance of cases. There was no subgroup non-I non-II strain identified.

The reactivity pattern of our isolates with MAbs directed to inner capsid protein clearly shows the relationship between the subgroup, genotypes and RNA electropherotypes. The subgroup I specificity represented serotype G2 or G8 with short RNA electropherotypes whereas subgroup II specificity exhibited serotype G1, G3, G4 or G9 with long RNA electropherotype. The results were comparable with studies described by Kalica et al. (1981), White et al. (1984) and Steele and Alexander, (1988). The ratios between the optical density readings obtained with MAb were determined, even though the results showed high OD values.

6.13 Restriction enzymes length polymorphism

Recent data have suggested that genetic complexity within the rotavirus VP6 gene are more extensive than previously anticipated such that some rotavirus strains fail to react with subgroup specific MAbs (Ramig, 1997; Desselberger, 1996a; Tian et al., 1993; Coulson et al., 1985). As changes in VP6 epitopes
may have resulted from point mutations, RT-PCR RFLP analysis was tested as an alternative to serological identification of VP6 subgroups. This method was used to test VP6 amplicons from representative strains including G1, G2, G3, G9, G8 and G12 using restriction endonucleases Aci I, Dde I and Rsa I. Eleven unique restriction profiles (A-K) were generated and analysed to show 95% agreement with VP6 MAb subgrouping, suggesting that RT-PCR RFLP analysis could replace routine VP6 MAb subgrouping. Restriction endonuclease RsaI could clearly distinguish between different subgroups without showing multiple restriction sites and partial digests. Our results indicate that restriction endonuclease RsaI is most suitable restriction enzyme to characterised VP6 subgroups. Further studies are needed to investigate the validity of restriction endonuclease RsaI in the full length VP6 product.

In addition, results showed concordance as to rotavirus strain specificity. Hereby, the two main rotavirus subgroups were identified in the study population: subgroup I and subgroup II. Genetic diversity of rotavirus strains was confirmed by nucleotide sequence analysis. The phylogenetic analysis revealed three genetic lineages designated as Lineage I-III, within Lineage III the VP6 sequences further exhibits five sublineages with no correlations to different genotypes. Iturriza-Gómara et al. (2002b) showed similar results. The VP6 sequence might indicate the interrelationships between different genotypes- showing Wa and DS-1 rotavirus strains. The RT-PCR RFLP assay could become a useful tool in genetic characterization of the VP6 subgroups and the assay is simple and relatively inexpensive.

6.14 Molecular characterization of rotaviruses

6.14.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of rotavirus genome has been considered to be the most widely used technique for epidemiological studies for
identification and characterization of rotaviruses. Two RNA migration patterns or electropherotypes have been described for group A rotaviruses – the so-called “long” and “short” electropherotypes. The study described molecular epidemiology of rotavirus strain by examining the RNA electrophoretic pattern. The application of the technique has revealed extensive genomic diversity of rotavirus genome. Variations in the electrophoretic patterns were particularly noticeable in segments 5, 6, segments 7, 8, 9 and segments 10 and 11 and reflected dominant electropherotypes changing from year to year. During the study period from 2003-2005, there were ten, six and seven different RNA electrophoretic patterns identified exhibiting 62% short electropherotypes, and 96 and 98% long electropherotypes, respectively. Our findings are consistent with previous studies in Africa including South Africa, Tunisia, Nigeria, Ghana, Guinea-Bissau and Kenya that describe the distribution of short and long electropherotypes (Steele and Alexander, 1987; Trabelsi et al., 2000; Pager et al., 2000; Pennap et al., 2000; Armah et al., 2001; Steele et al., 2003; Fischer et al., 2003). Mixed infection can be described by the presence of more than 11 segments in electropherotype profiles. Potentially mixed infection can lead to the formation of novel reassortant strains. The study shows a low frequency of electropherotypes indicating mixed infections strains (1%). Our findings confirm similar results by Beards (1982) in that a particular serotype may exhibit a significantly different RNA migration pattern and, conversely, the same electrophoretic migration pattern can exhibit different genotypes.

6.14.2 VP7 and VP4 genotypes

The epidemiological study describes the genetic diversity of rotavirus strains circulating in Ga-Rankuwa and Brits communities. Rotaviruses are classified into different G and P types based on the VP4 and VP7 proteins (Estes and Cohen, 1989; Hoshino and Kapikian, 2000). The proteins can independently elicit protective neutralizing antibodies and so a dual typing system has been established to describe the epidemiology of circulating rotavirus strains (Gouvea et al., 1990; Gentsch et al., 1992). Globally, rotavirus serotypes G1P[8], G2P[4], G3P[8], G4P[8], G9P[6] and G9P[8] are considered to be the
most common strains and represent >90% of rotavirus infections (Hoshino and Kapikian, 2000). The epidemiology of rotavirus strains in South Africa appears to be similar to other countries. The degree of diversity of co-circulating human rotavirus strains is high at the hospital showing a yearly change of predominant genotypes. The distribution of rotavirus strains followed a common global distributions and the data show that G1-G3 rotavirus genotypes are most frequently detected, associated with P[8], P[6] and P[4]. The P[6] genotypes of the rotavirus strains accounted for 30% of the typeable strains detected. The distribution of genotype P[6] was similar to most studies in Africa and India (Santos et al., 1994; Timenetsky et al., 1994; Steele et al., 1995; Ramachandran et al., 1996; Cunliffe et al., 1999; Steele et al., 2003; Peenze et al., 2008).

Rotavirus strain distribution varied considerably over time, in 2003, the majority of isolates were G2 (50%) followed by G1 (36%). In 2004, the situation differed, and reflected a high incidence of G1 (56%) types with a low percentage of G3 (25%). In 2005, there was a further increase in G3 (72%) strains with fairly low prevalence of G1 (9%) strains. The data clearly show the fluctuation of commonly circulating rotavirus genotypes where G1P[8] can be replaced and then re-established over time. On the other hand, the G3 rotavirus strain increased substantially from 26% in 2004 to 72% in 2005. The G2 rotavirus strains were more prevalent in 2003 (50%) and decreased over time and almost disappeared in 2004 (2%) and in 2005 were not detected. The G4 rotavirus strains were last detected in South Africa in 1988 by Steele and Alexander, (1988).

G8 and G9 rotavirus strains were also detected but at a low frequency of 3% and 4%, respectively. The G8 strains have been reported in many African studies (Adah et al., 1997; Cunliffe et al., 1999, Steele et al., 1999; Fischer et al., 2000; Armah et al., 2001). The G8 human rotavirus strains could have resulted from animal-human rotavirus reassortants. The situation may be
feasible, as the study population is comprised of urban, rural and informal settlements. In rural areas there is close proximity between animals and human population. The G9 rotavirus strains identified in the study were associated with P[6] and P[8] and showed a variety of long or short electropherotypes (4%), comparable to previous studies (Kirkwood et al., 2002; Steele et al., 2002a; Armah et al., 2003; Rahman et al., 2005b; Gentsch et al., 2005; Santos and Hoshino, 2005; Khamrin et al., 2006b; Lin et al., 2006; Rodrigues et al., 2007a). In South Africa, the G9 rotavirus strains were first reported in hospitalized children with diarrhoea by Steele et al. (2002a). Since then from 2003 to 2005, the low prevalence of G9 rotavirus strains with short or long electropherotypes were reported. The P and G type distribution of rotavirus infection in children hospitalized with diarrhoea has been demonstrated. The genotyping data indicated that both common and uncommon genotypes were co-circulating during the study period.

We identified strains, which appeared as intergenogroup reassortants, where strains from the DS-1 genogroup had acquired the VP4 gene from the Wa genogroup during co-circulation of the two-rotavirus strains. Thus, unusual combination of rotavirus G2 strains identified in the present study were G2 with P[4] and P[8] or G2 with P[4] and P[6] or G2P[6] exhibiting a short electropherotype pattern.

G12 human rotavirus strains have been detected all over the world in recent years including: Thailand, Japan, Korea, United Kingdom, Belgium, Argentina, Nepal, India, Bangladesh and Brazil (Griffin et al., 2002b; Pongsubwanna et al., 2002; Das et al., 2003; Shinozaki et al., 2004; Samajdar et al., 2006; Castello et al., 2006; Banerjee et al., 2007b, Rahman et al., 2007). The G12 strains have now been isolated in South Africa for the first time. These had long electropherotypes exclusively associated with P[6]. Comparison with other G12 strains from the Genbank has shown that the nucleotide sequences were closely related to Eastern India strains (SO23). The introduction of G12 strains
in South Africa could explain temporal variation of rotavirus serotypes circulation in other countries. These might be the occurrence of G12 strains under natural circumstances or due to changes in climatic and geographic conditions, immigrants, exports and imports. Emergence of serotype G12 rotavirus strains could be a threat to the currently available vaccines and emphasises the need to monitor current vaccines composition in response to changes in circulating rotavirus strains overtime.

Mixed infections of rotavirus strains were observed, but with a low prevalence rate of 8%. Similar results have been obtained in most developed countries, however studies described by O’Halloran et al. (2000) and Fischer et al. (2005) reported different results in Ireland and Denmark, respectively. A study by Bresee et al. (1999) also reported a high percentage of mixed infection. Improvements in typing methods such as RT-PCR, cloning, sequencing and RFLP have probably contributed to the reduction in the percentage of mixed infection and untypeables in this study. Untypeable strains were also observed, and further research is essential in order to determine whether their lack of identity was due to failure of primers to detect uncommon strains or due to other technical problems.

6.14.3 Sequence comparison and phylogenetic analyses

To gain further insight into the genetic variability of South African rotavirus strains, full length VP7 and fragments of the VP6 gene of the selected strains were sequenced. The evolutionary distances between our isolates and reference strains were investigated using pairwise comparison from multiple sequence alignments. BLAST searches identify the consensus nucleotide and deduced amino acid sequences to demonstrate the greatest homology and identity of (97-99%) with reference strains from South Africa, Vietnam, Bangladesh, East India, Republic of Congo, China, Russia, Thailand and
Japan. The results confirmed the genotyping results obtained by semi nested RT-PCR.

Phylogenetic analysis identified seven phylogenetic trees according to the most common genotypes observed during the study period, which includes G1-G3, G8- G9 and G12 rotavirus strains and international reference strains. The phylogenetic trees revealed different lineages, which were then subdivided into different sublineages according to their genetic relatedness.

The G1 strains formed an independent cluster with two different lineages, but revealed a close genetic relationship. The study either confirmed intratypic diversity of G1 genotypes or genetic evolutionary diversity of the viruses. The study supports previous studies that have shown distinct G1 lineages (Berois et al., 2003; Arista et al., 2006).

The BLAST search identified the consensus nucleotide sequences and deduced amino acid sequences of the VP6 gene that were closely related to G2P[4] subgroup I from Taiwan and G12P[6] genogroup DS-1 from Bangladesh, G1P[8], subgroup II strain from the United States and G9P[8] from Brazil, G3P[8] subgroup II strains from the United States and G12P[6] Matlab13, with nucleotide identity of 97-98%. The phylogenetic tree revealed four main lineages showing group A rotavirus subgroup specificity namely: subgroup I, subgroup II, subgroup I + II and subgroup non-I non-II. Subgroup II showed 5 sublineages and there was a correlation between genotypes and sublineages. Even though there was relatively little VP6 sequence data describing different rotavirus subgroups in the GenBank, representative strains clustered with reference strains in each lineage.
6.15 Conclusion

The three broad objectives of this study were achieved:

Firstly, to perform a hospital-based burden of rotavirus diseases in a tertiary and secondary health care facilities in the Pretoria area. The study provided essential evidence of the burden of rotavirus infection in South Africa. Overall, the prevalence of rotavirus infection ranges from 20-24% with an annual ratio of 1:4. Rotavirus infections displayed a distinct seasonal distribution during cooler and drier months of the year, with the highest proportions of (56–59%). The incidence rate of rotavirus diarrhoea was estimated that 5.5% (95%CI 5.1%, 6.0%) of all admissions were due to rotavirus infection. With an estimate of one in every 50 to 70 children less than 24 months of age in these areas were likely to be hospitalized for rotavirus gastroenteritis during the study period. Case fatality rate of rotavirus was estimated to 0.9–1.8% Rotavirus occurred most commonly in children less than 2 years of age (85%), with peak prevalence in age group 3-18 months. As the results, the study provided as a baseline data of rotavirus infection in South Africa.

Secondly, the study was to conduct molecular characterization of rotaviruses circulating in the Pretoria region. The study generated valuable data on rotavirus strains circulating in South Africa that will help to assess the vaccine effectiveness and inform any changes in circulating rotavirus strains in South Africa. The global common rotavirus strains G1P[8], G2P[4], G3P[8] and, G9P[8] or P[6] represented in the study was 62.9% of the rotavirus strains. These strains are included into the two currently licenced rotavirus vaccines. In South Africa, Rotarix® had been introduced from 1 August 2009 as part of the general (EPI) vaccine schedule. The Rotarix® is based on heterotypic protection as a result of cross-reactive antigens between genotypes. We assumed that the Rotarix® will offer 62.9% protection against the most common strains circulating. The challenge will be the uncommon strains representing about 24% of the rotavirus strains circulating in country. It is therefore important to continue with post marketing surveillance,
intussusception and possible other adverse events following vaccine introduction.

Thirdly, the study devised alternative molecular typing method to detect rotavirus VP6 subgroups. The RT-PCR RFLP analysis using restriction endonuclease *RsaI* was found to be a convenient alternative typing method to characterize rotavirus subgroups. The test is highly sensitive, specific, and simple to perform and may be regarded as a rapid diagnostic test for group A rotavirus subgroup.
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MRC DIARRHOEAL PATHOGENS RESEARCH UNIT
Rotavirus General Surveillance Form: Ga-Rankuwa Hospital

LAB NUMBER: I MRC I 2005 I I I I I I I

Basic Data:

Date of first visit: l l l l l l l DATE
Specimen date: l l l l l l l SDATE
Hospital No: l l l l l l l l l l l l l l HOSP_ID
Name: Last: __________________________ First: __________________________ SCNAM
Age (in months): ________ AGE
Date of birth: d d m m y y DOB
Sex: 1. M 2. F SEX

Address: __________________________________________

Weight at study start ________ Kg WEIGHT-S
Length (in cm) ________ cm LENGTH
Temperature ________ °C TEMP

History of current illness:

Please describe your child’s illness

Diarrhoea 1. Yes 2. No DIARRHOE
Diarrhoea with blood 1. Yes 2. No DIARBLD
Fever 1. Yes 2. No FEVER
Vomiting 1. Yes 2. No VOMIT
Cough/cold/difficulty in breathing 1. Yes 2. No AR
Measles/rash 1. Yes 2. No RASH
Other 1. Yes 2. No OTHER
Specify: __________________________
I.1 Duration of symptoms:

2.2.1 Vomiting Number of days
At its worst, how many times per day?
Has it stopped? 1. Yes 2. No

2.2.2 Diarrhoea Number of days
At its worst, how many times per day?
Has it stopped? 1. Yes 2. No

2.2.3 Fever Number of days
Has it stopped? 1. Yes 2. No

2. Feeding:

2.1 Is the child still breast fed?
1. Yes 2. No

2.1.1 If yes, how is the child fed?

2.1.2 If no, how is the child fed?

2.2 Has the child’s sucking increased?

2.3 Has the child’s sucking decreased?

2.4 Is the child mainly eating other food rather than milk?
1. Yes 2. No

2.4.1 If yes, what type of food does the child eat?

2.4.2 What is the child’s desire for foods like?
1. Increased 2. Decreased 3. Stayed the same 4. Not known

3. Treatment:

3.1 Did you take your child to a clinic for this illness?
1. Yes 2. No

3.1.1 If yes what is the name of the clinic?
1. Yes 2. No

3.2 Has the child had any medicine for this illness?
1. Yes 2. No

Antibiotics

Anti-motility (Molemo wa go emisa letshollo)

ORS (Motswako)

Herbal remedies (Molemo wa setso)
Vaccination record:

Does the child have an EPI card?

Record the dates of each vaccination from the vaccination record.
(Put 09/09/00 if EPI card is not seen put 07/07/00 if card is seen but no date to indicate that the particular antigen was given)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Date d</th>
<th>Date m</th>
<th>Date y</th>
<th>BCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>OPV 0</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>OPV 1</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>DPT 0</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>Hib 0</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>HBV 0</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>OPV 2</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>DPT 1</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>Hib 1</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>HBV 1</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>OPV 3</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>DPT 2</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>Hib 2</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>HBV 2</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
<tr>
<td>Measles</td>
<td>1. Yes</td>
<td>2. No</td>
<td>3. Unknown</td>
<td></td>
</tr>
</tbody>
</table>

BCG scar seen 1. Yes 2. No

Has the child been given any other vaccine?

Rotavirus vaccine trial
1. Yes 2. No

Influenza vaccine trial
1. Yes 2. No

Other:

Stool specimen


Mucus present: 1. Yes 2. No 3. Not applicable

Have any family members who live in the same house had diarrhoea in last month?

If yes please give:
Name: _______________________________ Age: __________ Relationship to child: _______________________________

1. Yes 2. No

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8.2 Appendix B

MRC DIARRHOEAL PATHOGENS RESEARCH UNIT
Rotavirus Clinical Surveillance Form

LAB NUMBER: I MRC I 2005 I     I     I     I     I     I  LABNUM

Basic Data:

Date of first visit:  I     I     I     I     I     I  DATE
Specimen date:  I     I     I     I     I     I     I     I     I     I     I     I     I  SDATE
Hospital No:  I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I     I  HOSP_ID
Name: Last: ___________________________ First: ___________________________

Examination: (To be completed by Paediatrician, Registrar or Intern:)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Maculopapular rash:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunken eyes:</td>
<td>1. No</td>
<td>2. Partially</td>
<td>3. Severe</td>
<td></td>
</tr>
<tr>
<td>Decreased skin turgor:</td>
<td>1. No</td>
<td>2. Partially</td>
<td>3. Severe</td>
<td></td>
</tr>
<tr>
<td>Dry mouth:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tears seen:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oedematous:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage dehydrated:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrition status normal:</td>
<td>1. Yes</td>
<td>2. No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Treatment

| Were ORS packets given to take home? | 1. Yes | 2. No |
| Did the child need to be resuscitated? | 1. Yes | 2. No |
| Was the child placed on a drip? | 1. Yes | 2. No |
| Was other medication prescribed? | 1. Yes | 2. No |
| Antibiotics | 1. Yes | 2. No | 4. Not applicable |
| Antipyretics | 1. Yes | 2. No | 4. Not applicable |
| Other | 1. Yes | 2. No |

Specify: ___________________________

Outcome of this visit: 1. Treated at POPD + discharged 2. Rehydrated in Wd 22 3. Admitted to Wds 19 or 23 4. Died

Weight at discharge: ___________________________ Kg