MOLECULAR EPIDEMIOLOGY OF ROTAVIRUSES, ASTROVIRUSES AND ADENOVIRUSES ASSOCIATED WITH GASTROENTERITIS IN CHILDREN IN NORTHWESTERN NIGERIA

BY

AMINU MARYAM
B.SC (ABU) 1991; M.SC (ABU) 2000; PGDE (ABU) 2002
(PhD/Sci/29897/01-02)

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DEPARTMENT OF MICROBIOLOGY, FACULTY OF SCIENCE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

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DECLARATION

I hereby declare that the work reported in this dissertation was carried out solely by me in the laboratory of the MRC/Diarrhoeal Pathogens Research Unit, Department of Virology, University of Limpopo, Medunsa Campus, Pretoria, South Africa, under the supervision of Dr John Dewar of the MRC/Diarrhoeal Pathogens Research Unit, Prof. A.A. Ahmad of the Department of Microbiology and Prof J.U. Umoh of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

This dissertation is an original work I carried out and no part of it has been presented elsewhere for the award of a degree or a diploma. The work of other researchers are duly acknowledged and referred to appropriately.

_____ _____

Maryam Aminu
Department of Microbiology,
Faculty of Science,
Ahmadu Bello University,
Zaria, Nigeria.

Date

CERTIFICATION

This dissertation, entitled "**MOLECULAR EPIDEMIOLOGY OF ROTAVIRUSES, ASTROVIRUSES AND ADENOVIRUSES ASSOCIATED WITH GASTROENTERITIS IN CHILDREN IN NORTHWESTERN NIGERIA" by Aminu Maryam,** meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

_____ _____

Prof. A.A. Ahmad Date
Chairman, Supervisory Committee
Department of Microbiology,
Ahmadu Bello University, Zaria.

_____ _____

Prof. J.U. Umoh Date
Member, Supervisory Committee
Department of Veterinary Public Health and Preventive Medicine,
Faculty of Veterinary Medicine,
Ahmadu Bello University, Zaria.

_____ _____

Dr. S.E. Yakubu Date
Head,
Department of Microbiology
Ahmadu Bello University, Zaria.

_____ _____

Prof. J.U. Umoh Date
Dean, Postgraduate School
Ahmadu Bello University, Zaria.

DEDICATION

This dissertation is dedicated to the memories of my late parents Mallam Mohammed Aminu Idodoh and Mallama Hauwa'u Aminu Idodoh, whom, by the Grace of Almighty God gave me the needed support and encouragement throughout my academic career, and stood by me thereafter until their departure to eternal rest. May their souls rest in perfect peace. Amin.

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ABSTRACT

Rotaviruses are associated with ~ 610,000 deaths worldwide and with 33,000 deaths in Nigeria in children < 5 years of age annually. A study of the epidemiology of rotavirus, astrovirus and adenovirus infection in children 0-5 years old with gastroenteritis was therefore undertaken in Northwestern Nigeria where there is little or no information. A total of 1063 (869 diarrhoeic and 194 non-diarrhoeic) stool specimens were collected from children attending clinics in Kaduna, Kebbi, Sokoto and Zamfara States from July 2002 to July 2004. Viral antigens were detected by enzyme immunoassay (EIA) and the different patterns of rotavirus genomic RNA by polyacrylamide gel electrophoresis. Rotavirus VP7 (G) and VP4 (P) genotyping was carried out by reverse transcription-polymerase chain reaction (RT-PCR). Rotavirus group A antigen was detected in 18% of the diarrhoeic and in 7% of the non-diarrhoeic specimens. Rotavirus infection occurred throughout the study period with slightly higher peaks in the dry season. Children < 2 years old were more vulnerable to rotavirus infection ($p < 0.01$). There were statistically significant associations between vomiting ($p<0.01$), feeding regimen ($p<0.05$) and rotavirus infection. There were, however, no statistically significant associations between sex of the child, type of stool, a child's socio-economic status and parents' level of education, type of drinking water and rotavirus infection.

Sixteen electrophoretic patterns were detected with the 'long' predominating (78%). Three strains exhibited the unusual combination of a VP6 Subgroup I specificity with a 'long' electropherotype. VP6 subgroup I + II strain predominated (36%) in this study. Only 54 (32%) of the 170 rotavirus strains could be assigned a single sero- and genotype. Of these, the predominant strain was G1 P[8] (46.3%). Six (3%) other strains could be assigned a single G or P type. Of the remaining 110 rotavirus strains, 47 (28%) could not be typed and 63 (37%) were mixed infections consisting of 37 different strain combinations. The most prevalent mixed combination was G1G8 P[6] (15.9%). Genotypes G9 and G4 occurred only as mixed infections. The most prevalent VP4 genotype was P[6]. Genotypes P[10] and P[14], detected for the first time in Nigeria as mixed infections, were found in 8.8% and 2.4% of cases respectively.

A further 282 specimens were randomly selected from the 1063 stool specimens and screened for astroviruses and adenoviruses using EIA kits. Astroviruses and adenoviruses were detected in 19% and 22.3% of the specimens respectively. Further analysis of the adenovirus EIA positive specimen detected Ad40/41 in 8% of the specimens. Astrovirus infection was more frequent among children 0-24 months old while adenovirus infection predominated in children 25-36 months old.

The present study indicates that the diversity of strains in Nigeria is much greater than was previously recognized. The detection of novel unusual VP4 genotypes (P[10] and P[14]) and the presence of unusual genotypes and mixed infections in this study underscores the need for a surveillance system for the detection of new rotavirus strains in Nigeria and in Northwestern region in particular. There is a further need to design primers specifically for the rotaviruses in Nigeria.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Diarrhoeal illnesses or gastroenteritis are constantly ranked as one of the top six causes of all deaths, one of the top three causes of death from an infectious disease, and one of the top two causes of death each year in the under-5-year age group in developing countries (Murray and Lopez, 1997a, 1997b; Black *et al*., 2003; Walker *et al*., 2005). The immediate causes of diarrhoeal illnesses are often of an infectious nature and involve a variety of pathogenic microorganisms that include bacteria, parasites and viruses (Estes, 2001a).

Viral diarrhoea is associated with severe disease and hospitalisation in children (Goodgame, 2001) and affects millions of persons of all ages worldwide (Wilhelmi *et al*., 2003). Watery diarrhoea, fever and vomiting are some of the common symptoms of viral diarrhoea that usually persist for more than three days (Belhorn, 1999). The major viral aetiological agents of gastroenteritis in humans are: rotaviruses (*Reoviridae*), astroviruses (*Astroviridae*), adenoviruses (*Adenoviridae*) and caliciviruses (*Caliciviridae*) (Estes, 2001a). The research focused on the first three of the viral pathogens, with particular emphasis on rotavirus, which is clearly the most important virus causing life threatening diarrhoea in infants and young children (Estes, 2001a; Wilhelmi *et al*., 2003; Medici *et al*., 2004; WHO, 2005a). Other viruses, such as the toroviruses, picobirnaviruses, picornavirus (the Aichi virus), and enterovirus 22, may play a role as well (Glass *et al*., 2001).

1.2 Rotaviruses: A Leading Agent of Infantile Diarrhoea

Viral agents of infantile diarrhoea can be ranked in the following order of importance based on literature reports. The least frequently isolated agents are the caliciviruses. The more frequently isolated agents are astroviruses. Second, to the most frequently isolated agents are the adenoviruses. The most frequently isolated agents are the rotaviruses, and researches into infantile viral diarrhoea are concerned more with rotaviruses than any other viral agent (Estes, 2001a; Glass *et al*., 2001).

It has been estimated that out of 130 million children who develop diarrhoea caused by rotavirus each year, 18 million experience moderate to severe dehydration (Institute of Medicine, 1986; WHO, 2005a) resulting in between 352,000-592,000 deaths (Glass *et al*.,

2005; Kapikian *et al*., 2005). Studies published between 1986 and 1999 indicated that rotavirus cause approximately 22% (range 17%-28%) of childhood diarrhoea hospitalizations. From 2000 to 2004, this proportion increased to 39% (range 29%-45%). Application of this proportion to the recent World Health Organization (WHO) estimates of diarrhoea-related childhood deaths gave an estimated 611,000 (range 454,000-705,000) rotavirus-related deaths (Parashar *et al*., 2006) indicating increase in the incidence of rotaviruses worldwide.

Rotaviruses remain major cause of morbidity and mortality in developing countries and are associated with approximately 400,000-600,000 deaths annually in infants and young children under 5 years of age (Miller and McCann, 2000; Medici *et al*., 2004; WHO, 2005a). In epidemiological studies conducted in developing countries, rotavirus accounted for a median of 8% of all diarrhoeal episodes, 28% of outpatients or clinic visits for diarrhoea, and 34% of hospitalizations of young children for diarrhoea (WHO, 2005a). In sub-Saharan Africa, recent estimations show that rotavirus infection is responsible for the death of approximately 110,000-150,000 children under 5 years old annually (Molbak *et al*., 2001; Jain *et al*., 2001; Glass *et al*., 2005). Rotavirus infection is associated with 33,000 deaths in children less than 5 years of age annually in Nigeria (Parashar *et al*., 2003). Nigeria was recently ranked third among the 10 countries with the greatest number of rotavirus disease-associated deaths per year in the same age group (Table 1.1) (Glass *et al*., 2005).

There are quite a number of studies regarding the epidemiology of rotavirus in West Africa (Asmah *et al*., 2001, Akran *et al*., 2002). Some hospital-based studies (Dossetor *et al*., 1979; Paul and Paul, 1986; Oyejide and Fagbami, 1988; Avery *et al*., 1992; Adah *et al*., 1997a; Gomwalk *et al*., 1990, 1993, 2002; Audu *et al*., 2002a; Pennap *et al*., 2002a) have shown rotaviruses to be associated with 3.6%-24.2% of childhood diarrhoea in Nigeria. While some community-based studies (Fagbami *et al*., 1987; Omoigberale *et al*., 1996) have indicated that rotavirus is associated with 5%-7.7% of childhood diarrhoea in Nigeria.

Table 1.1: Global estimates of and the 10 countries with the greatest number of rotavirus disease-associated deaths per year among children <5 years of age.

Country	No. of rotavirus disease-associated Deaths/year	Risk of rotavirus disease-associated death by 5 years of age[a]
India	101,000	1/250
China	35,000	1/540
Nigeria	33,000	1/140
Pakistan	25,000	1/220
Democratic Rep.of Congo	20,000	1/130
Ethiopia	20,000	1/150
Bangladesh	13,000	1/320
Afghanistan	12,000	1/90
Indonesia	10,000	1/450
Tanzania	8,000	1/180
Other Countries	163,000	1/360
Total	**440,000[b]**	**1/250[c]**

[a] Data presented as the No. of death/No. of children. Data were calculated by dividing the no. of children in the live birth cohort for each country by the estimated no. of rotavirus disease-associated deaths/year among children <5 years of age. Data are from parashar *et al*. (2003).
[b] Range 352,000-592,000 death/year.
[c] Range 1/86 to 1/313.

Adapted from Glass *et al*. (2005)

The severity of rotavirus disease could be substantially reduced by vaccination. The development and implementation of safe and effective vaccines to prevent the enormous health burden of rotavirus-associated disease is a global public health goal. Two rotavirus viral proteins (VP), namely VP7 and VP4, which constitute the outer capsid layer and the viral spikes, respectively, are known to induce neutralizing antibodies responses. These antigens confer immunity and are targets for vaccine development. Some rotavirus vaccines strategies are based on the need for a polyvalent vaccine candidate encompassing the four epidemiologically important group A rotavirus VP7 genotypes (G1-G4) in order to protect against rotavirus disease in a type-specific manner. Recent reports from rotavirus surveillance studies have shown an increase in rotavirus strain diversity with the appearance of unusual and the re-emergence of "new" (G8 and G9) genotypes (Ramachandran *et al*., 1996; Cunliffe *et al*., 2000; Iturriza-Gomara *et al*., 2000a; Palombo *et al*., 2000a; Adah *et al*., 2001; Audu *et al*., 2002b).

In Nigeria, some studies (Adah *et al*., 1997a, 2001) have shown that there is always an unexpected diversity among human rotavirus strains (HRVS) isolated. Mixed infection genotypes in most of the children from the northeastern part of the country were also shown, demonstrating the potential for re-assortment events among different rotavirus genogroup in Nigeria (Adah *et al*., 1997a).

Since the discovery of human rotavirus in 1973, several vaccine candidates have been developed and tested in human population in different settings. However immune responses to these vaccines have produced differing results in different regions of the world (Gentsch *et al*., 2005). The reasons for this discrepancy have been attributed to difference in circulating strains, co-infection with other pathogens and differences in the epidemiology of infection in these settings (Vesikari *et al*., 1983; Clark *et al*., 2004).

Currently, however, several new rotavirus vaccine candidates that are based on heterotypic or senotypic specific immunity have been developed (Bresee *et al*., 2004; Gentsch *et al*., 2005) and have completed large-scale clinical trials (Glass *et al*., 2005). These vaccines could show reduced effectiveness in countries where "novel" rotavirus strains are common. The assessment of the efficacy and decision about new rotavirus vaccine introduction will require reliable data on disease impact. Therefore, there is need to have some baseline data on the aetiology of the disease and the epidemiology of infection. Thus detailed and

continuous worldwide epidemiological studies are required to identify rotavirus genotypes that cause severe diseases and to map out changes in strains in different communities (Fischer and Gentsch, 2004). Results can be used to select areas for vaccine trials and to serve as baselines for identification of new strains should they emerge.

Statement of Research Problem

1 In Northwestern Nigeria, there is no published information on the epidemiology of enteric adenoviruses which some studies have implicated as the second most important viral agent associated with gastroenteritis in children.

2. In Northwestern Nigeria, little or no information is available on human astroviruses, which have been identified as one of the viral causes of infantile gastroenteritis. Only one study in Northwestern Nigeria addressing this issue has been published (Pennap *et al*., 2002b).

3. In Northwestern Nigeria, little is known about the circulating strains and the prevalent VP4 and VP7 genotypes of rotavirus, which is the major cause of acute gastroenteritis in infants and young children. To date only one study in Northwestern Nigeria addressing this issue has been published (Pennap *et al*., 2000).

4. Differences in public health infrastructure with respect to potable water availability and proper disposal of human and animal waste have translated into differences in incidence rates in diarrhoea caused by bacteria in developed and developing countries. Higher incidences of bacterial diarrhoea are in developing countries compared to developed countries. However, rotavirus infects all children worldwide with similarity in incidence by the age of five in both developed and developing countries, indicating that disease transmission will not be limited by improvement in sanitation and hygiene. Hence it is generally agreed that vaccines offer the best hope of disease burden reduction, with regards to rotavirus infantile diarrhoea. Effective vaccine development requires knowledge of the prevalent genotypes of rotavirus, since there appear to be geographical compartmentalization of rotavirus genotypes worldwide. There is therefore the need to identify the prevalent genotypes of rotavirus in Northwestern Nigeria.

5. While knowledge of the prevalence of rotavirus genotypes at any given time aid the formulation of a vaccine with local relevance, the dynamics of change in dominance of prevalent genotype with time and the confounding variable of untypeable strains and

unusual G/P strain combinations requires continuous longitudinal studies of circulating rotavirus strains for updating vaccine profile with local relevance. Establishing a database for circulating rotavirus genotypes is therefore essential to give focus to attempt to monitor the genotypes with time.

1.4 Specific Objectives of the Study

This study was therefore designed to address the following objectives:

1. To investigate the epidemiology of rotaviruses in Northwestern Nigeria.
2. To characterize the circulating strains of rotaviruses by molecular methods.
3. To check for the emergence of unusual rotavirus strains.
4. To investigate the epidemiology of human astroviruses in Northwestern Nigeria.
5. To investigate the epidemiology of adenoviruses in Northwestern Nigeria.

1.5 Significance of Study

The study would provide data on the diversity of rotavirus strains required for the design of effective vaccine strategy. This would improve the prospects for effective vaccine development, which should contribute greatly to the global efforts of providing effective rotavirus vaccines of local relevance on a continuous basis. The data obtained would also be useful for future estimation of the disease burden of rotavirus in Northwestern Nigeria and the future management of viral diarrhoea in Nigeria. The study would improve our understanding of the importance of astrovirus and adenovirus considered to contribute less to viral diarrhoea episodes in children.

Reseach Questions

1 What is the prevalence of rotavirus infection in the two studied populations during the study period in Northwestern Nigeria?
2. What is the prevalence and distribution of rotavirus infection by state, sex and age?
3. What is the peak and seasonal variation of rotavirus infection in the region?

4. What are the most common stool type and clinical features associated with rotavirus infection?
5. What is the variation of rotavirus infection by feeding regimen, type of drinking water, and child's socio-economic background?
6. What are the different patterns of rotavirus genomic RNA variants present in the specimens?
7. What are the VP6 subgroup specificities of the rotavirus strains?
8. What are the circulating and novel genotypes of rotavirus in the region?
9. Are there unusual genotypes arising as a result of mixed infections in the region?
10. What are the prevalence of astrovirus and adenovirus infection in the populations studied during the study period in Northwestern Nigeria?
11. What is the prevalence and distribution of astrovirus and adenovirus infection by state, sex and age?
12. What is the peak and seasonal variation of astrovirus and adenovirus infection in the region?
13. What are the most common stool type and clinical features associated with astrovirus and adenovirus infection?
14. What is the variation of astrovirus and adenovirus infection by feeding regimen, type of drinking water, and child's socio-economic background?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diarrhoea

Diarrhoea is generally defined as the occurrence of three or more loose liquid or watery stools or at least one bloody loose stool in a 24-hour period (Gondwe *et al*., 2006). There are three major forms of diarrhoea namely: acute watery diarrhoea, acute bloody diarrhoea and persistent diarrhoea (Guerrant *et al*., 2001). Acute watery diarrhoea is diarrhoea with a high volume of watery stool occurring over a period of less than 14 days. It usually results in severe dehydration if intervention measures are not sought. Acute bloody diarrhoea is diarrhoea manifested by loose or watery stools with the shedding of red blood cells. Persistent diarrhoea is usually associated with loose or watery stools with or without visible blood occurring over a period of 14 days.

2.1.1 Agents of Diarrhoea

The immediate causes of diarrhoeal illnesses or gastroenteritis are often of an infectious nature and involve a variety of pathogenic microorganisms that include bacteria, parasites and viruses (Estes, 2001a).

Among the bacterial pathogens of diarrhoeal diseases, the most commonly implicated in the endemic form of childhood diarrhoea on a global scale are strains of *Escherichia coli* (*E. coli*) (Huilan *et al*., 1991; Iruka *et al*., 2003; WHO, 2005a). At least six groups of diarrhoeagenic *E. coli* are described to cause diarrhoea. These are enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC) (Gunzburg *et al*., 1993). Among these strains of *E. coli*, EPEC is an important cause of infantile diarrhoea in developing countries (Robins-Browne *et al*., 2004; WHO, 2005a), whilst ETEC is the most common recognized causes of diarrhoea in adult. Studies in developing countries have shown that ETEC are present in 15-30% of diarrhoea illnesses occurring in children in the community or in hospital settings (Guerrant *et al*., 1990). In Nigeria, EHEC and EAEC have been shown to be significantly associated with diarrhoea (Iruka *et al*., 2003). Other bacterial pathogens found to be associated with gastroenteritis are, *Campylobacter* (Koulla-Shiro et al., 1995), *Shigella, Salmonella, Yersinia and Vibrio* species (Guerrant and Bobak 1991; Iruka *et al*., 2003).

Three important protozoan species implicated in diarrhoea are, *Entamoeba histolytica, Giardia lamblia and Cryptosporidium parvum* (Mims *et al*., 1999). Among these parasitic causes of diarrhoea, *Cryptosporidium parvum* is increasingly recognised as the major pathogen responsible for parasitic diarrhoea in both developed and developing areas in immunocompetent as well as immunocompromised individuals (Wolfson *et al*., 1985). *Giardia lamblia* is usually associated with diarrhoea lasting longer than ten days. *Entamoeba histolytica* infections occur worldwide but it is most commonly found in tropical and sub tropical countries (Mims *et al*., 1999) where infections are associated with bloody or invasive diarrhoea in which faecal leukocytes are often absent (Guerrant *et al*., 1990; Iruka *et al*., 2003).

The major viral aetiological agents of gastroenteritis in humans are: rotaviruses (*Reoviridae*), astroviruses (*Astroviridae*), adenoviruses (*Adenoviridae*) and caliciviruses (*Caliciviridae*) (Estes, 2001a).

2.1.2 Diarrhoea as a Global Problem

Diarrhoea has long been known as a condition affecting many children all over the world (Mayatepek *et al*., 1993; Hoshino and Kapikian, 2000). However, accurate health statistics remained scarce and it was not until the 1960s that diarrhoeal diseases were identified as a major cause of morbidity and mortality in children less than 5 years of age (WHO, 1998a). Diarrhoea alone or in association with malnutrition constitutes major cause of morbidity and mortality among children throughout the tropical world. Diarrhoea is both a cause and effect of malnutrition according to data from the Gambia and Thailand (Guerrant *et al*., 1992; Rauyajin *et al*., 1994). The major determinants of diarrhoeal deaths are dehydration and malnutrition (Gondwe *et al*., 2005). The magnitude and severity of diarrhoeal disease problem however varies considerably from one continent to another. In the developed world, diarrhoeal diseases do not represent a serious problem either in terms of morbidity or mortality (WHO, 1998a; de Quadros and Santos, 2004). However, they have assumed a very special significance in the developing world, where they are responsible for 15-30% of deaths in children less than 5 years of age (Enzley and Barros, 1997; Ali *et al*., 2000, Lima *et al*., 2000; Kosek *et al*., 2003; WHO, 2005a). The impact of diarrhoea illnesses is more severely felt in sub-Saharan Africa where of the 25 million children born each year, 4.3 million are expected to die by the age of 5 years and about 20% of these deaths will be from

diarrhoea (Cunliffe *et al*., 1998). As a sub-Saharan African country, Nigeria not surprising has an appalling under-five mortality rate. The UNICEF ranked Nigeria in the 2000 as 17th by the under-5-year mortality rate with diarrhoea accounting for 20-26% of all mortality (Black *et al*., 2003; Parashar *et al*., 2003; UNICEF, 2005). More recent figures by WHO have indicated that under-5-mortality have worsened in recent years. The death rate in children <5 years of age was reported to be 200/197 (male/female) per 1,000 live births in 2003 (WHO 2005b).

The disease burden for diarrhoeal diseases is estimated at 62 451 000 Disability Adjusted Life Years (Daly) in 2001 (WHO, 2005a). In some developing countries, children have more than 12 episodes of diarrhoea per year and diarrhoeal diseases account for 15-34% of all deaths. Conservative estimates place the death toll from diarrhoeal diseases at 4 to 6 million deaths per year, with most of these occurring in young children (WHO 2005a). Diarrhoeal morbidity has also been shown to slow growth in children well beyond the weaning age (Torres *et al*., 2000). These suggest that diarrhoea is of great public significance and therefore increased attention should be given to its study in children aged less than 5 years.

2.1.3 Treatment

In most cases of diarrheoa, replacing lost fluid to prevent dehydration is the only treatment necessary. This is because treatment for serious diarrheoal infections is limited due to the fact that there is no effective treatment available. However, antibiotics, anti-protozoan, anti-diarrhoeal and herbal remedies have been used mainly in the developing countries for the treatment of diarrhoea. Until recently, it was not common knowledge that diarrhoea usually run a course unaffected by so-called anti-diarrhoeal drugs or antibiotics, or was it widely appreciated that oral rehydration therapy (ORT) and nutritional support alone could reduce the worst effects of a diarrhoeal attack. Studies in Nigeria (Okoro and Jones, 1995), Pakistan (Kasi *et al*., 1995) and India (Singh *et al*., 1995) have shown that ORT management of diarrhoea as a first line of action is usually followed by treatment with mostly antibiotics, followed by anti-protozoans, anti-diarrhoeal and herbal remedies. However, this is contrary to the recommendation of the CDD programme in paediatrics practice. This is because anti-diarrhoeals interfere with efficacy of other drugs while misuse of antibiotics leads to its resistance (Singh *et al*., 1995). Oral rehydration therapy has been

recognized as a simple, safe and effective treatment for most cases of diarrhoea because it replaces water and salts (Okoro and Jones, 1995).

2.1.4 Control and Prevention

Because diarrhoeal diseases are major contributors to the morbidity and mortality among infants and young children in the developing world, the Global Programme for the Control of Diarrhoeal Diseases (CDD) was establishment in 1980 (Feachem *et al*., 1983). Since then a total of 126 countries worldwide including Nigeria have created national programmes for the CDD (Enzley and Barros, 1997). The main activity of this programme is the development of intervention programs, based on their effectiveness, feasibility and cost effectiveness for the prevention and control of diarrhoea with an ultimate aim of reducing diarrhoea morbidity and mortality. Based on this, a four-part intervention strategy for prevention and control of diarrhoea have been developed and adopted. These four part intervention strategies are; (1) maternal and child health care, (2) interrupting transmission of diarrhoeal agents, (3) case management and (4) immunization.

2.1.4.1 Maternal and child health

Improved maternal and child health care, with particular emphasis on exclusive breast feeding for 4-6 months of life, weaning practices, and Vitamin A supplementation have contributed to a large extent in protecting infants in developing countries against morbidity and mortality due to diarrhoea (Feachem and Koblinsky, 1984a; Mahmood *et al*., 1989). It has been reported that, if breast-feeding is followed strictly, there will be a reduction in diarrhoea incidence by 8-20% and diarrhoea mortality by 24-27% in the first 6 months of life (Feachem, 1986).

Weaning practices such as; washing hands and preparing food in clean places, thorough cooking or reheating of food and using boiling water to prepare formula milk have also helped to reduce the incidence of diarrhoea. Better nutritional status, achieved through weaning educational programmes, has helped to reduce diarrhoea mortality by 2-12% in children less than 5 years (Ashworth and Feachem, 1985; Stephenson *et al*., 1994; Brown, 1997; Ashworth, 1998) Nutritional improvement with Vitamin A supplementation has been reported to reduce diarrhoea mortality in young children by at least 30% (Fawsi *et al*., 1993; Barreto *et al*., 1994).

2.1.4.2 Intervention methods

Sanitation: Improved use and maintenance of drinking water and sanitation facilities, and improved personal and domestic hygiene have all contributed in reducing the incidence of diarrhoea by limiting exposure to diarrhoea pathogens (Feachem *et al*., 1983; Huttly *et al*., 1997). It has been estimated that implementation of water systems including; deep-bore wells, protected spring water and piped water could reduce diarrhoea morbidity by 26%. Also, Enzley and Barros (1997) have reported that evidence from Asia, Africa and Latin America have consistently demonstrated that improvements in water supply can reduce the morbidity and mortality associated with diarrhoea. For instance, a study in Bangladesh found that installation of hand pumps resulted in the reduction of diarrhoea incidence by 27%. In Malawi, a 20% decline in diarrhoea mortality was also observed in households with piped water.

Promoting specific features of personal and domestic hygiene, such as hand washing have also led to a reduction in diarrhoea incidence. A study in Lesotho, Southern Africa found that the combination of ventilated pit latrines, and regular hand washing was associated with a 24% reduction in diarrhoea incidence (Daniel *et al*., 1990). Furthermore, in some studies coducted worldwide, the median reduction in diarrhoea incidence was 35% for hand washing and 26% for severe hygiene behaviours and hand washing (Feachem, 1984; Kaltenthaler and Drasar, 1996).

2.1.4.3 Case Management

Improved case management, with particular emphasis on the early use of ORT in acute diarrhoea and appropriate feeding during illness and convalescence have contributed to the reduction of diarrhoea mortality but have no significant impact on diarrhoea incidence (Feachem *et al*., 1983). The development and use of ORT over the past 25 years has had a huge impact on public health. Oral rehydration therapy has been shown to prevent death from diarrhoeal dehydration in more than 1 million children each year (Gore *et al*., 1992; Bhan *et al*., 1994). It has also been extrapolated that ORT could prevent death from diarrhoeal dehydration in a further 2.5 million children each year if it is made available and used effectively especially in Africa (Bhan *et al*., 1994; Gore *et al*., 1992).

2.1.4.4 Immunization

The diversity of bacterial and viral infections that may cause diarrhoea complicates accurate surveillance and diagnosis, especially in developing countries with little or no access to modern laboratory procedures. The specific disease burden attributable to a particular infectious agent is particularly complex given the multiplicity of these agents, their serotypes, and depends largely on laboratory facilities in endemic areas. If, in the long term, access to clean water, better hygiene, and improvement of sanitary measures would certainly have the greatest impact on diarrhoeal disease, then immunizations against specific diseases are the best hope for the short- and mid-term. This is particularly true for viral diseases such as rotavirus, present in both high and low hygiene level countries (WHO, 2005a).

2.2 Viral Agents of Gastroenteritis

The major viral aetiological agents of gastroenteritis in humans are: rotaviruses (*Reoviridae*), astroviruses (*Astroviridae*), adenoviruses (*Adenoviridae)* and caliciviruses (*Caliciviridae*) (Estes, 2001a). Other viruses, such as the toroviruses, picobirnaviruses, picornavirus (the Aichi virus) and enterovirus 22 may play a role as well (Glass *et al*., 2001).

2.2.1 Caliciviruses

The application of new molecular diagnostic tools has shown that human caliciviruses (HuCVs) constitute two of the four genera within the family *Caliciviridae* (WHO, 2005a; Widdowson *et al*., 2005a). The two HuCV genera are currently called Norovirus and Sapovirus (WHO, 2005a). All the calicivirus strains that infect animals have also been placed under these two genera. The other two genera Lagovirus and Vesivirus naturally only infect animals. HuCVs are small (27-40nm), non-enveloped viruses with a 7.2-8.3Kb, polyadenylated, positive sense, single stranded RNA (ssRNA) genome (Cubitt *et al*., 1995; Atmar and Estes, 2001). The name Calici comes from the Latin word calyx for "cup" describing the 32-cup shaped depressions on the surface of "typical" virions revealed by electron microscopy. These cup-like depressions are more prominent in some strains (particularly SLVs), leading to the characteristic " Star of David" appearance from which caliciviruses get their name (Appleton, 1987).

The genomic organizations of the two HuCVs are distinct from each other. The noroviruses formally called "Norwalk-like viruses" (NLVs) possess three different open reading frames (ORFs), consisting of a 5' ORF encoding for the non-structural proteins of 1738 amino acids with a molecular weight of 193.3K and RNA polymerase, a second ORF encoding for the major capsid protein of 530 amino acids with a molecular weight of 56.6K and a third ORF at the 3' end of the genome encodes a protein of 212 amino acids with a molecular weight of 22.5K (Clarke and Lambden, 1997). In sapoviruses formally called "Sapporo-like viruses" (SLVs), the first ORF codes for the non-structural protein as well as the capsid proteins, whilst the second and the third encode small highly basic proteins (Numata *et al*., 1997).

On the basis of genome analysis of the RNA-dependent RNA polymerase region and capsid protein region and differences in antigenicity, noroviruses have been classified into five genogroups (GI-GV), distinguishable from sapoviruses (Vidal *et al*., 2005; Widdowson *et al*., 2005a) with genogroup II (GII) prevailing in outbreaks worldwide (Gallimore *et al*., 2004; Vidal *et al*., 2005).

Calicivirus particles have been associated with infantile gastroenteritis (Blacklow and Greenberg, 1991) and are associated with the epidemic form of diarrhoeal illness (Girard *et al*., 2005; Widdowson *et al*., 2005b). Studies conducted among hospitalised children and children in day-care centres with diarrhoea in Britain and the US respectively, found approximately 3% of the children excreting calicivirus (Matson *et al*., 1989; Le Baron *et al*., 1990). The incubation period for caliciviral disease ranges from 1-3 days. Vomiting and diarrhoea are common, with upper respiratory symptoms and fever occurring less frequently. Infections in the elderly have also been documented (Le Baron *et al*., 1990; de Wit *et al*., 2001; Vidal *et al*., 2005). Person-to-person transmission of the virus is presumed to be essential for endemicity (Marks *et al*., 2003). Contaminated shellfish (Vidal *et al*., 2005), cold food and drinking water have also been implicated as vehicles (Cubitt, 1988; de Wit *et al*., 2003; Skraber *et al*., 2005).

Although noroviruses may play a role in endemic diarrhoea, diagnostic technology is not yet sufficiently developed to assess this aspect of their disease burden. Studies in the United States of America have shown that illness is most commonly associated with older children of school going age and adults, and they are responsible for ≤50% of all food-borne gastroenteritis (Widdowson *et al*., 2005b). They are also major contributor to illnesses in

nursing homes and hospital (Loopman *et al*., 2004) and are associated with chronic diarrhoea among transplant patients (Widdowson *et al*., 2005a). The few studies in developing countries have shown that antibodies to noroviruses are acquired at an earlier age (Greenberg *et al*., 1979). Recent study in Mongolia has shown 25% of children in the community shedding noroviruses (Hansman *et al*., 2005).

Infections due to noroviruses have an incubation period of 24-48 hours and the mean duration of illness is 12-60 hours. The clinical symptoms include nausea, which is prominent, with vomiting, non-bloody diarrhoea and abdominal cramps occurring in most cases. These symptoms are common in all age groups (De-Wit *et al*., 2001); however, diarrhoea is relatively more prevalent among adults. Infection is transmitted through water, food (shellfish and salads), aerosol, fomites and person-to-person contact (Hansman *et al*., 2005). Infectivity can last for as long as 2 days after resolution of symptoms (White *et al*., 1986). Noroviruses are commonly, resistant to chlorination and freezing and they persist in the environment and require only very low inoculum to infect (Widdowson *et al*., 2005a).

2.2.2 Astroviruses

Astroviruses were first discovered in 1975 from electron microscope (EM) studies of an outbreak of diarrhoea in a maternity unit and a survey of the aetiology of diarrhoea in children in a Scottish city (Appleton and Higgins, 1975). They have been identified by several studies as one of the frequent viral cause of infantile gastroenteritis (Steele *et al*., 1998; Pennap *et al*., 2002b; Mendez-Toss *et al*., 2004). The name "astro" is derived from their distinctive appearance under EM, showing a smooth or slightly indented outer electron dense shell with an inner five or six pointed star shaped core. Astroviruses genome consists of a single-stranded RNA of 6.8 to 7.2 kilo base (Kb) in length. Astroviruses have three open reading frames (ORFs), ORFla, ORFlb and ORF2. The first two ORFs contain amino acid motifs homologous to protease and polymerase proteins, respectively. The third ORF2 is found at the 3' end of the genome and encodes the capsid protein precursor.

Astroviruses are classified within the family *Astroviridae* (Jiang *et al*., 1993; Cubitt, 1996; Glass *et al*., 1996) with non-segmented, non-enveloped particles of size of 27-34nm in diameter. Eight recognized human serotypes (types 1-8) have been shown to affect the gastrointestinal tract of human causing diarrhoea in children, the elderly and

immunocompromised (Lewis *et al*., 1994; Glass *et al*., 1996; Taylor, 2002). Previous studies have shown serotype 1 as the most predominant type in circulation.

Astrovirus infections occur worldwide accounting for about 2-16% of diarrhoea in hospitalised children and 5-17% of diarrhoea in the community (Steele *et al*., 1998; Taylor, 2002; Oh *et al*., 2003; Mendez-Toss *et al*., 2004). They are also commonly associated with mixed infections with other enteric pathogens, particularly rotaviruses (Gaggero *et al*., 1998; Bon *et al*., 1999). There are limited data on astroviruses in Africa. However, Enzyme immunoassay (EIA) base investigations on paediatric diarrhoea specimens reveal prevalence of 2% in children in Kenya (Bos *et al*., 2002), 1.2% in Malawi (Taylor, 2002), 6.7% in Nigeria (Pennap *et al*., 2002b) and 9% in Ghana (Armah *et al*., 2002a).

The viruses have been found to be present in diarrhoeic stool during mild gastroenteritis outbreaks usually in children from infancy to seven years of age (Blacklow and Greenberg, 1991). Studies of hospitalised children have shown an infectivity rate of between 3-5% in all admission for diarrhoea (Le Baron *et al*., 1990). Clinically, these viruses cause similar symptoms to caliciviruses. The symptoms include vomiting, diarrhoea, fever and abdominal pains (Le Baron *et al*., 1990). In the developed world, astrovirus infections occur throughout the year with peaks in the winter months (Grohmann, 1985). The incubation period is between 24 and 36 hours, with illness lasting one to four days. Reports on astroviral outbreaks in Britain and Japan have pointed at institutional settings such as kindergartens (Konno *et al*., 1982), paediatric ward (Kurtz *et al*., 1977) and nursing homes (Gary *et al*., 1987).

Astroviruses are far more common than previously thought (Steele *et al*., 1998; Taylor, 2002; Walter and Mitchell, 2003) and their clinical significances continue to generate interest (Oh and Schreier, 2001). Therefore further epidemiological studies are required to determine the true burden of astrovirus diseases in Africa where there are differences in epidemiology to that of the rest of the world. Unfortunately, little or no information is available on human astroviruses in Northwestern Nigeria.

2.2.3 Adenoviruses

Although, adenoviruses are more often associated with respiratory infections (Rocholl *et al*., 2004), some serotypes, denominated enteric adenoviruses (EAds) are related to diarrhoeal disease. Enteric adenoviruses belong to the *Mastadenovirus* genus in the family *Adenoviridae* and are known to cause respiratory, ocular, and genitourinary infections. They also cause cystitis, diarrhoea, intussusception, meningoencephalitis and epidemic keratoconjunctivitis, (Baum, 2000). To date, 51 human adenovirus serotypes that are divided into six species (A to F) based on their ability to agglutinate various types of erythrocytes are distinguished (Rosen, 1960) and within each serotype there are genetically different strains. Adenoviruses are non-enveloped, regular icosahedral particles with a diameter of 80 to 110 nm (Horne *et al*., 1959). The virions are made up of 13% of DNA and 87% of protein with a 252 capsomeres protein shell surrounding a DNA-containing core (Shenk, 2001). The virion capsid is comprised of 240 hexon proteins arranged in 20 triangular facets on the panels of the icosahedron, 12 penton bases located on the vertices and 12 fibres embedded into the penton bases (Ginsberg *et al*., 1966).

Adenoviruses are often recovered from the stools of young children and are considered to be the second most important viral agent associated with infantile gastroenteritis (Uhnoo *et al*., 1984; Bates *et al*., 1993; Hársi *et al*., 1995; Grimwood *et al*., 1995; Audu *et al*., 2002a; Basu *et al*., 2003; Oh *et al*., 2003). Adenoviruses have been associated with 3.1% to 22.2% of cases of paediatric gastroenteritis in studies from Australia, Europe, and North America (Uhnoo *et al*., 1984; Brown, 1990; Cruz *et al*., 1990; Grimwood *et al*., 1995; Hársi *et al*., 1995). However, detection levels as low as 1.5% to 2.0% has been reported for enteric adenoviruses (species F) in Thailand, Argentina and Malawi (Herrmann *et al*., 1988; Giordano *et al*., 2001; Cunliffe *et al*., 2002b).

Infection is transmitted from person to person (Le Baron *et al*., 1990) and faecal-oral transmission accounts for most infections in young children. However, the isolation of "fastidious" enteric adenoviruses from hospitalised infants suggests that species F adenoviruses can be transmitted by nosocomial infections (Flewett *et al*., 1975; Lin *et al*., 2000). Peak incidence of adenoviral illness occurs among children under the age of two and particularly in the first year of life (Kotloff *et al*., 1989), although older children and adults may be infected with or without symptoms (Le Baron *et al*., 1990). A high incidence of recurrent shedding in stool specimens has been observed (Brown, 1990) and large numbers of completely asymptomatic adenovirus infections occur (Cruz *et al*., 1990). Several studies also indicate that adenoviruses occur throughout the year with no seasonal distribution (Kotloff *et al*., 1989; Cruz *et al*., 1990; Bates *et al*., 1993). However, summer and autumn peaks in adenovirus shedding has been reported in South Africa and the United Kingdom (Kidd *et al*., 1986; Tiemessen *et al*., 1989; Cooper *et al*., 2000).

The adenoviruses most commonly associated with gastroenteritis in young children include those of species F (types 40 and 41) and to a lesser extent, species A (types 12, 18 and 31) (Hammond *et al*., 1987). Established adenoviruses belonging to species A to E have also been associated with diarrhoea (Cooper *et al*., 2000) with clinical symptoms that include watery diarrhoea accompanied by high fever and respiratory symptoms. However species F (types 40 and 41) primarily affect the gut, contributing to 5-20% of hospitalisations for childhood diarrhoea in developed countries (Uhnoo *et al*., 1984). In Nigeria, available studies have associated adenoviruses with 3.8% (Nimzing *et al*., 2000) and 6.7% (Audu *et al*., 2002a) of paediatric diarrhoea.

Clinically the incubation period is between 3-10 days, with illness lasting more than one week (Uhnoo, 1984). The clinical symptoms include watery diarrhoea accompanied by vomiting, low-grade fever and mild dehydration (Le Baron *et al*., 1990). The mean duration of gastric symptoms for Ad40 infection is 8.6 days. Adenovirus type 41 causes more protracted diarrhoea with a mean duration of 12.2 days and is more frequently accompanied by abdominal pain. Reported outbreaks of adenovirus infection involving children have been reported in hospitals and day care settings (Chiba *et al*., 1983).

The EAd is a relatively unknown entity in gastroenteritis epidemiology in Africa with the exception of South Africa where their etiological importance in paediatric gastroenteritis has been established (Steele *et al*., 1990; Moore *et al*., 1998, 2000). However, indications are that the detection of adenovirus in diarrhoeal stools is probably under-reported due to low or lack of specificity of commercially available EIA for all circulating adenovirus strains (Moore *et al*., 1998). This is speculated to be true for most of African adenovirus strains (Moore *et al*., 2000).

2.2.4 Rotaviruses

2.2.4.1 Historical Aspect of Rotavirus

Although infantile diarrhoea or gastroenteritis of viral aetiology had been a global problem, it was not until the 1960s when a viral agent with distinct morphological features was identified by 3 independent group of investigators in the intestinal specimens of mice and monkeys and stools of calves with diarrhoea (Adams and Kraft, 1963; Malherbe *et al*., 1963; Mebus *et al*., 1969). The breakthrough finally came in 1973, when the first human

associated rotavirus was discovered and reported by Bishop *et al*. (1973). The viruses were visualized by electron microscopy in intestinal biopsy specimens and stools of children with acute gastroenteritis. Within a few years of its detection in humans, numerous epidemiological studies soon indicated that rotaviruses are important agents of severe infantile diarrhoea, and responsible for 35-50% of hospitalisations in young children and infants (Kapikian and Chanock, 1990). To date, rotaviruses have been isolated in humans, monkeys, cattle, pigs, sheep, horses, dogs, cats, mice, rabbits and birds (Kapikian and Chanock, 1990; Nakagomi and Nakagomi, 1993).

2.2.4.2 Properties of Rotavirus

Morphology and Structure: Rotaviruses lend their name from the Latin word "rota" which means wheel. Intact rotavirus particles have a distinctive appearance of a well-defined rim, with short spikes radiating from a wide hub when observed by negative stain electron microscopy (EM). The intact virus is made up of 3-capsid structure: an inner core, an intermediate capsid and an outer capsid with short radiating spikes. Rotaviruses posses' dsRNA enclosed in a core structure. Three types of rotavirus particles are recognized under the electron microscope, these are (a) the complete infectious particle, (b) the single-shelled or double-layered particles and (c) the core or single-layered particles.

The Complete Infectious Rotavirus Particle: This is a rotavirus particle with all the three components, core, inner and outer capsid intact. It measures 70nm, non-enveloped and consists of a double-shelled icosahedral protein capsid and an inner core containing 11 double-stranded (ds) RNA segments (Flewett *et al*., 1974). The complete rotavirus particles have also been designated "smooth" particles because the outermost margin of the outer capsid layer has a well-defined, smooth circular appearance when observed under the EM (Rodger *et al*., 1975).

The Single-Shelled or Double-Layered Particles: These are rotavirus particles without the outermost capsid. They measure about 55nm in diameter and are sometimes referred to as "rough" particles because of the absence of the smooth outer layer and the projection of the trimeric subunits of the inner capsid to the periphery giving a circular "bristly" appearance.

The Core or Single-Layered Particles: These are rotavirus particles without both the outer and inner capsids. They measure about 37nm in diameter and appear hexagonal under the EM (Estes, 1990).

Further, studies of the three-dimensional structure of triple and double-layered rotavirus particles by electron cryomicroscopy, computer image processing and X-ray crystallography techniques (Prasad *et al*., 1988; Prasad *et al*., 1990) have revealed much clearer details of the ultra-structure of rotaviruses. These studies have established an icosahedral structure with an overall diameter of 1000Å. The capsid design is built on an icosahedral structure with a triangulation number (T) of 13 lattices. The viral structure has two distinct surface features made up of spikes and channels. The channels are located at the 5- and 6- co-ordinated position of the T = 13 lattice. Three types of channels have been identified. The type I channels are located at the fivefold axes, type II channels surround the icosahedral 5-fold axes, and type III neighbour the threefold axes. Each viral particle has 132 channels. There are 12 type I, 60 type II and 60 type III channels. There are 60 spikes, located at one edge of the type II channels, in each particle. The spikes are approximately 120Å in length with a knob at the distal end. They extend from the smooth surface of the outer shell inward into the virion and interact with inner structures. Removal of the outer layer by using chelating agents exposes the intermediate layer protein, which also has the same T (13 lattice) structure and consists of 260 trimers. These trimers register radially with the outer capsid glycoprotein trimers such that the channels in the outer glycoprotein layer continue into this layer. The intermediate layer protein is made up of two domains; the distal domain with an eight-stranded antiparallel B-barrel structure which interacts with outer layer glycoprotein, and the lower domain that interacts with the inner VP2 layer that is predominantly α-helical (Prasad *et al*., 2001).

2.2.4.3 Physicochemical Properties of Rotaviruses

The infectious double-shelled rotavirus particles have a buoyant density of 1.36g/cm3 in Caesium Chloride (CsCl) and a sedimentation coefficient of 520-530s. The double-layered or single-shelled particles have a density of 1.38g/cm3 with sedimentation coefficient of

380-400s. The density and sedimentation coefficient of the single-layered or the core particle have been determined to be 1.44g/cm3 and 280s respectively (Tam *et al*., 1976; Bican *et al*., 1982). Infectivity of the rotavirus particle depends on the presence of the outer layer and is deactivated by treatment with trypsin (Espejo *et al*., 1981; Dryden *et al*., 1993). The stability of the rotavirus outer capsid glycoprotein depends on the presence and concentration of calcium and is specific for each rotavirus strain (Ruiz *et al*., 1996). Calcium-chelating agents such as ethylene diamine tetra acetic acid (EDTA) that remove the outer capsid proteins (VP7 and VP4) have been shown to destroy the infectivity of the virus (Estes *et al*., 1979). Similarly, chaotropic agents such as sodium thiocyanate or high concentrations of Calcium Chloride disrupt the inner capsid protein inactivating the infectivity of the rotavirus particles (Almeida *et al*., 1979).

Rotaviruses are indefinitely stable at -70°C and show a relatively slow loss of infectivity even at ambient temperature. However, infectivity and haemagglutinating activity are lost by repeated freeze thawing (Estes *et al*., 1979). Because rotaviruses are non-enveloped, they are normally resistant to ether, chloroform and other lipid solvents and have been found to retain infectivity for months at pH ranges of 3-9 and temperature of 4°C (Shirley *et al*., 1981; Weiss and Clark, 1985).

2.2.4.4 Classification

Rotaviruses belong to the family *Reoviridae* that consist of eight other distinct genera: *Orthoreovirus, Orbivirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus and Oryzovirus* (Kapikian and Chanock, 1996). Three of the genera, *Rotavirus, Orthoreovirus and Orbivirus*, infect humans and animals, whilst *Coltivirus* infects insects, rodents and humans, Aquareovirus infect fish, *Cypovirus* infects only insects and *Fijivirus, Phytoreovirus and Oryzovirus* infect plants (Desselberger, 1998a; Nibert and Schiff, 2001).

The members of the family *Reoviridae* are medium-sized viruses with similar structural features. They possess a segmented ds-RNA genome and are all non-enveloped icosahedral viruses with two-protein capsid. They have an average diameter of about 85nm with similar replicative strategies (Nibert and Schiff, 2001).

Rotaviruses are classified by both serological and molecular method. Serologically, rotaviruses are classified into multiple groups (serogroups) based on the VP6 antigenicity,

each of which may contain multiple serotypes. Rotaviruses comprise of seven distinct groups (A to G) (Table 2.1) and members of each group share VP6 antigenic similarities. Human rotavirus (HRV)-associated gastroenteritis infections are predominantly caused by group A rotaviruses, although groups B and C rotaviruses have occasionally been associated with human illness (Kapikian and Chanock, 1996). The group B rotavirus, also known as the adult diarrhoea rotavirus (ADRV) have been detected in outbreaks in China in the 1980s and in patients with diarrhoea in India (Krishnan *et al*., 1999), whilst the group C rotaviruses have a global distribution (Kuzuya *et al*., 1996) and are associated with both sporadic diarrhoeal illness (Rasool *et al*., 1994; Jiang *et al*., 1995) and outbreaks of diarrhoea (Caul *et al*., 1990; Maunula *et al*., 1992).

Only viruses within each group are capable of genetic reassortment. The group antigenic determinants or common antigens are found on most of the structural proteins and probably on some of the non-structural proteins as well. Under the electron microscope the different groups of rotavirus appear the same, however, they diverge genetically.

The VP6 protein also determines classification of rotaviruses based on subgroup specificity. This mode of classification has been used to characterize the antigenic properties of various rotavirus strains in epidemiological surveys. Subgroup classification is based on the reaction of specific monoclonal antibodies to the VP6 protein and so far four subgroups, subgroup I, subgroup II, subgroup I and II and non-subgroup I or II, have been described. Most HRVs belong to either Subgroup I or subgroup II (Estes, 1996; Kapikian and Chanock, 1996).

Table 2.1: Host range of rotavirus antigenic groups.

Rotavirus Group	Hosts or Reservoirs

A	Human, primate, horse, pig, dog, cat, rabbit, mouse, cow, birds
B	Human, pig, cow, sheep, rat
C	Human, pig, ferret
D	Chicken
E	Pig
F	Chicken
G	Chicken

The polyacrylamide gel electrophoresis (PAGE) of genomic RNA has also helped in the classification of the various subgroups. Three groups can be identified by their RNA patterns in PAGE. The 11-segmented RNA segments of group A rotaviruses migrate in clusters 4-2-3-2 bands. The RNA segments of group B and C rotaviruses migrate in clusters

of 4-2-2-3 and 4-3-2-2 respectively (Figure 2.1) (Kapikian and Chanock, 1996). Furthermore the migrating pattern of gene segment 10 further classifies the viral strain into `Long' and `Short' electropherotypes.

Within each group, rotaviruses are again classified into serotypes, which were originally defined by cross-neutralization, using hyperimmune antisera in plaque reduction or fluorescent focus reduction assays. When it was shown that both outer capsid proteins, VP4 and VP7, elicit neutralizing antibodies and that the genes that encode these proteins (gene 4 and 7, 8 or 9) can segregrate independently by virus reassortment, it became necessary to classify rotaviruses by a binary system (Hoshino *et al*., 1985a). Using this system of classification, serotypes determined by use of VP7 are termed G (for glycoprotein) types, and those based on VP4 are termed P (for protease-sensitive protein) types (Estes, 1996). Using neutralization assays with hyperimmune antisera, fourteen rotavirus G serotypes, ten in humans (G1-4, G5, G6, G8, G9, G10 and G12), have been identified (Table 2.2 and 2.3). Rotavirus strains of animals and human origin may fall within the same serotype.

Classifications of VP4 (P-types) serotypes have been hampered by the lack of readily available typing serum or monoclonal antibodies to different VP4 types (Larralde and Gorziglia, 1992). However, with the use of sequence analysis and serologic analysis based on neutralization test the properties of VP4 protein have been studied and at least 20 different genotypes, nine in humans (3, 4, 6, 8, 9, 10, 11, 12 and 14), have been identified, whereas 13 different P-serotypes of VP4 (including subtypes) with eight in humans (IA, 1B, 2A, 3A, 3B, 4, 5, and 8) have been identified (Table 2.4) (Sereno and Gorziglia, 1994; Mphahlele and Steele 1995; Estes, 1996; Koopmans and Brown, 1999).

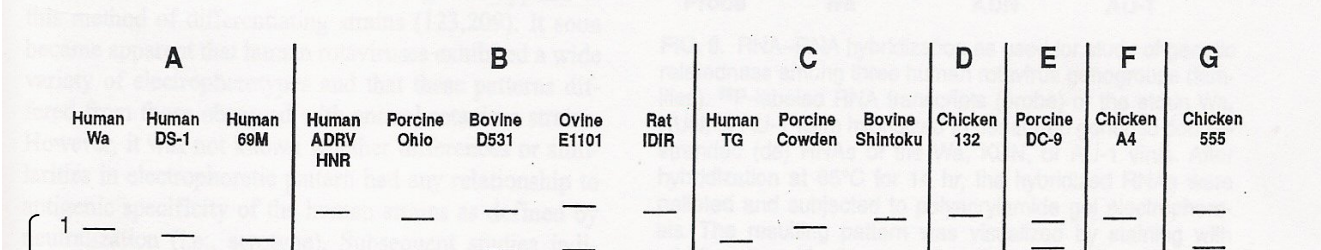


Figure 2.1: Schematic diagram of electrophoretic migration patterns of RNAs of representative rotaviruses belonging to groups A to G

Table 2.2: Serotypic and genotypic classification of human group A rotavirus VP7

Serotype	Strains
1	Wa, KU, D, M37, K8
2	DS-1, S2, KUN, RV-5, 1076
3	P, MO, YO, RV-3, Ito, M, AU-l, McN13, HCR3
4	Hochi, Hosokawa, ST3, ST4, 57M, VA70
5	IAL28
6	PA151, PA169, MG6
8	69M, B37, HAL1166, HMG89
9	W161, F45, 116E, Mc345, US 1205
10	A64, A28, Mc35
12	L26, L27

Modified from Kapikian *et al*. (2001)

Table 2.3: Serotypic and genotypic classification of animal group A rotavirus VP7

Serotype	Strains
1	T449, C60, S7, S8
3	SA 11, SAII-4fm, RRV, K9, H-2, EL,
4	Gottfried, SB-1 A
5	OSU, A34, A46, A134, CC117, S8
6	NCDV, UK, WC3, B-60,
7	Ch2, Ty-1, PO-13
8	678, A5, 26/94, NCDV-cody
9	ISU-64, S8, LRV
10	B223, 61A, A44, CR129, V1005
11	YM, A253
13	L338
14	F123, FR4, CH3

Modified from Kapikian *et al*. (2001)

Table 2.4: Serotypic and genotypic classification of group A rotavirus VP4

Genotype	Serotype	Human rotavirus strains	Animal rotavirus strains

1	6		C486, NCDV, A5, SAI1-4fm,
2	5B		SA11, C13, SAII -S EM
3	5B		RRV, FRV64
	5A	Ro l845, HCR3	K9, CU-1, RS15, Cat97
4	1B	RV-5, DS-1, S2, L26, KUN, E210	
5	7		UK, W C3, B641, 61A678, V1005
6	2A	M37, RV-3, ST3, US 1205	
	2B		Gottfried, BEN-144, S5
7	9		OSU, A821, YM, C134, CC117
8	1A	W a, KU, W 161, Hochi, Hosokawa	S8, LRV
9	3	K8, AU-1, M 318, AU228	FRV-1, Cat2
10	4	69M , 57M	
11	8	116E, 1321	B223, A44, KN-4, CR129
12	4		H-2, 69, 124, EQ431, K311,
13	13		M DR-13A46, C lon8
14	11	PA 169, HAL1166, M c35, M G6	ALA, C-11, BAP-2, Cap455
	11?		R-2
1 5			Ip l4, Ip 16
16	10		Eb, EW, EC
17			PO-13, 993/83, Ch-1, Ty-1, Ty-3
18	12		L338
19			4F
20			EHP

Adapted from Estes (2001b)

2.2.4.5 Molecular Characteristics of Rotavirus

The Rotavirus Genome: Rotaviruses have an 11 segmented gene encoded in the inner core. These genes are separated on polyacrylamide gel as 11 distinct genes with different migrating properties as shown in Figures 2.1. The dsRNA segments are readily transcribed by RNA dependent RNA polymerase into 6 Structural proteins, VPI, VP2, VP3, VP4, VP6, VP7 and 6 non-structural proteins NSPI, NSP2, NSP3, NSP4, NSP5 and NSP6 (Figure 2.2). Some of which are involved in immune reaction and viral replication. The rotavirus particle is made up of multiple protein layers enclosing the genome that consists of 11 discrete segments of dsRNA, contained within the virus core capsid. The dsRNA segments range in size from 667 bp (segment 11) to 3,302 bp (segment 1) in length, with an estimated molecular weight (MW) ranging from $2x10^5$ to $2.2x10^6$ daltons (Estes and Cohen, 1989; Mattion *et al*., 1994).

Nucleotide sequence analyses of all 11 discrete segments of dsRNA of several rotavirus strains have shown general features about the structure of each of the genome segments (Figure 2.2). Each positive-sense RNA strand starts with a 5'-guanidine followed by a set of conserved sequences that form part of the 5'-noncoding sequences. Each of the 11-dsRNA segments contains an open reading frame (ORF) flanked by variable non-coding sequences at both 5' and 3' ends. The lengths of the 3'- and 5'- non-coding sequences vary for different genes and no polyadenylation signal is found at the 3' end of the genes. The first initiation codon, AUG, in all the genes (except VP7 gene) is a strong initiation codon based on Kozak's rules (Kozak, 1986). Some of the genes possess additional in-phase (genes 7, 9 and 10) or out-of phase (gene 11) ORF's and all the genes except gene 11 are monocistronic (Mattion *et al*., 1991). The rotavirus gene sequences are A-T rich (58%-67%) and the ds-RNA segments are based-paired end to end with the positive-sense strand containing a 5' cap sequence m^7 $GpppG^{(m)}GPy$ (McCrae and McCorquodale, 1983). The strong conservation of terminal sequences in genome segments suggests that they contain signals important for transcription, RNA transport, replication, or assembly of the viral genome segments (Patton and Spencer, 2000).

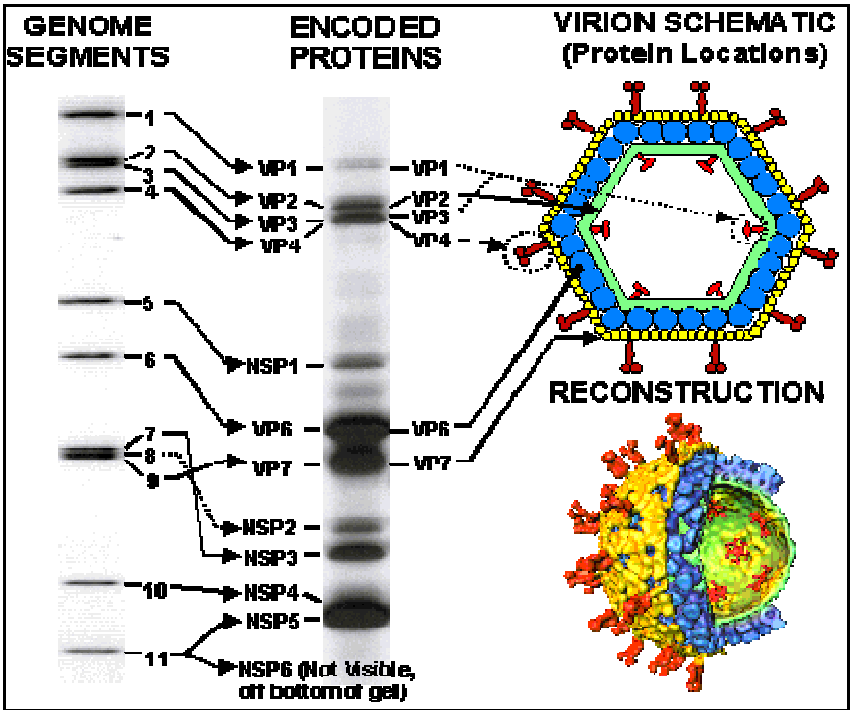


Figure 2.2: Gene coding assignments and virion locations of rotavirus proteins and three-dimensional structure of the rotavirus particle.

Genome segments: Shows separation of the 11 segments of the rotavirus SA11 genome as seen on PAGE.

Encoded proteins: Shows intracellular proteins synthesized by SA11 and genome segment in which they are encoded.

VP (Viral structural proteins) and **NSP** (non-structural proteins).

Source: Mossel, E., Estes, M. and Ramig, F. http://www.iah.bbsrc.ac.uk/dsRNA

2.2.4.6 The Concept of Electropherotype

The 11 ds-RNA segments migrate differently when electrophoresed on polyacrylamide gels. The RNA migration pattern, which is both consistent and characteristic for particular strain or group, defines the viral electrophoretype. The 11 gene segments migrate in four groups or clusters as shown in Figures 2.1 and 2.2 (Kalica *et al*., 1978). Gene segments 1-4 migrate as cluster 1, segments 5-6 as cluster II, segments 7-9 as cluster III and segments 10-11 as cluster IV. The clustering is however different for other rotavirus groups.

Analysis of the electrophoretypes is a relatively easy, rapid and popular technique for rotavirus detection and has been used for epidemiological studies to monitor virus outbreaks and transmission (Rodger *et al*., 1981; Holmes, 1996). It is very objective and has provided the first insights into the degree of variety among rotaviruses. It has been used to delineate changes both within and between annual epidemics in the human populations in studies in some countries (Konno *et al*., 1984).

Despite the extensive diversity in electrophoretypes observed among group A rotaviruses, two distinct RNA migration patterns are obvious. These include the "Short or Super short "and "Long" (the fast-moving bands). These fast moving bands are due to faster mobilities of gene segment 10 and are caused by deletions in the gene. The slowly migrating bands (short or super short) on the other hand represent high molecular weight linked repeats of sections of the genomic dsRNA (Donelli and Superti, 1994).

2.2.4.7 Genetic Variation in Rotaviruses

It has been proposed that new rotavirus strains can evolve by any of these mechanisms: antigenic drift, antigenic shift or genome rearrangement (Coulson *et al*., 1985; Tian *et al*., 1993; Desselberger, 1996; Ramig 1997). These mechanisms as well as the introduction of animal rotaviruses into human population, provide a large pool of genes, which may allow the diversity of rotaviruses to increase.

Antigenic Drift: Antigenic drifts are caused by successive intragenic mutations such as deletions, substitutions and insertions. RNA replication is error prone and hence the rate of mutation within rotavirus genes is relatively high (Ramig, 1997). As is the case of most RNA viruses with segmented genomes, rotaviruses can reassort in doubly infected host cells as long as their genetic relation is sufficiently close i.e. the progeny viruses carry genomic segments from both parent viruses in various combinations. For group A rotaviruses reassortment seems to occur in vivo during circulation under natural conditions. Shuffling of genes can occur and is influenced by the host cell, the immune status of the host and the gene combinations produced (Ramig, 1997).

Genome Rearrangements: Genomic rearrangements or antigenic shift in rotavirus genome have resulted in an open reading frame (ORF) with an extended 3'-UTR (untranslated regions), a truncated ORF or an ORF extended by partial duplication (Desselberger, 1996). In most cases, the rearrangement is due to duplication of portions of RNA gene segment 11. However, it is not clear why genome rearrangement occurs more commonly in gene segment 11 and whether it has a selective advantage (Estes, 1990). Rearrangement has also been documented also in gene segment 5 (Hua and Patton, 1994), segment 6 (Shen *et al*., 1994) and segment 7 (Xin *et al*., 1993). Gene rearrangement can also occur as a result of large genomic concatemerization or deletion. For example, Tian and his group (1993) have observed a deletion as large as 308bp in gene segment 5 resulting in a shorter defective polypeptide.

2.2.4.8 The Concept of Genogroups in Rotaviruses

Genetic relationships among human rotaviruses have been studied by RNA-RNA hybridization under high stringency conditions and have been termed "genogrouping" (Nakagomi *et al*., 1989). Studies of Flores *et al*. (1982, 1985) demonstrated that genomic RNAs from subgroup I human rotaviruses do not form hybrids with the total genomic RNA probes derived from the prototype subgroup II human rotavirus strain Wa (G1 serotype, Long RNA pattern). Similarly, the genomic RNAs from subgroup II human rotaviruses did not form hybrids with the probes prepared from the prototype subgroup I human rotavirus strain DS-1 (G2 serotype, short RNA pattern). However strains belonging to the same subgroup share a high degree of genetic homology as determined by high number of RNA-RNA hybrids. These observations led to the suggestion that there are at least two distinct

"Families" of human rotaviruses, represented by the prototype strains Wa and DS-1 (Flores *et al*., 1985).

Later on, human rotaviruses with subgroup I and those with subgroup II specificity were shown to exhibit a short and a long RNA patterns respectively (Kalica *et al*., 1981). The majority of animal rotaviruses belong to subgroup I, yet they show a long RNA migration pattern. The AU-1 strain isolated in 1982 was the first HRVS found to belong to subgroup I, but exhibiting a long RNA electropherotype typical of animal rotaviruses (Nakagomi *et al*., 1987). Soon after, it became apparent that other human rotaviruses shared properties similar to those of the AU-1 strain (Steele *et al*., 1988; Gerna *et al*., 1990a).

Molecular analysis by RNA-RNA hybridization assays revealed that the AU-1 and some of these similar strains are much more genetically related to each other than to human rotaviruses belonging to groups represented by either the DS-1 or WA "Family" (Nakagomi *et al*., 1989). On the basis of these molecular observations, the AU-1 strain was designated to represent the AU-1 "genogroup" instead of the AU-1 "family", so that rotavirus classification conforms to the taxonomic hierarchy (Nakagomi *et al*., 1989). By definition, the term genogroup suggests that this classification is a genetic parallel to the serologic classification (subgroups and serotypes) of rotaviruses (Nakagomi *et al*., 1989). Members of a genogroup share a high degree of genetic relatedness but have significantly less genetic homology with members of other genogroup (Nakagomi and Nakagomi, 1993). These studies have extended the earlier observations of Flores *et al*. (1982, 1985) and to date three distinct genogroups have been established among human rotaviruses represented by the prototype strains Wa, DS-1 and AU-1.

Different genogroups have been identified among other animals' namely porcine, bovine and canine (Midthun *et al*., 1987; Nakagomi *et al*., 1989). Genetic reassortment between members of the Wa and the DS-1 genogroup to form inter-genogroup reassortants has also been described. This type of inter-genogroups reassortments could occur between rotaviruses derived from different species (Ward *et al*., 1990).

2.2.4.9 Gene Coding Assignments of Rotavirus

Gene coding assignments have been completed for several strains of rotaviruses and have been determined by in-vitro translational studies using mRNA or denatured dsRNA (Dyall-

Smith and Holmes 1981; Mason *et al*., 1983), and by analysis of reassortant viruses (Greenberg *et al*., 1983; Liu *et al*., 1988). The gene coding assignments, the properties and locations of the proteins encoded by each of the 11 segments are shown in (Figure 2.2 and Table 2.5). Although the gene coding assignments of the genome have been described for a number of rotavirus strains, only the proteins of simian rotavirus strain SA-11 have been studied in detail because of the easy propagation of virus in cell culture (Kapikian and Chanock, 1996). These studies indicated that the 11 dsRNA segments code for six structural and six nonstructural proteins (Prasad *et al*., 2001). The proteins have been designated viral proteins (VP) followed by a number designating the gene segment. Proteins generated as a result of cleavage of a larger precursor are indicated by an asterik. For instance, the VP4 protein is cleaved to produce two subunits, VP5* and VP8* (Estes *et al*., 1981). Similarly the non-structural proteins are designated as NSP, followed by a number. The non-structural proteins, which were formally called NS53, NS34, NS28, NS26, and NS20, are now known as NSP1 (encoded by gene 5), NSP3 (gene 7), NSP 2 (gene 8), NSP4 (gene 10) and NSP5 and NSP6 (both encoded by gene 11) respectively (Gonzalez *et al*., 1998).

Table 2.5: The dsRNA segments and proteins of simian rotavirus A/SAll

[a]Genome segment	Genome size (bp)	ORFs	Gene products	Location in virus particle	Copy number/ Particle	Proteinsize amino acid (Da)	Functions and Properties
1	3302	18-3285	VIP(Pol)	Inner capsid, 5-fold axis	<25	1088 (125005)	Possess a fully conservative RNA-dependent RNA polymerase
2	2690	16-2662	VP2	Inner capsid	120	880 (102431)	Inner capsid structural protein, non-specific RNA-binding activity
3	2690	49-2557	VP3 (cap)	Inner capsid, 5-fold axis	<25	885 (98120)	Guanylyltransferase, basic protein, Methyltransferase, part of virion transcription complex
4	2362	9-2340 - -	VP4 VP5* VP8*	Outer capsid spike	120	776 (86782) 529 (247-776) [60000] 247(1-247) [28000]	Form outer capsid spike protein, Virus infectivity enhanced by trypsin cleavage of VP4 into VP5* and VP8*, Hamagglutinin, cell attachment protein, P-type neutralization antigen, VP5* permeabilities membranes
5	1611	30-1518	NSP1	Nonstructural	0	495 (58654)	Associate with cytoskeleton, two conserved cysteine-rich zinc-finger motifs, RNA binding
6	1356	23-1217	VP6 (T13)	Middle capsid	780	397 (4816)	Major virion protein subgroup, antigen.
7	1049	25-976	NSP3	Nonstructural	0	315 (34600)	Specifically binds 3, end of rotavirus mRNA, binds elF4G1, Involved in translational regulation
8	1059	46-1000	NSP2 (VIP)	Nonstructural	0	317 (36700)	Nonspecific ssRNA-binding, involved in viroplasm formation, NTPase activity. Bind NSP5 and VPI, regulates NSP5 autophosphorylation.
9	1062	48-1029	VP7	Outer capsid glycoprotein	780	326 (37368)	Outer capsid structural glycoprotein G-type neutralization antigen, N-linked high mannose glycosylaton and trimming, RER transmembrane protein, cleaved signal sequence
10	751	41-569	NSP4	Nonstructural	0	175 (20290)	Enterotoxin, receptor for budding of double layer particle through ER membrane, Ca++/Sr++ binding site
11	667	20-618	NSP5	Nonstructural	0	198 (21725)	Interacts with NSP2 and NSP6, O-linked glycosylation, binds ssRNA, autocatalytic kinase activity.
		80-355	NSP6	Nonstructural	0	92 (11012)	Interacts with NSP5, localizes in the viroplasm

[a]Segment number based on migration of SA11 genome segments in SDS-PAGE gel.
[b]Protein structure and function: RNA polymerase = A (Pol), capping enzyme = A (CAP), Inner virus structural protein with T = 13 symmetry = A (13), viral inclusion body or viroplasm matrix protein = A (VIP).
Source: Mossel, E., Estes, M. and Ramig, F. http://www.iah.bbsrc.ac.uk/dsRNA

2.2.4.10 Rotavirus Structural Proteins

2.2.4.10.1 Core and Inner Capsid Structural Proteins

VP1: The VP1 is a viral protein enclosed by the inner layer. The protein VP1 is encoded by genome segment 1 and constitutes only 2% of all virion protein (Estes and Cohen, 1989). VP1 is a relatively hydrophobic and slightly basic protein and has a high sequence homology with the RNA polymerase of other RNA viruses. It has been shown to be the RNA-dependent-RNA polymerase for rotaviruses (Cohen *et al*., 1989). The VP1 does not have a known important structural function, but it may be part of an enzymatic complex (Estes and Cohen, 1989).

VP2: The VP2 protein forms the inner layer of rotavirus enclosing VP1 and VP3 (Estes, 1996). It is encoded by genome segment 2 and is the most abundant structural protein found in the core particle (Bican *et al*., 1982) and the third most abundant protein in the double-shelled particles. The protein is known to be highly immunogenic, and it been found that serum antibodies to this protein are a good indicator of prior infection (Conner *et al*., 1988). The VP2 has also been shown to possess nucleic acid binding activity in RNA overlay-protein blot experiments (Boyles and Holmes, 1986). Sequence analysis of this protein shows that the gene codes for one open reading frame (ORF) of 880 amino acids and the NH_2-terminal half of the protein exhibits two stretches of sequences containing leucines at every seventh position from amino acids 536 to 665 (Leucine Zipper). This section of the protein is been reported to bind to DNA or dsRNA. The VP2 protein is myristilated (Clark and Desselberger, 1988).

VP3: This inner capsid protein is encoded by genome segment 3. It is a minor structural protein and sequence analysis of the gene indicates that this segment codes for a protein of 835 amino acids with a predicted molecular weight of 98,120 daltons. In addition the deduced amino acid sequence indicates that it is a basic protein that contains multiple repeats of amino acids. The protein has a high homology with RNA polymerase from other viruses and hence is thought to be involved in RNA replication (Liu *et al*., 1988; Estes and Cohen, 1989).

2.2.4.11 Intermediate Capsid Structural Protein

VP6: The VP6 protein is encoded by gene segment 6 and is located on the intermediate capsid of the double-shelled particles (Kapikian and Chanock, 1996; Rao *et al*., 2000). It has a molecular weight of 44, 816 dalton and constitutes 51% of the virion protein and is the most abundant antigenic component of the rotavirus virion.

Biochemical characterization of the VP6 protein has revealed that it is a trimer located on the surface of viral particles (Prasad *et al*., 1988). This protein when synthesized in the absence of other viral proteins forms tubules and trimers (Estes and Cohen, 1989) and is associated with viral RNA transcriptase activity, although it has no polymerase activity itself. The removal of VP6 protein from single-shelled particles yields core particles that are transcriptionally inactive. Reconstitution of the core with purified VP6 protein restores the transcriptase activity (Kohli *et al*., 1992). However, it is not known whether the VP6 protein plays a structural or functional role in transcription (Sandino *et al*., 1986) although it is known to help maintain the conformation of the viral core structure (Clark and Desselberger, 1988).

The VP6 protein is highly antigenic and immunogenic and it is the most frequently targeted protein in diagnostic assays for detection of rotavirus particles (Estes and Cohen, 1989; Kohli *et al*., 1992). It contains common epitopes shared by other group A rotaviruses, as well as subgroup antigens (Svensson *et al*., 1988). The VP6 protein contains two distinct domains. The first domain is made up of common group-specific epitope shared amongst all rotaviruses of a given group. This group-specific antigenic site is located between amino acid residues 48 and 75 (Kohli *et al*., 1992). The second domain, which has been defined serologically by ELISA, complement fixation, immune adherence hemagglutination assay and immune electron microscopy (Taniguchi *et al*., 1984) is antigenically polymorphic and called the subgroup antigens. These epitopes are the targets for antigenic classification of rotavirus subgroups.

Monoclonal antibodies to the VP6 antigen have been generated (Greenberg *et al*., 1982), and extensively used in epidemiologically studies of both humans and animal rotaviruses (Steele and Alexander, 1988). Subsequently, a lot of studies have been conducted globally to determine the subgroup specificities of rotavirus strains from different locations. The use of VP6 protein as an epidemiological marker was questioned when strains of rotavirus reacting with both subgroup specific monoclonal antibodies and the common VP6

monoclonal antibody were detected among human and horses in Japan. Also, it has been demonstrated that both subgroup II and subgroup I antigens reside on the intermediate capsid protein of these strains (Urasawa *et al*., 1990). Steele and Alexander (1988) also speculated that both epitopes exist on all rotaviruses and that monoclonal antibodies used to differentiate subgroup specificity possibly reacted with overlapping conformational determinants formed by interaction with other viral proteins. Currently, four subgroup specificities have been recognized These are; subgroup I (found mostly in animals and rarely in human rotavirus strains), subgroup II (found mostly in humans porcine and Lapine rotavirus strains), subgroup I and II (found in murine rotavirus strains but rarely in humans and animals) and neither subgroup I nor II (found in avian rotavirus strains but rarely in humans and animals).

Gorziglia *et al*. (1988a) clearly showed that there are 2 distinct "families" of rotavirus, when deduced amino acid sequences of VP6 protein of all four different rotavirus subgroup were compared. The gene segment 6 was found to encode a protein of 397 amino acids in all strains except the H-2 (non I/II) strain, which encoded a protein of 399 amino acids. Region A (amino acid 45-56), and region C (amino acid 114-120) was speculated to define subgroup I specificity, while regions B (amino acid 83-92), D (amino acid 312-319) and E (amino acid 341-352) were thought to define subgroup II epitope. Monoclonal antibodies specific for VP6 epitopes shared by all rotaviruses reacted with monomeric and trimeric forms of VP6, while monoclonal antibodies for subgroup I or II epitope react only with the trimeric form of VP6, suggesting that the subgroup antigenic specificity is determined by conformational epitopes produced by the folding of VP6 or the interaction between VP6 monomers. Comparisons of the amino acid sequences revealed more than 90% amino acid conservation between subgroup I and subgroup II rotaviruses. A high degree of homology (95% or more) was found between subgroup I and non I or II rotavirus strains and it was also found that subgroup I and II rotavirus strains were more closely related to subgroup II strains. The results indicated that common epitopes were continuous determinants while subgroup specific antigenic sites were dependent upon interaction between VP6 monomers for their mature configuration (Gorziglia *et al*., 1988a).

The subgroup II human rotaviruses have been found to possess a long electropherotype and the subgroup I strain, short electropherotype. However, there have been reports of the

isolation of several subgroup I rotaviruses with long electropherotypes from human (Nakagomi *et al*., 1985; Steele and Alexander, 1988).

2.2.4.12 The Outer Capsid Structural Proteins

Two viral proteins, VP7 and VP4 make up the outer capsid. VP7 is the major neutralization antigen and the VP4 is the minor neutralization antigen (Haffejee, 1995).

The VP7 protein: Apart from being the major neutralization antigen of rotavirus recognized by hyperimmune antiserum, VP7 protein serves as the basis for identification of rotaviruses serotypes. It is a glycoprotein containing one to three potential sites for N-linked glycosylation, and has a molecular weight of 34, 000 dalton. It constitutes 30% of the virion protein and forms the smooth external surface of the outer shell of the rotavirus. This makes the VP7 the second most abundant rotavirus protein and major constituent of the outer capsid. Being the external surface protein, the VP7 has been shown to be responsible for attachment of the virus to the host cells (Estes and Cohen 1989; Kapikian and Chanock, 1996).

Comparisons of the deduced amino acid sequence of the VP7 protein encoded by the seventh, eight or ninth gene (depending on the rotavirus strain) from a number of animal and human strains has revealed interesting and potentially biologically relevant features about the VP7 protein. The entire VP7 protein consists of 1062 nucleotides, with two potential ORF beginning with an initiation codon AUG at nucleotide positions 49-51 or 136-138 and with a stop codon UAG, at nucleotide positions 1027-1029 (Taniguchi *et al*., 1988). It is estimated that the ORFs have a coding capacity for a VP7 protein of 297 or 326 amino acids. Each of which begins with an initiation codon with a weak consensus sequence. The second in-frame initiation codon with strong consensus sequence lies 30 codon downstream of the first. Each of these first two initiation codons precedes two regions of hydrophobic amino acid (Hl and H2), which can act as the signal sequence to direct VP7 to the endoplasmic reticulum. A third initiation codon found 42 nucleotides downstream from the second hydrophobic region have also been reported in some strains (Kouvelos *et al*., 1984). Consistent with this view, two bands of the VP7 protein could be seen in some studies after polyacrylamide gel electrophoresis (Chan *et al*., 1986).

Several sets of VP7-serotype specific monoclonal antibodies have been developed and incorporated in ELISAs for the detection of rotavirus serotypes (Padilla-Noriega *et al*., 1990). To date more than 24 neutralizing monoclonal antibodies to human rotavirus has been developed, 15 of these antibodies are serotype specific and are directed to each of the four common human rotavirus serotypes (G1, G2, G3, and G4) and 7 to other rotavirus serotypes (Taniguchi *et al*., 1987).

The sequence of VP7 rotavirus genes of several human and animal serotypes has been determined (Glass *et al*., 1985; Gorziglia *et al*., 1986; Green *et al*., 1987; Coulson and Kirkwood, 1991). Comparisons of these sequences have identified divergent regions in the protein. All together, six serotype-specific regions have been identified. These six discrete divergent regions corresponding to amino acids, 39-50, 87-101, 120-130, 143-152, 208-221, and 233-242 respectively have been designated antigenic regions A, B, C, D, E and F. Furthermore, amino acid homology has been documented to exist in the six discrete regions among similar rotavirus serotypes, but differs considerably among members belonging to different serotypes. The regions encompassed by amino acid 87-99, 145-150 and 211-223 have been deduced as neutralization epitopes. Furthermore, the serotype of a given rotavirus isolate could be predicted from the sequence of two of these divergent regions (Gouvea *et al*., 1990a).

VP4 Protein: The VP4 protein is the second outer capsid protein and is encoded by the RNA segment 4. It is a non-glycosylated protein and has a molecular weight of 88,000 daltons. It constitutes about 1.5% of the total virion protein (Liu *et al*., 1988). Because of the initial failure to identify a protein product from RNA segment 3, the RNA segment 4 product was called the VP3 protein. However, it was renamed VP4 when a protein was identified for the gene 3. The VP4 protein is present as a series of sixty short spikes protruding from the smooth surface of the VP7 protein (Figure 2.2). Each spike is about 12nm in length and l0nm in diameter and possesses a knob-like structure at the distal end to which antibodies bind (Prasad *et al*., 1988). It is an important neutralizing antigen and is associated with restriction of growth of certain rotavirus strains in tissue culture cells and can enhance plaque formation in the presence of protease. In addition, it is an important determinant of virulence or pathogenicity in human (Burke and Desselberger, 1996; Estes, 1996).

The entire VP4 protein is protease sensitive and is cleaved by proteolytic enzymes, such as trypsin into two polypeptides components, VP5* and VP8*. The VP5* portion has a molecular weight of 60,000 daltons, while the VP8* portion has a molecular weight of approximately 28,000 daltons (Espejo *et al*., 1981). The two-trypsin cleavage sites in human rotavirus VP4 molecule are at arginine 240 or arginine 246 (Gorziglia *et al*., 1988b). The cleavage of the VP4 protein has been implicated in the enhancement of viral infectivity (Espejo *et al*., 1981), by enhancing penetration of the virion into the cells of the host, but not the rate of attachment of the virus to the cells (Kalijot *et al*., 1988). The VP4 protein is involved in stimulating neutralizing antibody and inducing protective immunity to rotavirus challenge (Girard *et al*., 2005). A complete nucleotide sequence of the fourth gene which encodes the outer capsid VP4 protein of several human symptomatic and asymptomatic rotavirus strains has revealed that the VP4 gene is 2359 base pairs in length and possesses 5'- and 3'-non-coding regions of 9 and 25 nucleotides respectively. There is one VP4 initiation codon (AUG) at the nucleotides 10-12 which conforms to Kozak's consensus sequence for a strong initiation codon (Kozak, 1986; Gorziglia *et al*., 1988b).

In HRVS, the VP4 gene possesses a single long ORF of 2325 bp that is capable of coding for a protein of 775 amino acids. Consequently, the VP8* subunit of certain human rotaviruses is one amino acid longer, that is 241 instead of 240 amino acids after proteolytic cleavage. Two distinct hypervariable regions have been described in VP4, one at amino acid 71-208 in VP8* subunit and the other at amino acid 559 and 617 in the VP5* subunit (Lopez *et al*., 1991; Isa and Snodgrass, 1994). This hyperdivergent region has been used to distinguish the different gene 4 alleles of rotavirus strains by sequence analysis (Qian and Green, 1991) and probe analysis (Larralde and Flores, 1990, Steele *et al*., 1993b). The VP8* subunit possessed the major antigenic sites at amino acids 87-89, 100, 115, 148-150 and 188 and are responsible for serotype specificity, while the VP5* possessed sites responsible for cross-reactivity between variety of VP4 proteins at amino acids 305, 388, 392, 433 and 439 (Larralde *et al*., 1991).

Larralde and Gorziglia (1992) further analysed the VP8 * subunit and three different peptides were expressed. All three peptides were analysed by immunoblot assays. They later demonstrated that peptide B (amino acids 84 to 180) contained VP4 serotype and subtype specificities and that, Peptide A (amino acids 1 to 102) and peptide C (amino acids 150 to 246 from VP8* and amino acids 247 to 251 from VP5*) showed cross-reactivity

between the different VP4 proteins. In conclusion, they suggested that serotype and subtype specificities were located in a region covered by residues 102 to 149 of VP8* subunit.

2.2.4.13 Non-Structural Proteins

These are proteins present only in infected cells but absent in the mature particles (Liu *et al*., 1988). Six non-structural proteins have been detected in rotaviruses. These are NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6 and are coded by gene segments 5, 8, 7 or 8 or 9 (depending on rotavirus strain), 10, and 11 (codes for both two proteins) respectively (Prasad *et al*, 2001).

NSP1 (NS53): This is a non-structural protein encoded by genome segment 5 and has a molecular weight of 53 000 daltons (Kapikian and Chanock, 1996). The protein has been found in infected cells but not in virus particles. The sequence analysis of gene 5 revealed that NSPI had two regions at amino acid positions 42-79 and 315-328 characteristic of zinc fingers. The amino-terminal half of NSP1 has been shown to display conserved cysteines ..and histidines among all rotavirus strains, although the entire sequence shows extensive sequence diversity (Mitchell and Both, 1990).

Alignments of 17 NSP1 sequences revealed that only 88 amino acid positions (17.6%) were absolutely conserved among the rotavirus strains (Xu *et al*., 1994). On the basis of deduced amino acid sequence, the NSP1 is a basic protein and has three basic regions (amino acids 10-39, 79-93, 111-126) with a net positive charge of 9 at pH 7.0 (Bremont *et al*., 1987). Nucleic acid hybridization studies also indicated that the nucleotide sequence of the NSP1 protein may be least conserved (Schroeder *et al*., 1982) and rearrangements occurring within gene 5 can alter the ORF such that the NSP1 product contains a c-terminal truncation, a duplication or deletion (Tian *et al*., 1993, Hua and Patton, 1994). With such results, Okada *et al* (1999) concluded that the NSP1 is not essential for virus replication. So far, the largest of the sequence duplications and deletions that have been described are 1112 and 500 nucleotides long respectively and occur in gene 5 (Hua and Patton 1994; Okada *et al*., 1999). The biological function of NSP 1 is currently not known.

NSP2: The NSP2 protein has a molecular weight of 35000 daltons (Kapikian and Chanock, 1996). It is a basic protein coded by gene segment 8 and has been shown to localize in the

viroplasms (Petrie *et al*., 1984; Fabbretti *et al*., 1999), which are proposed sites of genome replication. The NSP2 has been suggested to play a role in RNA replication or packaging.

Due to the presence of a helix-turn-helix motif in NSP2, it has been suggested to interact with nucleic acids (Estes and Cohen, 1989), and shown to have affinity for single-stranded RNA and dsRNA in a sequence-nonspecific manner. Comparison of several mammalian virus NSP2 sequences revealed that they exhibited high levels of amino acids conservation of >83% (Patton *et al*., 1993). In addition, a basic and conserved region was detected at amino acid 205-241 and it is suggested to be an RNA-binding domain. Conserved cysteines were also found at sites as 6, 8, 85 and 285 (Patton *et al*., 1993). The first 75 nucleotides in the 5'-end and the last 28 nucleotides in the 3'-end were found to be nearly identical among the mammalian rotavirus sequences and were suggested to be able to produce a stem double-loop structure, which is thought to serve as a packaging signal for the assortment of NSP2 mRNA into replicase particles (Aponte *et al*., 1996).

NSP3: It is a 37 000 daltons molecular weight non-structural protein encoded by genome segment 7, 8 or 9 depending on the virus strain (Kapikian and Chanock, 1996). For example, genome segments 8, 9 and 7 encode SA11, RRV and bovine UK virus respectively (McCrae and McCorquodale, 1982; Gombold and Ranug, 1986). The deduced amino acid sequence indicates that the NSP3 protein is basic and is localized in association with viroplasms in infected cells (Petrie *et al*., 1984). NSP3 has been detected in subviral particles isolated from infected cells and characterized to possess replicase activity (Helmberger-Jones and Patton, 1986). The NSP3 protein is an RNA-binding protein, which specifically recognizes the 3' translation enhancer sequence (Poncet *et al*., 1994). It has also been shown by the two-hybrid system and by pull down assays to interact with eIF4G1 (Piron *et al*., 1998). Therefore, NSP3 can be considered to be a functional homologue of PABP (poly-A-binding protein) in that both specifically binds to the 3' end of mRNAs and both have affinity for a cap-associated initiation factor. Based on these properties, NSP3 has been proposed to catalyse the circularization of rotavirus mRNA within polysomes, thereby increasing translation efficiency (Patton, 2001).

Rao *et al*. (1995) reported that there is a relatively conserved amino acid sequence with identity of over 75% among mammalian NSP3s. The lengths of the 3' non-coding regions varied among rotaviruses, but a stretch of 80 nucleotides in the 3'-untranslated region was

highly conserved in the NSP3 gene among the 13-rotavirus strains compared. In addition, a conserved basic region amino acid 81-150, in the N-terminal half of the protein has been proposed as the RNA-binding domain of NSP3.

NSP4 (NS28): The NSP4 protein is a non-structural endoplasmic reticulum-specific glycoprotein encoded by genome segment 10 and has a molecular weight of 28,000 daltons (Estes, 1996). The NSP4 protein functions as a viral enterotoxin capable of inducing diarrhoea in young mice. It has been suggested that NSP4 may be a key determinant of rotavirus pathogenicity and could be a target for vaccine development (Lee *et al*., 2000). Sequence analysis of the rotavirus enterotoxin NSP4 from human and a limited number of animal rotavirus strains revealed the presence of four distinct genetic NSP4 alleles represented by NSP4A (genetic group I or DS-1 allele), NSP4B (genetic group II or Wa allele), NSP4C (genetic group III or AU-1 allele), and NSP4D (genetic group IV or EW allele) (Cunliffe *et al*., 1997; Horie *et al*., 1999; Kirkwood and Palombo, 1997; Ciarlet *et al*., 2000). NSP4 has been shown by several researchers to serve as an intracellular receptor for double-layered rotavirus particles and interacts with viral capsid proteins during viral morphogenesis (Au *et al*., 1989; Tian *et al*., 1995). A peptide consisting of 21 amino acids (aa), 114 to 135 on NSP4 has been shown to trigger a signal transduction pathway that increases intracellular calcium levels in cells by mobilizing calcium from the endoplasmic reticulum, thus stimulating chloride secretion (Tian *et al*., 1994; Dong *et al*., 1997). Furthermore, amino acid changes in this region have been associated with alterations in the toxigenic activity of NSP4 and virulence of rotavirus (Zhang *et al*., 1998).

The 175aa backbone of NSP4 consists of an uncleaved signal sequence, three N-terminal hydrophobic domains and a predicted amphipathic a-helix that overlaps a folded coiled region (Taylor *et al*., 1996). NSP4 also possesses a membrane distabilization activity when incubated with liposomes that stimulate endoplasmic reticulum (ER) membranes, and it has been hypothesized that this activity may play a role in the removal of the transient envelope from budding particles (Tian *et al*., 1996). Also, NSP4 may facilitate cell death and virus release from cells (Newton *et al*., 1997).

In addition, polarized cells treated with NSP4 undergo reorganization of filamentous action and lose transepithelial cell resistance due to alterations in tight junctions (Tafazoli *et al*., 2001). The NSP4 protein possesses the ability to stimulate net secretion in intestinal

segments in the absence of inducing histological changes, thus confirming its functions as an enterotoxin (Estes *et al*., 2001).

Deduced amino acid sequence analyses of virulent and avirulent pairs of viruses detected sequence changes in the NSP4 gene in aa residues 136 and 138 and expressed NSP4 with these mutations shows reduced enterotoxin activity (Zhang *et al*., 1998). These results also indicated that the enterotoxin domain extends beyond as 114-135 and help explain why the initial NSP4 114-135 peptide was not as active as the full-length protein (Horie *et al*., 1999).

The most recent finding on the NSP4 enterotoxin peptide is that it is secreted from rotavirus-infected cells (Levy *et al*., 2000; Zhang *et al*., 2000). The secreted cleavage product of NSP4 represented amino acid 112-175 and when purified was shown to increase intracellular calcium mobilization in intestinal cells, and hence was able to cause diarrhoea in neonatal mice (Levy *et al*., 2000). Cleavage of NSP4 was observed only when infections were carried out in the presence of trypsin. Also cross-linking studies revealed that NSP4 functions as oligomeric forms, mainly as tetramers (Maass and Atkinson, 1990). Finally, it has been reported that antibody to NSP4 can induce protection against disease caused by infection with either simian or a highly virulent murine rotavirus. Protection consists of a reduction in the number of pups that develop diarrhoea, the severity of disease and the duration of the diarrhoea.

NSP5: NSP5 is a non-structural protein coded by segment 11 in the rotavirus SA-11 strain. The protein is hydrophilic and rich in serine and threonine (Ward *et al*., 1985). It is the only rotavirus segment, in which more than one ORF has been found (Mitchell and Both, 1988). It has two overlapping ORFs; the longer codes for the NSP5 protein of 198 aa and the out-of-phase ORF (+1) codes for the protein NSP6 of 92aa (Petrie *et al*., 1984). Ericson *et al*. (1982) further reported that in addition to the 26 K-dalton product of NSP5, a 28 K-dalton product is related to it by protease mapping. Gonzalez and Burrone (1991) found the NSP5 to be modified by addition of single O-linked residues of N-acetylglucosamine. Additional forms of NSP5, varying between 32-34 K-dalton, have been found. Treatment of these different forms of NSP5 with phosphatases produced a single band of 26 K-dalton, indicating that the level of phosphorylation affected migration of the protein in gel (Afrikanova *et al*., 1996). The forms of NSP5 with the highest molecular weight were more

heavily phosphorylated and appeared to contain very little or no O-glycosidic residues (Afrikanova *et al*., 1996; Poncet *et al*., 1997).

Results suggesting the presence of autophosphorylation activity associated with NSP5 were obtained from in-vitro experiments with NSP5 expressed in *E. coli* (Afrikanova *et al*., 1996; Poncet *et al*., 1997). Despite the autophosphorylation of NSP5, its fully phosphorylated form has not been detected in expression systems. Since the localization of NSP5 in infected cells is different from that of the *in-vitro* expressed NSP5, correlation between the localization of NSP5 in viroplasms and its protein kinase activity has been proposed. The necessity of cellular or viral cofactors was suggested but it is unclear whether cellular protein kinases also participate in NSP5 phosporylation (Poncet *et al*., 1997). The protein from the out-of-phase ORF of NSP6 has been shown to be expressed in rotavirus-infected MA104 cells using monospecific sera prepared against NSP6 (Mattion *et al*., 1991). The existence of multimerization site located at the C-terminus of NSP5, which forms a helical structure, has been reported and the binding sites of NSP6 for NSP5 protein were found to overlap with the multimerization domain (Torres-Vega *et al*., 2000). Since phosphorylation was poor in mutants with deletion in the C-terminus, multimerization was proposed to be important for the activity of NSP5, and NSP6 was suggested to play a regulatory role in its self-association (Torres-Vega *et al*., 2000). Afrikanova *et al* (1998) reported that NSP5 could be chemically cross-linked with VP1 and NSP2 in living cells. In transient transfection assays, NSP5 phosphorylation was enhanced by co-expression of NSP2. A 33-aa N-terminal deletion mutant was shown to become phosphorylated, but to be insensitive to NSP2 activation, suggesting that NSP2 may bind to the N-terminus of NSP5 (Afrikanova *et al*., 1998). Both the N- and C- terminal domains of NSP5 were found to be essential for viroplasm formation when NSP5 deletion mutants were expressed in combination with NSP2 (Fabretti *et al*., 1999).

2.2.4.14 Genome Rearrangements of NSP5 (gene 11)

Several genome rearrangements of gene 11 have been reported, and they group roughly into two types of rearrangements: either a partial duplication of the ORF region or an AT-rich insertion in the 3'- non-coding region. The longer segment 11 has been easy to visualize by its slower migration in gel, while it is normally the fastest moving RNA segment of rotavirus. The RNA patterns occurring with rearranged segment 11 are called either short or super-short electropherotypes in contrast to the normal long electropherotypes.

Observations common to all rearrangements are that one complete ORF region is preserved and normal NSP5 protein appears to be produced (Mitchell and Both, 1988). Rearrangements with duplications contain a complicated ORF region in a head-to-tail orientation following the normal ORF region, with the exception that it lacks bases from the 5' end including the AUG initiation codon and this is non-coding. This type of rearrangement was found in swine rotaviruses (Gonzalez *et al*., 1989), the rabbit rotavirus Alabama strain (Gonzalez *et al*., 1989), a bovine rotavirus (Scott *et al*., 1989), the human rotavirus strain Mc323 (Kojima *et al*., 1996) and a rotavirus strain isolated from a chronically infected immunodeficient child (Palombo *et al*., 1998).

The following mechanisms for rearrangements have been proposed: rotavirus RNA-dependent RNA polymerase pauses during RNA synthesis due to the secondary structure of the template or other cause. The RNA polymerase dissociates from the template or slips back along it, reattaches to the same template without releasing its nascent transcript and reinitiates transcription to the normal 3'- end of the RNA (Gorziglia *et al*., 1989; Scott *et al*., 1989, Kojima *et al*., 1996). Nearly identical sequence of six bases has been found adjacent to the reinitiating site and the recombination site in rearranged segment 11. The orientations of these direct repeats indicated that the rearrangement had occurred at the stage of plus-strand synthesis (Kojima *et al*., 1996). The short electropherotypes of human rotaviruses represent the rearrangement type of gene 11 in which the 3' non-coding region contains an AT-rich insert (Nuttal *et al*., 1989; Giambiagi *et al*., 1994). The AT-rich region (148-158 bp) is highly homologous among the strains, although it is not similar to any other rotavirus gene sequence. The short electropherotypes are stable circulating strains that usually show VP6 subgroup specificity of subgroup I, VP7 serotype of G2 and VP4 serotype of P[4], although exceptions have been found.

2.2.5 Epidemiology of Rotaviruses

Rotavirus infection is primarily a disease of infants and young children but both symptomatic and asymptomatic rotavirus infection can affect neonates (Haffejee, 1991; Bhan *et al*., 1993; Brussow *et al*., 1993) and adults (Soenarto *et al*., 1981; Hrdy, 1987).

2.2.5.1 Neonatal Rotavirus Infections

Neonates generally have asymptomatic or very mild infections and clinical illness affects less than 10-20% of infected babies (Haffejee *et al*., 1990). Rotavirus infections in nursery

are common because of the stability of the virus. Studies have shown that rotavirus is quite resistant to the common detergents and have been found in nurseries for approximately two years (Grillner *et al*., 1985). The reasons for the asymptomatic nature of most neonatal rotavirus infections are far from being clear. However, transplacentally acquired maternal serum antibodies, anti-rotavirus antibodies and trypsin inhibitors in breast milk, immature pancreatic enzymes in the neonates and the inherently attenuated nature of the "nursery" rotavirus strain have all been implicated in the asymptomatic characteristic of neonatal rotavirus infection (Hoshino *et al*., 1985a; WHO, 1988a; Brussow *et al*., 1993). Although the immature neonatal proteolytic enzymes are not able to cleave the VP4 of virulent rotavirus strains, thereby rendering them infective, they do permit the nursery strains to replicate at a rate slow enough to produce diarrhoea (Haffejee, 1995).

Studies in Australia, Bishop *et al*. (1979), have shown that the nature of neonatal rotavirus infection appears to be related to the type of nursery in which the neonates are residents. Asymptomatic infections were mostly common where room-in with the mother was practiced. Whereas in the case of communal nurseries and especially special care units, clinical illness was more common and directly related to close proximity to other neonates and frequency of handling by adults. Circulating strains in neonatal nurseries are fairly consistent (Rodger *et al*., 1981) as compared to changes in community strains, which have been found to have periodicity of 2-3 years (Grillner *et al*., 1985). In studies in Australia, 93% of asymptomatic neonates in nurseries excreted serotype G3 between 1974 and 1986, whereas the dominant rotavirus causing clinical illness in the same city belonged to serotype G1 and to a lesser extent, serotype G4 (Bishop *et al*., 1991). Also, neocleotide sequence analysis revealed that the VP7 and VP4 genes of the nursery rotaviruses were distinct from those of the strains circulating in the community (Lee *et al*., 2001). This finding supports the hypothesis that endemic nursery infection may influence the epidemiology of rotavirus serotypes in the same community (Bishop *et al*., 1991). Whilst generally neonatal infections are asymptomatic, they can be life threatening in some cases. Severe cases of neonatal illness with dehydrating diarrhoea, abdominal distension, necrotizing enterocilitis and even death have been reported (Bryden *et al*., 1982).

2.2.5.2 Rotavirus Infections in Infants and Children

Rotaviruses are ubiquitous and 95% of children worldwide are infected by three to five years of age (Parashar *et al*., 1998). Because natural infection confers some immunity to

disease and protection increases with each subsequent exposure, the highest rates of rotavirus disease occur early in life, between three months and two years of age (Kim *et al*., 1989; Raul Velazquez *et al*., 1996; WHO, 2005a). Infection can however occur earlier in regions where exposure is intense (Kapikian and Chanock, 1990). Haffejee (1995) reported that infants in the developing countries tend to acquire the infection much earlier than their counterparts in the developed countries.

Although it is well known that breast-feeding does not necessarily protect against rotavirus diarrhoea, the highest incidence of rotavirus diarrhoea generally coincides with the peak period of weaning (Haffejee, 1995). However, some other studies have found the peak incidence at 6-12 and 9-14 months (Haffejee and Moosa, 1990; Huilan *et al*., 1991). In Argentina, a peak was found in the less than 6 months old (Muchinik *et al*., 1981). In the United States, 13% of children admitted to the hospital because of rotavirus gastroenteritis are younger than 3 months of age (Matson and Estes, 1990). In Bangladesh, the peak incidence of rotavirus disease occurs before the age of one year, when almost all infants are still being breast-fed (Black *et al*., 1982; Crawley *et al*., 1993). Sequential infections occur in all age groups. They can be caused by both the same serotype as the original infection or a different one (O'Ryan *et al*., 1990).

2.2.5.3 Rotavirus Infections in Adults

Rotaviral diarrhoea has been reported in 11-70% of infected adults (Hrdy, 1987). These high rates of clinical illness are probably related to differences in rotavirus virulence, hygiene and sanitation (Hrdy, 1987). Rotavirus is now a common etiological agent of diarrhoea in immunocompromised individuals and is usually associated with severe disease. Outbreaks of rotavirus infection have also been reported in closed adult communities like geriatric wards, nursing homes and cardiology wards (Cubitt and Holzel, 1980; Holzel *et al*., 1980).

Rotavirus associated diarrhoea traced to fecal contamination of water sources have also been reported from USA and China (Hopkins *et al*., 1984; Fang *et al*., 1989). In these situations, the attack rates were higher in adults than in children, possibly due to the inoculum effect (adult tend to consume larger quantities of contaminated water). Group A and B rotaviruses caused the nationwide outbreak in China. However in the Chinese epidemic group B rotavirus infected only adults further highlighting the difference in

infection from group A rotavirus infections, which infect predominantly children. The epidemic was characterized by both asymptomatic and severe symptomatic infection, with rapid person-to-person spread, suggesting that emphasis on preventing waterborne transmission may not control the introduction of group B rotavirus into new cases (Fang *et al*., 1989).

Many investigators have implicated rotaviruses to be the cause of travelers' diarrhoea. The infection rate is approximately 25%. Asymptomatic rotavirus infections also occur among tourists. Since much of the diarrhoea afflicting visitors to tropical or subtropical countries are associated with enterotoxigenic Escherichia coli, the rotavirus may not be the most important cause of travellers' diarrhoea but may instead play a role in the spread of infection (Ryder *et al*., 1981; Smith *et al*., 1983).

2.2.5.4 Nosocomial Rotavirus Infections

Nosocomial diarrhoea is diarrhoea acquired at least 3 days after being admitted in the hospital and is an important cause of childhood morbidity and mortality. Rotavirus is one of the most common causes of nosocomial infection especially in pediatrics hospitals (Ford-Jones *et al*., 1990) and it has been estimated that about one of every five-rotavirus infection appeared to be hospital-acquired. Several studies have demonstrated the frequent occurrence of nosocomial rotavirus infections in hospital wards (Oelofsen *et al*., 1985; Gaggero *et al*., 1992; Steele *et al*., 1993a; Abiodun and Omoigberale, 1994). Although nosocomial infections are usually subclinical, nosocomial outbreak of rotavirus gastroenteritis have been reported in newborn nurseries in Italy (Gerna *et al*., 1990b) and among pediatric cardiology patients in the United States (Ratner *et al*., 2001). Nosocomially acquired rotavirus infections have also been reported to be associated with severe diarrhoea in adult renal transplant recipients (Peigue-Lafeuille *et al*., 1991). Rotavirus strains isolated from nosocomially-infected newborns have been electrophoretically identical to those from toddlers and older children (Vial *et al*., 1988).

2.2.5.5 Seasonality

Rotavirus infections display a distinct seasonal pattern in temperate climates with epidemic peaks occurring predominantly in the cooler winter months of the year. The monthly peaks may vary from country to country and from year to year (Parashar *et al*., 1998). The seasonal nature of rotavirus infections is not universal and in countries with tropical

climates, rotavirus disease have been reported to occur year round, with seasonal peaks occurring during the dry cool months of the year (Armah *et al*., 1994). These different seasonal patterns observed in tropical and temperate settings may lead to differing patterns of transmission and perhaps to different reservoirs of infection. In addition, it may also have an influence on the age of infection seen in the different climatic settings. Rotavirus infection is predominantly seen in older children (> 12 month) in temperate countries compared to the younger age (< 6 months) in tropical countries (Armah *et al*., 1994; Molbak *et al*., 2001). The seasonality observed in temperate countries may result more from the occurrence of unfavourable conditions for transmission of rotavirus in the summer months, which are characterized with higher ambient temperatures, low relative humidity and less crowding. In tropical settings the risk of exposure to rotavirus infection is higher due to the presence of all the conditions that favour the transmission of rotavirus. In a nutshell, the mechanism underlying this seasonal variation is unclear but involves a variety of factors such as survival of virus in the environment, physiological effects on the host and level of crowding of susceptible children (Bishop, 1996).

2.2.5.6 Transmission of Infection

Rotavirus diarrhoea is reported to be contagious and transmission occurs rapidly within a susceptible community. The fecal-oral route has been reported as the most likely mode of spread of rotavirus infection (Parashar *et al*., 1998). However, it must be noted that evidence for this mode of spread of rotavirus infection is largely circumstantial and not conclusive. Evidence from other studies seems to suggest that close physical interpersonal contact is important for both symptomatic and asymptomatic infection in families (Grimwood *et al*., 1983). A person with rotavirus diarrhoea may excrete approximately 1 trillion infectious particles/millilitre of stool. Furthermore, since the infective dose in a child can be as few as 10 particles (Ward *et al*., 1986), person-to-person transmission probably perpetuates endemic disease. Galil and co-workers (1986) reported a closed outbreak in Kibbutz, Israel in which the rotavirus infection spread serially to other infants, mothers, nursery staff, adolescents, and fathers in that order. This was a direct proportion to the degree of close contact with the index cases.

Another mode of rotavirus spread that has been implicated is the respiratory route (Cook *et al*., 1990). Evidence for this is circumstantial and rests on the following:

- The rapid acquisition of rotavirus antibodies in the first few years of life in all settings, regardless of hygienic standards (Elias, 1977).
- Few large outbreaks in which fecal-oral spread could not be documented (Foster *et al*., 1980).
- The occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis (Gurwith *et al*., 1981).

For example, during two outbreaks, one in an American Indian reservation (Santosham *et al*., 1985) and the other in a group of pacific Islanders (Foster *et al*., 1980), the infection spread rapidly with limited personal contact, in each situation. The affected population groups were widely separated geographically. Each had its own water supply, and there was little or no contact between the various groups. Attempts to isolate rotavirus from respiratory secretions in patients with rotavirus diarrhoea have so far given inconsistent results (Goldwater *et al*., 1979). There have been scattered reports of detection of rotavirus in the upper respiratory tract. One such report is where the study subjects had pneumonia and no gastroenteritis (Santosham *et al*., 1983). However, the predominance of data from other studies suggests that this is not the usual mode of transmission (Wright *et al*., 1991; Zheng *et al*., 1991).

Resistance to physical inactivation may contribute to the efficient transmission of the human rotaviruses. This conclusion is drawn from the observed stability of various human and animal rotaviruses at ambient temperatures (Ansari *et al*., 1991; Fischer *et al*., 2002). Other observations that also suggest environmental contamination as a source of infection include: the persistence of rotavirus infection in certain newborn nurseries and the high frequency of nosocomial rotavirus infection in hospitals (Kapikian and Chanock, 1996).

The ability of rotaviruses to survive on various surfaces under different conditions may also contribute to the rapid spread of these agents (Wilde *et al*., 1992). Although rotaviruses have been detected in raw or treated sewage, Smith and Gerba (1982) reported that it is unlikely that contaminated water plays an important role in transmission of group A rotaviruses. Also, high relative humidity of approximately 80% results in an increase loss in human rotavirus infectivity (Ansari *et al*., 1991). Effective disinfection of contaminated material and careful hand washing constitutes important measures to contain rotavirus infection, especially in a hospital or institutional setting (Vaughn *et al*., 1986; Wilde *et al*.,

1992). The spread of rotaviruses from animals to human have been speculated and this speculation has been intensified by the following observations:

- Certain animal rotaviruses share a neutralization antigen with human rotaviruses and
- Certain naturally occurring animal rotavirus strains may infect humans (Nakagomi *et al*., 1990; Nakagomi *et al*., 1992; Nakagomi and Nakagomi, 1993; Taniguchi *et al*., 1994). However, this type of interspecies transmission appears to be a rare event.

2.2.5.6.1 Interspecies Transmission and Intergenogroup Reassortment

The diversity of rotavirus strains around the world is due to the genetic reassortment occurring between human and animal rotavirus strains and this can lead to the generation of new strains. Nakagomi and Nakagomi (1996) made two major observations during genogrouping of a large number of human and animal rotaviruses. Firstly, they observed that, in some instances, the genogroup was conserved between the strains from different host species. Strains of different host species are linear and were not shown to exhibit any significant homology. This was hence taken as evidence of interspecies transmission of rotaviruses. Similarly, the observation that some genomic RNA segments are similar to genomes of specific genogroups was considered an evidence of intergenogroup reassortment.

In 1989, Nakagomi and co-worker, first reported evidence which exemplified the sharing of a genogroup between human rotaviruses belonging to the AU-1 genogroup and feline rotavirus FRV-1, thus supporting molecular evidence of interspecies transmission of rotavirus strains. However, Iizuka *et al*. (1994) further observed that, rotavirus strains belonging to the AU-1 genogroup were not limited to the AU-1 and FRV-1 isolates alone, but more strains were being included with the improvement of molecular techniques and assays.

2.2.6 Clinical Features

The clinical symptoms of rotavirus infection range from mild to severe and occasionally, fatal dehydrating illness if not treated (Kapikian *et al*., 2001). It has been reported that rotavirus diarrhoea is frequently associated with dehydration (El Assouli *et al*., 1995), vomiting and fever (Mangia *et al*., 1993). Where death occurs, the main cause is believed to

be dehydration and electrolyte imbalance. Kapikian and Chanock (1996) noted that dehydration is 14 times more frequent in children with rotavirus diarrhoea than among those with the disease from other causes. Matson (1994) also observed that rotavirus is more likely to cause severe dehydration than other viral gastroenteritis pathogens. In contrast, Unicomb *et al*. (1997) observed that children infected with rotavirus were more likely to have watery stools and severe vomiting, but less dehydration than children infected with other pathogen.

In their work, Nishimura *et al*. (1993) observed chronic diarrhoea associated with prolonged viral shedding in immunodeficient children. The authors also found the virus in the serum and spinal fluid of patients with gastroenteritis and convulsion. In 1992, Gilger *et al*. demonstrated the viral antigens in sections of the liver and kidney at a postmortem. Respiratory symptoms have been associated with the viral infection but, apparently, are not caused directly by the virus (Mangia *et al*., 1993). Dodet and associates (1997) noted that in developed countries it just coincides with the peak of respiratory infections.

Mangia *et al*. (1993) in a study in Brazil reported the most common clinical features as dehydration (54%), vomiting and fever (50%), vomiting (32.6%), fever (13%), respiratory infection (13%), bloody diarrhoea (2.1%). Another study in Poland by Kolakowska *et al* (1993) reported the significant association of rotaviral infection with pharyngitis and rhinitis. Further laboratory investigations showed a greater tendency of rotavirus positive diarrhoea for hypokalaemia, metabolic acidosis and white blood cells count of below 10,000. These features were observed in 77.5% of the patients. Offit (1996) observed that rotavirus infection usually resolve within the first week of the onset of symptoms where the fever lasts for 3 days, vomiting 2-3 days, and diarrhoea 5days. Rotavirus has been reported in Japan, to be characterized by a milky white stool (Nagaoshi *et al*., 1980). Reports from Cairo showed the preponderance of watery stools, a high frequency of vomiting and respiratory symptoms (El Mangia *et al*., 1989) while in Malawi it is likely to produce a yellow watery loose stool (Pavone *et al*., 1990). However, in a recent development, Dodet *et al*. (1997) observed vomiting as one major difference between rotavirus and bacterial diarrhoea.

In neonates, as reported by Haffejee in 1991, the infection is associated with a large spectrum of clinic manifestations like severe diarrhoea, necrotizing enterocolitis, bowel

perforation, abdominal distension and even death. Rotaviruses can also lead to chronic symptomatic infection or serious illness and prolong shedding of the virus in immunodeficient children such as bone marrow transplantation patients (Wood *et al*., 1988).

2.2.7 Diagnosis

The clinical manifestations of the illness are not sufficient to allow for diagnosis. Therefore, it requires the detection of virus or viral antigen and/or demonstration of a serologic response (Estes, 1996b). The accurate diagnosis of rotavirus infection is important not only for rapid identification of the patient with rotavirus diarrhoea, but also for the identification of potential sources of infection (Dennehy *et al*., 1999). The most common specimen used in diagnosis is the stool, although the virus has also been found in sera, cerebrospinal fluid and throat swabs (Ushijima *et al*., 1994). The antibody response is not routinely determined for diagnostic purposes as it may provide only a retrospective diagnosis. However, some studies of the antibody response in serum, saliva, duodenal fluid, and feaces have shown that specific lgM and lgA antibodies in serum may occur in response to infection. They usually show patterns comparable to those measured in intestinal secretions and so may give a reliable indication of recent infection (Gerna *et al*., 1990). Feacal specimen from day 1 to day 4 of illness is ideal for viral detection and maximum rotavirus excretion occurs on the third and fourth day from onset of the disease (Middleton *et al*., 1977).

The most commonly applied method for viral detection as reported by Olive and Sethi (1989) are electron microscopy (EM), enzyme immunoassay (EIA) techniques such as enzymes linked immunosorbent assay (ELISA) and polyacrylamide gel electrophoresis (PAGE). For the EM, the specimen is stained with phosphotungstic acid (PTA) and examined directly. This permits the viral detection in 80-90% of positive specimen. It has the advantage of high specificity and is therefore frequently used as a confirmatory test when discrepancies occur with other techniques. However, the method of choice in most laboratories is ELISA because it is highly sensitive, does not require specialized equipment, and has a built in control for non-specific reactions. They are available commercially as ELISA kits (Dennehy *et al*., 1999).

Various electrophoretic profiles of the virus are obtained by the use of PAGE. It can show both inter group and intra group polymorphisms. In this method, developed by Herring *et al*. (1982), a 10% polyacrylamide gel is silver-stained after electrophoresis because silver

ions form a stable complex with nucleic acids. The virus if present will appear showing the 11 bands corresponding to the RNA segments.

Fernandez *et al*. (1992) reported the use of dot blot hybridization based on in situ hybridization of labeled ssRNA transcript on heat denatured viral RNA immobilised on a nitrocellulose membrane as more sensitive than ELISA. The individual laboratory determines which method to adopt considering its needs on the basis of patient population, pricing and technical requirement (Dennehy *et al*., 1999).

2.2.8 Pathogenesis

Although the virus has a limited tissue tropism and the outcome of infection is age related, it is highly infectious and replication can occur with as little as 10 TCID 50 in the upper small intestine. This is followed by a release of infectious particles (> or $=10^{10}$/g) found in feaces (Bishop *et al*., 1996).

In their review of several studies on rotavirus pathogenicity, Burke and Desselberger (1996) noted that it all depends on viral and host factors like dose, capacity to spread in specific cell populations, capacity to cause damage, hosts' presence of viral receptors, age, immune status (i.e. memory from previous exposure), nutritional status and genetic make up. They also noted that the virus infects mature enterocytes in the mid and upper villus epithelium of the host's small intestine, although they have also been demonstrated in goblet cells, epithelial endocrine cells, regional lymph nodes and macrophage in the lamina propria. Hall *et al*. (1993) observed that in calves, low pathogenicity rotavirus causing sub-clinical infections show preferential colonization. They are usually found in the proximal small intestine, while the high pathogenicity strains causing diarrhoea infect the whole small intestine and show a difference in their rates of replication and cytopathogenesis. They suggested that with the pathogenic strain, the ability to replicate rapidly may outstrip the ability of the intestine to replace damaged cells, thus the disease condition (diarrhoea).

Similarly, Burke and Desselberger (1996) reported that in gnotobiotic piglets, the pathogenic strain was excreted 1 or 2 days earlier than the apathogenic strain. This further gave credence to the hypothesis that the rate of replication may affect pathogenicity. They also noted that apathogenicity of nursery strains has not been fully proven because of host factors like the effect of maternal antibodies and difference in intestinal structure or function in neonates has not been ruled out as contributory factors. Steele *et al*. (1995)

noted that these antibodies could be passively acquired via breast milk or transplacentally. In their contribution Dodet *et al*. (1997) reported less gastroenteritis in newborns as a result of fewer viral receptors on intestinal cells and reduced protease concentration in the intestinal lumen resulting in reduced VP4 cleavage (i.e. host factors that help select for virulence).

There are also speculations that interspecies transmission and gene reassortment among rotaviruses could have led to the emergence of strains with reduced pathogenicity. For example, the VP4 of asymptomatic human neonatal viral stains are most closely related to that of the porcine Gottfried strain (Gorziglia *et al*., 1990) and that of bovine B223 strains (Gentsch *et al*., 1993).

Infected cells are distinguishable by the presence of enlarged cisternae of the endoplasmic reticulum, fewer and shorter microvilli. The virus kills and sloughs off epithelial cell thus the stunting of the villi (aberrant villi) and thickened mucous layer in the lumen of the small intestine (Dodet *et al*., 1997). This pathological state leads to malabsorption especially of D-xylose, carbohydrate and nitrogen which all contribute to diarrhoea. Another diarrhoeagenic factor is the state of hyper secretion which arises as a result of damage to and lysis of the villous tips of cells with the consequent replacement of functional enterocytes by mature crypt cells which is accompanied by hyper secretion. Thus, there is movement of water from the affected segment of the lumen to the extra cellular fluid (ECF) and increased sodium influx from the extra cellular fluid into the lumen. There is also a loss of potassium, bicarbonate, chloride, phosphates, zinc and iron. There are also changes in serum albumin and hydroxyproline leading to edema in some instances. In this condition, vitamins are used up faster and the depletion of zinc leads to a negative effect on cell-mediated immunity, while that of iron in recurrent infections contributes to aneamia. Protein is also lost as a result of enteropathy caused by this infection (Dodet *et al*., 1997; Angel *et al*., 1998).

Malnutrition has been noted as important in increasing the severity of the clinical manifestation of human rotavirus infection. It has been observed that repeated diarrhoea enhances malnutrition by damaging intestinal mucosa thus making absorption difficult over an extended period (Mata *et al*., 1982).

Generally this viral infection shows a high morbidity but relatively low mortality rates. This fact can be attributed to the development in the 1940s of replacement for body fluids and electrolytes usually lost in course of the illness. This improvement in therapy showed a marked decrease in childhood diarrhoea mortality rates (Kapikian and Chanock, 1996). However, Dodet *et al*. (1997) concluded that the greater severity of infection in developing countries is associated with concomitant malnutrition, malabsorption, zinc and vitamin A deficiencies and the synergistic action of mixed infection with other pathogens.

2.2.9 Treatment

The first line of action in treating rotaviral diarrhoea is the replacement of fluids and electrolytes lost through vomiting and diarrhoea. Many formulae of oral rehydration salts (ORS) have been reported to be effective in this direction. The standard WHO glucose electrolyte formula is made up of the following:

1 liter of water

3.5g	sodium chloride
2.9g	trisodium citrate dehydrates (or 2.5g sodium bicarbonate)
1.5g	potassium chloride
20g	glucose (anhydrous)

The use of this regimen replaces fluids, electrolytes and helps maintain a daily fluid requirement (Sack and Rabbani, 1994). Oral rehydration can be intravenous when dehydration is severe (Desselberger, 1998b) and is an inexpensive and effective treatment for serious, dehydrating rotavirus diarrhoea (Keusch and Cash, 1997). The use of human milk, which may contain rotavirus antibodies, has also been advocated during diarrhoea. It was successful in treating immunodeficient children with chronic rotavirus infection. However, colostrums from dams immunized with human rotavirus were not 100% effective when used to treat children with acute rotavirus gastroenteritis, but resulted just in a decrease in viral shedding (Losonsky *et al*., 1985).

Another study by Guarino *et al*. (1994) observed that administering human serum immunoglobulin by alimentary route to 2 infants with prolonged rotavirus diarrhoea was effective in clearing the virus and the associated diarrhoea. However, the latest approach to control is the use of vaccines of which there are quite a good number that have completed

large scale trial (Bresee *et al*., 2004; Gentsch *et al*., 2005; Glass *et al*., 2005). The goal is to prevent diarrhoea, which can lead to dehydration and death (Hoshino and Kapikian, 1994).

2.2.10 Immunity

Both active and passive immunity occur in human rotavirus diarrhoea. The former is acquired during an infection or artificially by immunization while the latter is acquired naturally when maternal antibody passes to the child via the placenta, milk or artificially by administering sera containing antibodies from animals or human beings (Gerna *et al*., 1990b). Many studies have identified local immune mechanisms as key determinants of protection from rotavirus infection and disease (Matson *et al*., 1993; Burns *et al*., 1995; McNeal and Ward, 1995). A recent study has shown that passively transferred antibodies can suppress or delay rotavirus infection (Westerman *et al*., 2005).

Each of the outer capsid proteins (VP4 and VP7) independently induces the production of antibodies that neutralize the virus *in vitro* and protect against infection *in vivo*, and antibodies to either is sufficient to neutralize the virus (Matson, 1994). So far, it has been reported that antibodies induced by the major inner capsid protein (VP6) lacks any protective effect (Unicomb *et al*., 1999).

A major problem in studying rotavirus specific immune response to either natural infection or immunization has been that of distinguishing primary from subsequent infection. Antibodies are transferred transplacentally to the newborn before birth and may obscure the capacity to determine whether or not a child has had a previous exposure to the virus. In their study, Bernstein *et al*. (1991) reported transplacental maternal antibody to a circulating strain. Presumably, neither IgA or IgM nor lymphocytes cross the human placenta (Offit *et al*., 1993), but Ward *et al*. (1996) posits that probably IgG is taken into the intestine by transcytosis where it neutralizes rotavirus prior to infection of the enterocytes. However, it has been observed that antibodies appear rapidly in serum and mucosal secretions (feaces, saliva and small intestine) after the onset of infection in a homologous host. In children with diarrhoea, rotavirus specific IgG and IgA can be detected in serum after 7-20 and 28 days, respectively after onset of symptoms. On the other hand IgM and IgA can be detected in saliva, duodenal contents and feaces by 7-28 days after disease onset. Rotavirus specific IgM, which is the first to appear, disappears after the 6th week. It is a sign of recent

infection in the absence of IgG, which appears only after convalescence (Elaraby *et al*., 1992).

There is a lot of evidence indicating the protective role of secretory IgA (sIgA) against mucosal pathogens. While they form the first line of defense against many pathogens, the actual mechanisms of how they protect is not very clear (Ruggeri *et al*., 1998). However, these may include prevention of pathogens' contact with epithelial surfaces, formation of immune complexes and intracellular neutralization with or without neutralizing antibodies (Kaetxel *et al*., 1991). Secretary IgA can be transported via transcytosis across certain epithelial cell types lining mucosal surfaces, and then released into the mucosal membrane (Burns *et al*., 1996). Secretory IgA has been demonstrated in colostrums and mature milk and can also be transplacentally transferred (Offit *et al*., 1993; Guarino *et al*., 1994; Yolken *et al*., 1994). When found in breast milk, it is said to coat the virus in the gut of breastfed babies. This is one of the reasons why rotavirus diarrhoea is uncommon in the breastfed neonates (Simhon, 1978; Elaraby *et al*., 1992).

The concentration of sIgA produced in mammary glands is higher in colostrums than in milk produced later, and was found in 60%-100% of lactating mothers, except in South Africa where the prevalence rate was only 3% and 13% among Asian and Black mothers, respectively (Haffejee and Moosa, 1990). Also the neutralization antibody in the colostrums of mothers of infants who were infected with the virus was lower than in those whose infants did not become infected. Breast milk contains specific IgA to rotavirus and non-specific inhibitors to the viral replication like mucin and antitrypsin (Rennel, 1996).

In a general review, Haffejee (1991) reported that studies in Britain, Australia, Japan, Costa-Rica and Israel reported a lower rate of rotavirus infection in breastfed than formula fed newborns. This is in contrast to another study in Australia, Britain and United States where breast-feeding was not associated with any reduction in rotavirus morbidity. However, some researchers still observed that the clinical disease was milder, with a lower concentration of virus shedding and decreased frequency of vomiting in breast fed infants. On the whole, the difference was attributed to the following reasons:

i. The presence of trypsin inhibitors in breast, which may prevent viral VP4 cleavage.

ii. A non-specific inhibitory factor thought to be a glycoprotein with a wide range of antiviral activity present in milk.

iii. The rich cellular components of breast milk like lymphocytes and macrophages might play an active role in cell mediated immunity especially during early lactation.

iv. Breastfed infants unlike formula fed ones develop a transient bacterial flora, which consists of about 99% bifidobacteria.

Although there is still a controversy over the protective effect of breast milk, Haffejee (1991) noted that it protects infants from rotavirus diarrhoea, which even if it occurs is milder, and resolves quickly. Contrary, Kapikian and Chanock (1996) argued that even if breast milk transfers immunity to newborns and young infants, its protective effect seems to be very little and the role of cell-mediated immunity is still being studied. Fecal IgA has been used to predict the presence of duodenal IgA with an accuracy of 86% and 92% at one and four months respectively after onset of diarrhoea. Its local secretion is the most important effector mechanism of mucosal immunity since more than 40 mg of IgA/kg of body weight can be secreted into the gut lumen during mucosal stimulation.

Moser *et al*. (1998) reported that protection against challenge infection is mediated by the presence of virus-specific IgA at the intestinal mucosal surface at the time of challenge in both immunocompetent and immunodeficient mice. This is corroborated by many studies that identified local immune mechanisms as key determinants of protection from rotavirus infection and disease (Mc Neal *et al*., 1994; Burns *et al*., 1995; Mc Neal and Ward, 1995). Shaw and Hempson (1996) in their contribution suggested that active viral replication in the mucosa may provide factors that enhance antibody response like cytokines of the epithelial origin which may promote specific immunoglobulin secretion. Anti VP8 antibodies prevent infection by preventing cell attachment, while anti VP7 antibodies can inhibit previously cell-bound virus. Thus, viral replication is an important determinant of mucosal IgA response. This is in contrast to an earlier report that its presence in feaces or serum of infants was not a reliable correlate of protection against the disease in vaccine trial (Johansen *et al*., 1994; Bernstein *et al*., 1995). Johansen and Svensson (1997) noted that antibodies are also directed against the inner proteins, VP2 and VP6.

The increasing risk of exposure to infection and the waning of transplacentally acquired immunity might be responsible for the gradual risk with age of rotavirus positive response (Elaraby *et al*., 1992). However, many authors reported that oral immunoglobulin had a beneficial effect in decreasing the severity of symptoms in low-weight infants infected with the virus (Guarino *et al*., 1994). It also aids the disappearance of the virus from stool of immunodeficient children with protracted rotavirus diarrhoea and faster recovery from acute gastroenteritis (Guarino *et al*., 1994). Similarly, Clark *et al*. (1996) demonstrated that children immunized orally even with non-human rotavirus immunoglobulin are protected against relatively severe disease when challenged with human rotavirus.

Although natural rotavirus infections do not provide full protection against subsequent illness, there are reports that such infections have been protective to some extent. For example, in a longitudinal study in Australia, Bishop *et al*. (1983) concluded that neonatal infection was not protective against re-infection, but induced some resistance against the diarrhoeal illness and complete protection against severe diarrhoea during re-infection. Bernstein *et al*. (1991) and Ward *et al*. (1994) also reported that infants who had symptomatic or asymptomatic natural infection were protected against subsequent illness. Similarly, Das *et al*. (1994) in their study in India observed that asymptomatic neonatal infection reduced the frequency and severity of subsequent rotavirus diarrhoea by 46%. While Guarino *et al*. (1994) also reported that infection at birth with a persistent nursery strain without any detectable modification conferred partial protection against disease when re-infected with other strains likely to even include a variety of serotypes.

After a primary infection, children develop a higher serum (homotypic) neutralizing antibody to the G type of the infecting strain than to G types of other strains (heterotypic). Similarly during natural infection, children are more likely to be protected against challenge by a strain with similar than with different G types (Offit, 1996). An efficacy trial of RRV-TV rotavirus vaccine across the United States showed 57% protection against all rotavirus gastroenteritis, while natural rotavirus infection in placebo recipients was 93% protective (Ward and Bernstein, 1994) and was even protective against un-infecting serotypes (Offit *et al*., 1993; Ward and Bernstein, 1994).

Despite the limited information from human studies, it appears that homotypic immunity predominates following the first natural or vaccine induced rotavirus infection and is

mediated by antibodies to VP4 and VP7 (Gouvea *et al*., 1990a). It expands to include other serotypes following a subsequent natural or vaccine induced re-infection with the same or other serotypes. Adults develop a significantly greater number of heterotypic reponses than infants less than 6 months of age (59% vs. 1.2% respectively) when vaccinated with RRV (Gouvea *et al*., 1990a). This is expected since one or more neutralization epitopes on VP4 or VP7 are shared among viruses of serotypes 1, 2, 3, and 4. Therefore, re-infection with a virus of the same serotype will surely amplify cross-reactive neutralizing antibody response (Gouvea *et al*., 1990a; Offit *et al*., 1993).

It has been shown that homotypic (serotype-specific) protection may be more effective than heterotypic protection, thus the basis for the RRV-TV vaccine (Unicomb *et al*., 1999). The relationship between serum neutralizing antibody titers and protection against the virus is still unclear (Parashar *et al*., 1998), but serotype specific serum neutralizing antibody responses have been used in determining vaccine trial rates as a potential proxy to vaccine efficacy (Ward, 1996). Both VP7 and VP4 are found to induce homotypic as well as heterotypic neutralizing antibodies. Genotype G1 frequently induced a response to both G1 and G3, while G3 and P1A [8] induced mostly homotypic responses. There have also been reports that intestinal IgA level measured against the viral antigen correlated best with protection after a heterologous rotavirus immunization in the mouse model; though it was not clear which specific protein (s) was the target of the protective immunity (Feng *et al*., 1994; Ishida *et al*., 1996). McNeal *et al*. (1994) also reported that levels of rotavirus antibody correlated with protection against re-infection in the model.

The apparent ability of asymptomatic neonatal infection to elicit protective immunity (Bishop, 1983) has led to the evaluation of the asymptomatic neonatal strain M37 in vaccine trials, where it has been shown to be immunogenic and of low virulence in the majority of vaccines. Muccins (mucous glycoprotein) derived from human milk and others (adult human and mice intestines) have been demonstrated to possess a viricidal effect. Mucins found in adults are different from those in infants and this might be a reason for the age related susceptibility to rotavirus gastroenteritis (Willoughby and Yolken, 1990; Yolken *et al*., 1992, 1994).

2.2.11 Rotavirus Vaccines

In spite of widesprSead use of ORS, severe rotavirus gastroenteritis still causes up to a half-million deaths in children younger than 5 years of age worldwide (Parashar *et al*., 2003). Studies published between 1986 and 1999 indicated that rotavirus cause approximately 22% (range 17%-28%) of childhood diarrhoea hospitalizations. From 2000 to 2004, this proportion increased to 39% (range 29%-45%). Application of this proportion to the recent WHO estimates of diarrhoea-related childhood deaths gave an estimated 611, 000 (range 454, 000-705, 000) rotavirus-related deaths (Parashar *et al*., 2006). In epidemiological studies conducted in developing countries, rotavirus accounted for a median of 8% of all diarrhoeal episodes, 28% of outpatients or clinic visits for diarrhoea, and 34% of hospitalizations of young children for diarrhoea (WHO, 2005a).

Severity of rotavirus disease can be substantially reduced by vaccination. Two rotavirus capsid proteins, VP7 and VP4, which constitute the outer capsid layer and viral spikes, respectively, are known to induce neutralizing antibodies responses. These antigens are responsible for conferring immunity and hence are a target for vaccine development. The high mortality and morbidity associated with rotavirus diarrhoea, coupled with the fact that improvement in water supply, sanitation or hygiene has not had a significant impact on disease, (Perez-Schael *et al*., 1997; Glass *et al*., 2005; WHO, 2005a), has been the main reasons why the WHO, the Institute of Medicine and the Global Alliance for Vaccine Initiative (GAVI) made the development of a vaccine against rotavirus a high priority.

Since the discovery of human rotavirus in 1973, several vaccine candidates have been developed and tested in human population in different settings. However, immune response to these vaccines has produced differing results in different regions of the world (Gentsch *et al*., 2005). The most widely tested vaccine was the RIT 4237, a bovine rotavirus strain, which had been attenuated by multiple passages through tissue culture after cold adaptation. Whilst the RIT 4237 was effective with a protection rate of 88% against severe diarrhoea in the developed world (Vesikari *et al*, 1983), it was rather disappointing with protection rates of zero and 33% in trials in Rwanda and the Gambia (De Mol *et al*., 1986; Hanlon *et al*., 1987) respectively.

The reasons for this discrepancy have been attributed to difference in circulating strains, co-infection with other pathogens and differences in the epidemiology of infection in these settings. These reasons have been buttresed with recent reports from rotavirus surveillance

studies in developing countries showing an increase in rotavirus strains diversity with the appearance of unusual genotypes and the re-emerging of new strains other than the common types G1, G2, G3 and G4. These include genotype G8 in Malawi and Nigeria (Cunliffe *et al*., 2000; Audu *et al*., 2002b), G5 and G9 in Brazil (Gouvea *et al*., 1994a; Santos *et al*., 2001) and G9 in India, Kenya and Nigeria (Ramachandran *et al*., 1996; Cunliffe *et al*., 2001; Adah *et al*, 2001).

The other widely tested vaccine was the rhesus rotavirus vaccine-tetravalent (RRV-TV) (RotaShield, Wyeth-Ayerst Pharmaceuticals, Wayne, Pa). The RotaShield was the first vaccine to be licensed by the Food and Drug Administration (FDA) in 1998 (CDC, 1999a). After several trials of the RotaShield in different settings in the United States, Finland and Latin America, the vaccine proved efficacious. However, RotaShield was withdrawn from the United States market within 1 year because of reports of intussusception among vaccinated infants (Kapikian *et al*., 1996; CDC, 1999b; Glass *et al*., 2004) leading to suspension of further development of this vaccine in other settings. Hence, the only schedule trials of the vaccine in Africa (South Africa and Ghana) were not completed.

According to Cunliffe *et al*. (2002a), Glass *et al*. (2005) and WHO (2005a), the world health community believes that vaccines offer the best hope for protection against rotavirus despite the withdrawal of RotaShield due to the following reasons:

- Naturally acquired rotavirus infections provide protection against disease. A vaccine could also stimulate immunity.
- Rotavirus transmission rates seem unaffected by improvements in sanitation and hygiene (infections are universal in children during the first few years of life), one of the principal public health approaches to controlling disease.
- Life-saving intravenous treatment to rehydrate children with severe rotavirus diarrhoea is unavailable to many of the developing world's 575 million children under age five.
- For many countries of both the developed and developing world, a vaccine will be the most cost-effective way to stop rotavirus.

Currently, however, several rotavirus vaccine candidates that are based on heterotypic or serotypic specific immunity have been developed (Bresee *et al*., 2004; Gentsch *et al*., 2005) and on last phase of trials. This has prompted the constitution of rotavirus surveillance

system networks with the collaboration of the US Centers for Disease Control and Prevention and WHO to estimate; the hospital-based disease burden of RV gastroenteritis in children less then 5 years of age, the baseline incidence of intussusception (IS) and to constantly update the frequency and characteristics of circulating strain. This latter aspect is of tremendous importance for the development of a rotavirus vaccine and for studying the possible vaccine selective pressure and the emergence of new strains in the vaccinated populations (WHO, 2005a).

2.2.12 Global Diversity of Rotavirus VP4 and VP7 Genotypes

The serotyping of rotavirus in the early 1980s and 1990s by monoclonal antibodies-ELISA left rotavirus G1, G2, G3 and G4 as the predominate strains in circulation worldwide. This was probably because of the availability of monoclonal antibodies specific for GI, G2, G3 and G4 serotyping resulting in approximately 20% to 30% of isolated strains remaining untypeable (Gouvea *et al*., 1990b). There was therefore the urgent need to develop new serotype specific monoclonals to increase the serotyping, which culminated in the development of monoclonal antibodies specific for G5, G6, G8, G9, G10 and G12 (Hoshino and Kapikian, 1994). The development of the RT-PCR and molecular methods further greatly improved strains typing and characterization of field isolates. The combination of these 2 methods i.e. monoclonal antibody based-ELISA and RT-PCR assays for typing human rotavirus field isolates in various epidemiologic surveys worldwide has generated very important information and improved our understanding of the epidemiology of rotavirus infection and the circulation of rotavirus strains. Globally it has been shown that rotavirus G serotype 1, 2, 3 and 4 constitute more than 90% of all G serotypes detected and that serotypes 1, 3 and 4 are most commonly associated with P-types lA[8], whereas G serotype 2 is commonly associated with P-types 1B[4]. It has also shown that multiple G and P-types can co-circulate within the same region, the prevalence of rotavirus serotypes in different regions within same country can differ during the same year and that individual serotypes in the same region can show a yearly change.

Recently, the use of molecular methods have illustrated that novel rotaviruses with unusual combinations of VP4 and VP7 types are common (Griffin *et al*., 2000, Hoshino and Kapikian, 2000a). Some of the unusual genotypes detected are rotavirus strains with VP7 G5, G6, G8, G9, G10 and G12 as well as strains with the P-types P2 A[6], 3[9], 4[10], 5A[3] and 11[14] (Gentsch *et al*., 2005). The enormous diversity and capacity of HRVs for

change suggest that RV vaccines must provide good heterotypic protection to be optimally effective.

2.2.12.1 Diversity of G and P-Types in America

North America: There is a lot of data on the circulating genotypes in the USA. The methods used have either been by antigenic methods using serotype specific monoclonals or by molecular methods with genotype specific primers. In these studies, the common strains G1-G4 have been consistently stated as the most common circulating strains. G serotyping of HRV positive samples collected from children at different hospitals and laboratories in USA from 1990 to 1999 showed that serotype G1 was the most prevalent in circulation, followed by G3, G2, G4, and G9 (Griffin *et al*., 2000). Rotaviruses of the different G and P genotypes co-circulate. However, the predominant G/P types were G1 P[8], G2 P[4], G3 P[8], and G4P[8]. The incidence of these strains have been observed also to change with seasons as was seen in genotyping studies carried out between 1990 to 1997 where the predominant G/P types was G1 P[8] followed by G3 P[8] instead of G2 P[4] as was the case with the 1990 to 1999 study. Rotavirus strains with unusual G/P combinations have also been isolated in the USA. In studies carried out by Santos *et al* (1994) and Ramachandran *et al* (1998) strains with the unusual G1 P[6], G2 P[6] and G9 P[6] were found in circulation.

Central America: In surveillance studies involving several different cities in Central America, from 1979 to 1992, the G serotypes characterization of HRV positive samples collected from hospitals and day care centres showed that serotype G1 was the most predominant, followed by serotype G3 whilst serotypes G2 and G4 were either rarely seen or were very low in circulation (Gouvea *et al*., 1990a; Matson *et al*., 1990; O'Ryan *et al*., 1990; Woods *et al*., 1992). Changes in predominance of strains were also observed in the region.

In Mexico, one study involving 132 HRV positive samples from 1984-1987 showed that serotype G1 was the most prevalent serotype in circulation, whilst serotypes G2, G3 and G4 were circulating at almost the same level (Padilla-Noriega *et al*., 1990). Two years later in 1990 serotype G3 was reported as the most predominant strain in circulation, followed by serotype G1 and G2 (Woods *et al*., 1992). The predominant VP7 genotype in circulation in

Central America has always been either a G1 or G3 genotype. In another survey, the G and P genotypes of 257 HRV positive samples obtained during three epidemiological seasons from 1994-1997 from children with diarrhoea in several states of Mexico showed that G1 P[8] genotype was the most prevalent followed by G3 P[8] and G2 P[4] (Castillo *et al*., 2000).

South America: In South America, the diversity of HRV is a mirror image of what is seen in Central America except for the fact that, the detection of some animal rotavirus strains in human has made the diversity of HRV rotavirus very complex. In several studies involving both antigenic and molecular characterization of HRV positive samples collected from children in different cities in South America between 1981 to 1999 showed that serotype G1 was the most predominant followed by G2, G4, G3, G5, G9 and either G8 or G10 (Flores *et al*., 1988; Gouvea *et al*., 1994a; Timenetsky *et al*., 1994; Leite *et al*., 1996; Mascarenhas *et al*., 1998; Santos *et al*., 1998; Arguelles *et al*., 2000; Cardoso *et al*., 2000; Araujo *et al*., 2001; Bok *et al*., 2001a,b; Santos *et al*., 2001). The most predominant G/P constellation seen was G2 P[4] followed by G1 P[8], G4 P[8] and G3 P[8]. Also found in circulation were unusual combinations such as, G5 P[8], G1 P[4], G2 P[8], G4 P[6], G10 P[8], G9 P[6] and G4 P[4].

2.2.12.2 Diversity of G and P-types in Australia

In Australia, several community and hospital based studies involving the characterization of rotavirus in 9, 329 HRV positive samples collected from newborns, children and adults from 1973 to 2001 have used serotype specific monoclonals or RT-PCR. These studies showed that serotype G1 has always been the predominant strain in circulation followed by serotype G4, G2 and G3 as revealed by studies conducted from 1973 to 1989. In addition, a single study conducted from 1974 to 1986 showed serotype G3 as the most prevalent strain in circulation. Furthermore, surveillance studies conducted from 1996 to 2001 showed serotype G1 as the most predominant followed by G9, G2, G3 and G4. Also detected but at low levels in circulation are the unusual serotypes; G5 and G8. Combining the G and P-types, showed that more than 95% of typeable strains were identified as G1 P[8] or G2 P[4]. Also identified were two unusual strains, G1 P[6] and G2 P[6] (Birch *et al*., 1988; Unicomb *et al*., 1989; Bishop *et al*., 1991; Liddle *et al*., 1997; Masendycz *et al*., 2000, 2001; Bishop *et al*., 2001). The strain diversity in Australia is similar to what is seen in

Central and North America except for the simple fact that the second most important strain in Australia is an unusual G9.

2.2.12.3 Diversity of G and P-types in Europe and the Mediterranean

Most studies on HRV diversity in Europe have made use of serological methods to characterize strains. These methods include both polyclonal and monoclonal antisera in solid-phase immune electron microscopy and monoclonal-based ELISA (Gerna *et al*., 1987; Noel *et al*., 1991; Beards and Graham, 1995). Recently, RT-PCR methods have been incorporated in the characterization of rotavirus strains. Generally in Europe and the Mediterranean, more than 90% of HRVs Group A that have been typed is of serotypes G1-G4 (Koopmans and Brown, 1999).

Similar to studies reported in Australia, North, South and Central America, studies conducted in Europe and the Mediterranean from 1981 to 1998 showed that serotype G1 was the most predominant strain in circulation followed by serotypes G4, G2 and G3. Furthermore, unusual G types such as G5, G7, G8, G9, and G10 were also found in circulation but at low levels (Gerna *et al*., 1985, 1987; Beards *et al*., 1989; Arista *et al*., 1990; Noel *et al*., 1991; Beards and Graham, 1995; Silberstein *et al*., 1995; Szucs *et al*., 1995; Gault *et al*., 1999; Iturriza-Gomara *et al*., 1999, 2000c; Johansen *et al*., 1999; Koopmans and Van Asperen 1999; O'Mahony *et al*., 1999; Vesikari *et al*., 1999; Bon *et al*., 2000; Cilla *et al*., 2000; Cubitt *et al*., 2000; Fruhwirth *et al*., 2000; O'Halloran *et al*., 2000; Cascio *et al*., 2001; Van der Heide and Van der Poel, 2001). Combining the G and P types also showed strains G1 P[8] as the most common strain circulating throughout Europe and the Mediterranean. Also detected in circulation across Europe and the Mediterranean but at low levels are some unusual strains such as; G9 P[6], G1 P[4], Gl P[9], G1 P[6], G3 P[4], G2 P[8] and G8 P[8]

2.2.12.4 Diversity of G and P-types in Asia

A review on G and P types of HRV positive samples collected from Asian children from 1984 to 2000 showed that serotypes Gl-G4 are the most common pathogenic agents of diarrhoea among Asian children. This is a similar picture seen in Europe and the Mediterranean, Australia, and USA. The predominant serotype was G1 followed by G2, and either G3 or G4. Also, the only uncommon serotype seen in circulation was serotype

G9 whilst no G8 serotype was reported in the reviewed papers. The P type P[8] was also the most predominant VP4 genotype in circulation followed by P type P[4] and P[6]. Unusual VP4 types detected but rarely are P [9] and P[11] (Taniguchi *et al*., 1992; Ushijima *et al*., 1994; Husain *et al*., 1996; Ahmed *et al*., 1999; Qiao *et al*., 1999; Unicomb *et al*., 1999; Fang *et al*., 2000; Koshimura *et al*., 2000; Maneekarn and Ushijima, 2000; Nishio *et al*., 2000a,b; Seo and Sim, 2000; Tsai *et al*., 2000, Zhou, *et al*., 2000, 2001; Jain *et al*., 2001; Lee *et al*., 2001; Das *et al*., 2002; Fang *et al*., 2002). Although the most common G/P constellation seen in circulation was strain G1 P[8] followed by G4 P[8], G3 P[8] and G2 P[4], strains with unusual combinations such as; G9 P[6] and G9 P[11] were the most predominant detected in circulation in 1993 and 1986 to 1993 in India respectively. Recent studies conducted between 2001 and 2003 have shown G4 P[6] (Kang *et al*., 2005b), G3 [P8] (Fang *et al*., 2005) and G9 [P8] (Chen *et al*., 2005) to predominate in South Korea, China and Taiwan.

2.2.12.5 Diversity of G and P-types in Africa

Whilst there have been a lot of studies on the characteristics of circulating rotavirus strains in Europe, the Americas and Asia, there are but a few studies on strain types in Africa. Most studies on rotaviruses have focused on the epidemiology of the disease, mainly on incidence, seasonality, age distribution and prevalence. A few studies have focused on the sero-epidemiology of rotavirus infection in community settings (Biritwum *et al*., 1984; Georges-Courbot *et al*., 1988a; Olusanya and Taiwo, 1989; Tswana *et al*., 1990; Cassel-Beraud *et al*., 1993). All these studies have documented a high incidence of rotavirus infection in children (20-35%) with peak incidence during the cool dry months in West, Central and East Africa and during the winter months in North and South Africa.

The mid 1990s saw an increase in surveillance studies on rotavirus. The easy availability of typing reagents, the introduction of molecular methods and the birth of the African Rotavirus network, with the base at Medical University of Southern Africa (MEDUNSA), now University of Limpopo, South Africa, saw a rapid increase in information on rotavirus genotypes in Africa. These studies have shown a huge diversity of circulating rotavirus strains across the African continent as well as large repertoire of unusual strain types.

North Africa: North Africa has a genotype distribution close to what prevails in Europe. Three hospital and community based studies conducted in Egypt, Tunisia and Libya,

involving 162 HRV positive samples from children, showed that the predominant genotype was G1 rotavirus strains. The characterization of diarrhoeic stools collected from hospitalized Tunisian children from 1995 to 1999 showed that serotype G1 was the most predominant strain, followed by G4 and G2 (Trabelsi *et al*., 2000). The prevalent VP4 genotype in circulation was P[8]. In addition, strain G1 P[8] was the most common, followed by G4 P[8] and G4 P[6]. In Egypt, an epidemiological study of rotavirus diarrhoea in a population-based cohort of children under 3 years of age between 1995-1996 also showed serotypes G1 and G2 as the most prevalent (Naficy *et al*., 1999). Similar results were obtained in a neonatal study carried out by Radwan *et al*. (1997). In this neonatal study, serotypes G1 and G4 were the strains predominating in all age groups, followed by G3 and G2. A study of the G and P-types from fecal samples obtained from Libyan children in the year 2000 showed similar trends. The predominant G type was G1 and the most common strain was G1 P[8] followed by strains bearing the G9 P[8], GI P[6] G/P characteristics (Cunliffe *et al*., 2001a) (Tables 2.6a and 2.6b).

Central Africa: In Central Africa, a single survey on rotavirus strains conducted among children in the Central African Republic by Georges-Courbot *et al*., (1988b) from 1983-1985, showed that serotype G1 was most prevalent followed by G2 and G3 (Table 2.6a).

East Africa: The distribution of rotavirus serotype in East Africa is different from what is seen in North, Central and West Africa. Two studies conducted in Kenya from 1982 to 1994, involving 333 HRV positive samples collected from diarrhoeic children aged 0 to 5 years showed that serotype G4 was the most predominant strain in circulation followed by serotype G1, G2, G8 and G3. The VP4 genotype P[8] was the most predominant followed by P[4] and P[6] (Urasawa *et al*., 1987; Nakata *et al*., 1999). Another study on the G and P types from 337 HRV positive samples obtained from Kenya children from 1991 to 2000 showed a totally different picture of the circulating strains. From 1991-1994, the strain G4 P[8] was the most predominant strain in circulation followed by G1 P[8] and G2 P[4]. But in the year 2000, the strain with serotype G1 specificity was the most prevalent followed by an unusual strain G9 P[8], G9 P[6], G8 P[4] and G1 P[6] (Nakata *et al*., 1999; Cunliffe *et al*., 2001b) (Tables 2.6a and 2.6b). Furthermore an epidemiological study of the VP7 rotavirus serotypes from young children with diarrhoea in 1999 in Tanzania, showed that the most prevalent strain was serotype G1, followed by serotype G2 (Mngara *et al*., 2002).

West Africa: West Africa has been termed the "hotbed for rotavirus genetic recombination". This genetic recombination makes the diversity of rotavirus strains in West Africa very complex. Hospital, rural and urban community based studies involving HRV positive samples collected from children from Nigeria, Ghana, Burkina Faso and Ivory Coast between 1989 to 2000, showed serotype G1 to be the most prevalent followed by G3, G2, G8 and G9. Interestingly no serotype G4 was detected. VP4 genotype P[6] was also the most predominant in circulation. The distribution of rotavirus varies from one country to another. Two separate studies in Ghana involving 96 HRV positive samples collected from children <5years old from 1998 to 1999, showed that serotype G3 and VP4 genotype P[4] were the most predominant strains in the rotavirus season of 1998 whilst in the season of 1999, serotype G2 and VP4 genotype P[6] were the most common in circulation (Armah *et al*., 2001; Asmah *et al*., 2001).

Furthermore, unusual strains G3 P4] and G2 P[6] were the most prevalent in the 1998 and 1999 rotavirus season respectively. In Nigeria, four epidemiological studies involving 484 HRV positive samples obtained from diarrhoeic children from different regions from 1989 to 2000 showed that serotype G3 was the most predominant followed by G1 and G2, whilst no G4 was identified. The VP4 genotype P[6] was the most common in circulation followed by P[8] (Avery *et al*, 1992; Adah *et al*., 1997a,b; Adah *et al*., 2001: Audu *et al*., 2002b). In addition, strains G1 P[8] and G2 P[6] were the most predominant followed by G1 P[6] and G3 P[6]. In a community based study in Guinea-Bissau, during 1996 to 1998, strain G2 P[6] was the most prevalent followed by G2 P[4] and G1 P[8] (Fischer *et al*., 2000). In addition, unusual strains such as G1 P[6], G3 P[4], G3 P[6], G3 P[9] and G4 P[9] were also found in circulation (Tables 2.6a and 2.6b). However, in 1999, serotype G9 predominated and G4 was identified for the first time in a single case in Nigeria (Steele *et al*., 2002). The unusual strain, G2 [P6] was the most prevalent in a study conducted in 2001 (Salu *et al*., 2003).

Southern Africa: Rotavirus strain diversity in Southern Africa is a mirror image of what is seen in Central and North America. In Southern Africa, both hospital and community based studies conducted from 1984 to 1999 using 2,484 HRV positive samples collected from children in South Africa, Zambia and Malawi revealed that serotype G1 was the most predominant followed by G3, G4 and G2. VP4 genotype P[8] was the most common type in circulation followed by P[6] and P[4]. In four hospital-based studies conducted in South

Africa from 1984 to 1993, a total of 683 HRV positive samples collected from children with diarrhoea were examined. The results showed that serotype G1 was the predominant strain in circulation followed by, G4, G2 and G3. On the other hand, VP4 genotype P[8] was the most common type detected followed by P[4], P[6], P[9] and P[12] (Mnisi *et al*., 1992; Steele *et al*., 1993b; Mphahlele and Steele, 1995; Steele *et al*., 1995). A review of 3 published studies on the G and P types from fecal samples obtained from South African children between 1986 to 1989, showed that strain G1 P[8] was the most common in circulation followed by G2 P[4] and G4 P[6] strains. In addition, unusual strain such as G4 P[6] was also detected at very significant level (Pager *et al*., 2000) (Tables 2.6a and 2.6b). Steele *et al*. (1998b) typed the G and P types of 69 rotavirus strains recovered from different infants and young children from Zambia using hybridization analysis. VP7 type G1 strain was the predominant followed by G2 and G4. In addition, the VP4 genotype P[8] was the most prevalent followed by P[4]. Strain G1 P[8] was the predominant, followed by G2 P[4] and G4 P[8].

In Malawi, the G and P types of 514 HRV positive samples collected from children with acute diarrhoea from 1997 to 1999 revealed that G8 was the most predominant followed by G1, G3, G4 and G9. VP4 genotype P[8] was also the most common followed by P[6], P[4], P[9] and P[12]. Strain G8 P[6] was the most predominant followed by G3 P[6], G8 P[4] and G9 P[6]. In addition, strains G P[6], G3 P[4], G1 P[6] were also found in circulation but at low levels (Cunliffe *et al*., 2001a) (Tables 2.6a and 2.6b).

Table 2.6a: Percentages of rotavirus G-genotypes detected in Africa.

Country	Year	No tested	G1	G2	G3	G4	G8	G9	Mix	Non Typeable	References
C.A.Republic	1983-85	178	57	12	11	-	-	-	-	20	Georges-Courbot *et al*., 1988b
Nigeria	1989	13	62	0	0	0	-	-	-	38	Avery *et al*., 1992
Nigeria	1993-95	314	0	0	63	0	0	0	64	9	Adah *et al*., 1997a
Nigeria	1999-2000	112	39	18	2	0	28	0	12	2	Adah *et al*., 2001
Ghana	1998	50	2	12	80	0	0	0	6	0	Asmah *et al*., 2001
Ghana	1999	46	0	54	0	0	7	0	15	24	Armah *et al*., 2001
Kenya	1982-83	16	44	25	13	0	-	-	0	18	Urasawa *et al*., 1987
Kenya	1991-94	317	24	17	<1	42	2.2	0	0	15	Nakata *et al*., 1999
Tunisia	1995-99	65	57	3	0	14	0	0	5	22	Trabelsi *et al*., 2000
Egypt	1992-93	62	18	3	7	18	0	0	16	39	Radwan *et al*., 1997
South Africa	1989	94	43	13	0	10	0	0	0	34	Mnisi *et al*., 1992
South Africa	1989	14	43	21	0	29	0	0	7	0	Steele *et al*., 1993
South Africa	1988-89	418	51	22	2.2	23	0	0	1.4	27	Steele *et al*., 1995
Zambia	-	69	64	12	0	1.4	0	0	-	23	Steele *et al*., 1998
Malawi	1997-98	100	0	0	38	5	51	3	1	2	Cunliffe *et al*., 1999
Malawi	1997-99	414	30	0	27	6	35	<1	1.2	0	Cunliffe *et al*., 2001a

C.A = Central Africa

Table 2.6b: Percentages of rotavirus G/P constellation detected in Africa

Country	Year	No Tested	G1P(8)	G2P(4)	G3P(8)	G4P(8)	Others (%)	Mix	Non Typeable	References
Nigeria	1993-95	45	22.2	0	4.4	0	G3P(6) (18) G1P(6) (4.4)	31	20	Adah *et al*., 1997
Nigeria	1999-02	112	9	0	0	0	G1P(6) (9) G2P(6) (11)	2	0	Adah *et al*., 2001
Guinea-Bissau	1996-98	167	8	11.4	0	0	G2P(6) (24) G1P(6) (2) G8P(2) (1)	4	16	Fischer *et al*., 2001
Ghana	1998	50	0	2	2	0	G3P(4) (74) G2P(6) (12) G3P(6) (7) G1P(6) (2)	0	0	Asmah *et al*., 2001
Ghana	1999	46	0	0	0	0	G2P(6) (50) G8P(6) (4.3)	0	7	Armah *et al*., 2001
Kenya	1991-94	317	24	17	1	42	G8P(4) (2.2)	0	15	Nakata *et al*., 1999
Kenya	2000	20	60	0	5	0	G9P(4) (10) G9P(6) (5) G8P(4) (5) G1P(6) (5)	0	59	Cunliffe *et al*., 2001a
Tunisia	1995-99	65	50	-	-	-	G4P(6) (?)	-	-	Trabelsi *et al*., 2000
Libya	2000	35	46	3	0	0	G9P(8) (34) G1P(6) (11)	0	52	Cunliffe *et al*., 2001b
South Africa	1989	14	38	29	0	0	G4P(6) (29)	0	0	Steele *et al*., 1993
South Africa	1988-89	377	56	22	<1	11	G4P(6) (10) G3P(9) (1)	0	34	Steele *et al*., 1995

Malawi	1997-98	100	0	0	20	2	G1P(6) (6) G8P(6) (27) G3P(6) (10) G8P(4) (9)	2	9	Cunliffe *et al*., 1999
Malawi	1995-99	414	27	0	23	5	G8P(6) (27) G8P(4) (8) G3P(6) (3)	4	0	Cunliffe *et al*., 2001a

In one of the most extensive rotavirus strain surveillance studies conducted in Africa from 1996-1999, a total of 3,000 rotavirus positive samples obtained from 15 different countries were characterized for their G and P-types. The predominant HRV strains were G1 P[6] and G3 P[6] (Steele *et al*., 2003). Here, a geographic difference in HRV strains diversity was revealed, with the HRV strains in West Africa having the most diverse nature. Mosaic viruses G3/8 and Gl/3 also occurred. G9 strains were identified in several countries indicating that the strain is emerging in Africa.

The increase in rotavirus surveillance studies globally has actually shown an increase in strain diversity worldwide and also brings to light the emergence of novel strains in Africa.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in four states (Kaduna, Kebbi, Sokoto and Zamfara) in Northwestern Nigeria (Figure 3.1). Northwestern region of Nigeria lies between longitude 4° and 10° East of the Greenwich Meridian and latitude 9° and 13° North of the equator. The region has a typical tropical continental climate and Northern dry savannah vegetation with alternating humid to wet and cool to hot dry season (April to September and October to march respectively). The seasons are governed by the movement of the intertropical discontinuity, a zone where warm, moist air from the Atlantic converges with hot, dry, and often dust-laden air from the Sahara known locally as the harmattan (Microsoft® Encarta® Reference Library, 2005). Temperatures are high throughout the year, averaging from 25° to 28°C (77° to 82°F). Northern Nigeria typically experiences greater temperature extremes than the south. Rainfall is especially variable over distance and time. The inhabitants of the region are mostly subsistence farmers who cultivate cereal such as rice, cotton and onions and produce livestock.

Kaduna State is located on the southern end of the high plain of Northwestern region, bounded by parallels 9°03′N and 11°32′N, and extends from the upper River Mariga on 6°05′E to 8°48′E on the foot slopes of the scarp of Jos Plateau. The seasonality in Kaduna state is pronounced with the cool to hot season being longer than the rainy season. Rainfall varies, decreasing from an average of about 1530mm in the southeast to about 1015mm in the northeast. The 1991 census provisional result puts the population of Kaduna State at 5,001,258 with a near 1:1 male/female ratio. Apart from a large two unit University Teaching Hospital, there are three large fully equipped government hospitals and two hundred and sixty nine (269) registered privately owned clinics located in the major towns. There are also comprehensive Primary Health Care Units (PHCU) in some selected rural areas established by the State Government together with the Federal Government and the World Health Organization. These PHCU cater for childcare, pre and antenatal health care. The agriculture and forest resources of the state are enormous. Cereal and cotton are intensively cultivated during the dry season and livestock are raised on small scale (Bello, 2000).

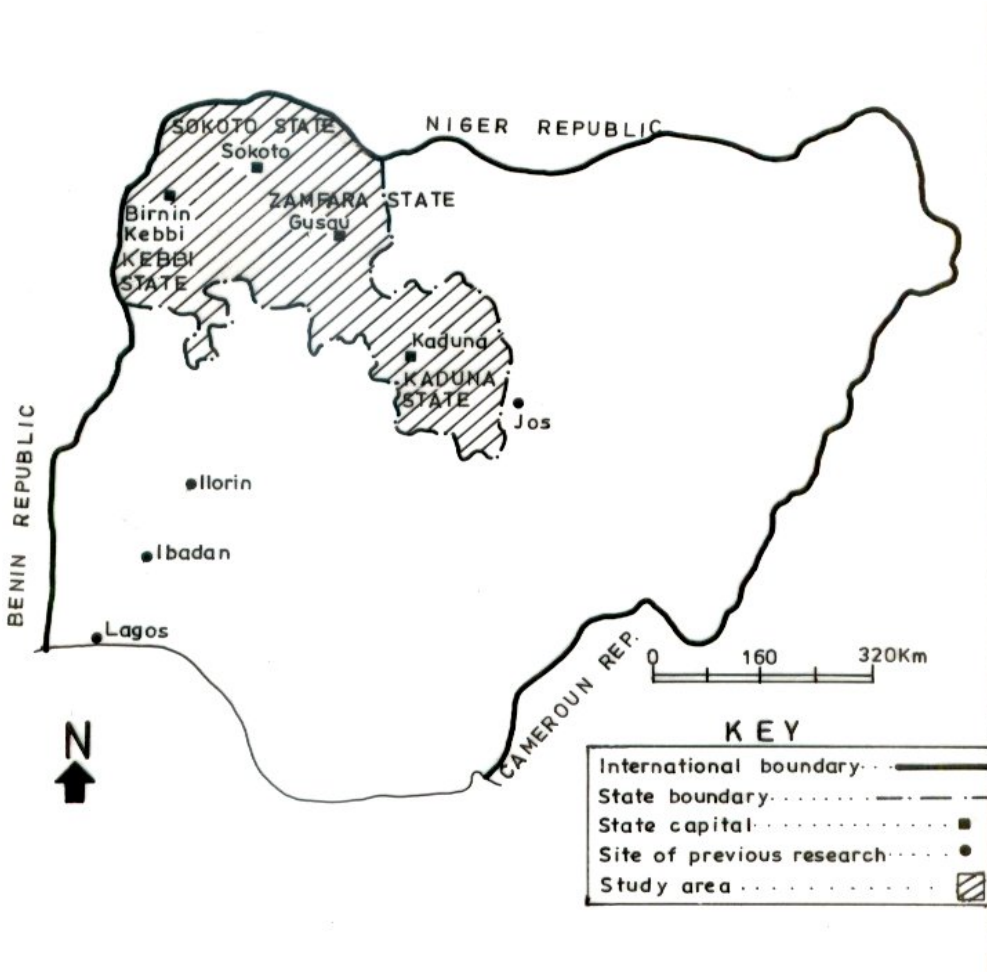


Figure 3.1: Map of Nigeria showing study area

Kebbi State has its headquarters in Birnin Kebbi and is located between latitude 10°8'N and 13°15'N and longitude 3°30'E and 6°02'E. The state is bounded by Sokoto State to the north and east, Niger State to the south, and Benin Republic to the west. It has a land area of 36,129 sq. km. Mean annual rainfall is about 800mm in the north and 1000mm in the south. Temperature is generally high with mean annual temperature of about 26°C in all location. However, during the harmattan season (December to February) the temperature can go down to 21°C and up to 40°C during the months of April to June. Farming is the major occupation of the people followed by trading, fishing, animal rearing and artwork, et cetera. The 1991 census provisional result puts the population of Kebbi State at 2,587 million. There are twelve General Hospitals, one Specialist Hospital, and twenty private clinics, most of which are located in Birnin Kebbi. There are eleven PHCU and 352 dispensaries in all the local government areas (LGA) put together. In all these institutions put together, there are a total of 2,032 beds. The state has abundant livestock hence; the major occupation of the people is livestock and hides and skins production (Iliya and Kwabe, 2000).

Sokoto town is the headquarters of Sokoto State located to the extreme of the Northwestern region between latitudes 12°'N and 13°58'N and longitudes 4°8'E and 6°54'E. The state shares common borders with Niger Republic to the north, Kebbi State to the southwest and Zamfara State to the east. The total land area is about 32,000 sq. km, while the total population in 1999 (projected from the 1991) is about 2,809 million. Much of the rain in Sokoto falls between June and September in the north and from April to October in other parts. The annual rainfall is between 500mm in the north and 1300mm in the south. The highest temperature is experienced in the months of March/April. As at June 1999, the state had a total of 440 health facilities located in different LGA of the state. Of this total, there are eight general hospitals, one each of teaching, psychiatry, army and specialist hospitals. Over 80% of the state's population is engaged in agriculture. Livestock production is another major occupation (Mamman, 2000a).

Zamfara State is located between latitudes 10°50'N and 13°38'N and longitudes 4°16'E and 7°13'E. The state covers a land area of 38,418 sq. km. It is bounded in the north by Sokoto State, Kebbi and Niger State to the southwest, Kaduna State to the southeast and Katsina to the east. The hottest months in Zamfara are the months of March and April while the peak of the rainy season is from July to September. Mean annual rainfall is about 579mm in the north and 1,020mm in the south. The 1991 census provisional result puts the population of

Zamfara State at 2,231,402 with a slightly female skewed sex ratio and composition. There are eight General Hospitals, six PHCU, three Basic Health Service Centres, one center for Vesico-Vaginal-Fistula, a mental home and a Federal Medical Centre mainly located in the state capital Gusau. Agriculture is the mainstay of the economy of the state and engages about 90% of the people while livestock is also a vibrant agricultural sub-sector (Mamman, 2000b).

All state capitals in Nigeria have Government run hospitals and PHCU including private clinics that provide health services to the population. The health care facilities of these state capitals serve all the surrounding villages in each state. The population uses either government or private health facilities, which are the two main health care systems in Nigeria. The number of patients attending each of this health care facility depends on socioeconomic status and the availability of drugs at low prices. Government hospitals and health centres that provide free health care with available drugs at low prices are usually much more frequented. In some Northwestern Nigerian States (Kebbi, Sokoto and Zamfara), in addition to the two main health services, the State Government runs Women and Children Welfare Clinics (WCWC) that provide almost free health services to only women and children. Hence these WCWC are much more frequented by women and children from all over the states. In Zamfara State, in addition to the free health services, free transport to and from nearby villages is provided. The clinics and hospitals selected for sample collection were based on the above and the advice of the primary health care director of each State.

3.2 Study Population

The study was hospital and clinic based. Approval was obtained from each State Ministry of Health and Ethics Committee of the hospitals before the study was conducted. The target population was infants and young children aged 0-5 years with or without diarrhoea presented at or hospitalized in the under listed health care facilities in the state capital of each of the four states except Kaduna state. Those without diarrhoea served as control. The health care facilities selected in each of the four states were:

Kaduna State

1. Ahmadu Bello University Teaching Hospital, Tudun Wada, Zaria.
2. Primary Heath Care Centre, Samaru, Zaria.
3. Primary Heath Care Centre, Kwata, Sabon Gari, Zaria.

4. Primary Heath Care Centre, Chikaji, Zaria.

Kebbi State

1. Fati Lami Women and Children Hospital Birnin Kebbi.
2. Primary Heath Care Centre, Jega.

Sokoto State

1. Maryam Sani Abacha Women and Children Hospital, Sokoto.
2. Specialist Hospital Sokoto.
3. Women and Children Welfare Clinic, Tudun Wada, Sokoto.

Zamfara State

1. Women and Children Hospital, Tudun Wada, Gusau.
2. Federal Medical Centre, Gusau.
3. General Hospital, Tsafe.

3.3 Inclusion and Exclusion Criteria

The inclusion criteria include: age 0-5 years of both sexes, diarrhoeic, not having diarrhoea in the three weeks preceding sample collection and given consent. Children who had been on antimicrobial agents were not excluded in the study.
The exclusion criteria include: age above 5 years of both sexes, no consent given.

A diarrhoeic case in this study was defined as a child passing loose, liquid, watery or a bloody loose stool three or more times in a 24-hour period as reported by parents. The control was any child presented for an illness other than diarrhoea and with no history of it on the day of, or in the three weeks preceding sampling. The socio-economic status of a child's parents was determined using education and earnings as indices. A child with low socio-economic background was any child whose parent earned low minimum wage and had a primary or secondary school education. A child whose parent earned low or medium wage but had tertiary school education was said to belong to a medium socio-economic background while those with parents with tertiary education who earned high wages were said to belong to a high socio-economic background.

3.4 Sampling

At the respective health care facility, arrangements were made with a doctor, matron or laboratory technician whereby the mother or caregiver of any child who satisfies the study inclusion criteria was asked to provide the child's stool specimen after consultation. Prior to collection of specimen, the mother or caregiver who consented to inclusion of his/her child in the study was interviewed using structured questionnaire designed to obtain demographic and clinical information about the child and his/her illness (Appendix 1). Samples were collected when available on each day from Monday through Sunday. Where possible, stool was collected on the spot with the child using provided potty lined with cellophane for each use. Otherwise, the mother or caregiver was asked to bring it the following day. All specimens were submitted to the health facilities laboratories and refrigerated where possible or put on ice in a cold box until collected for final storage.

3.5 Sample Collection and Storage

A total of 1063 faecal specimens were collected from the various health care facilities, 869 from cases of diarrhoea and 194 from controls. This was during the study period from July 2002 to July 2004. During the 25 months, 608 (57.2%) males and 455 (42.8%) females were recruited for the study. Three hundred and sixty six (366) were from Kaduna State, 96 from Kebbi State, 229 from Sokoto State and 372 from Zamfara State. The number of sample collected per state was largely dependent on its population and the number of health facilities in the state. About 7gm or 7ml of each faecal specimen was scooped with a wooden spatula or decanted respectively into clean, labelled screw capped polystyrene tubes, tightly screwed and were stored frozen (-20^{o}C) at the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria, until transportated to South Africa. Specimens within iceboxes were transported to the MRC/Medunsa Diarrhoeal Pathogens Research Unit, University of Limpopo, Medunsa Campus, Pretoria, South Africa, where a 10% fecal suspension of each specimen was prepared using a balanced salt solution for analysis.

3.6 Laboratory Method

3.6.1 Detection of Rotavirus Antigen in Stool Specimens

All the 1063 faecal specimens collected were analysed for rotaviruses. Rotavirus group A antigen was detected with commercially available EIA (Rotavirus IDEIA^{TM}, DAKO, UK) kit code Number K6020. The test was carried out according to the manufacture's instructions.

3.6.1.1 Test Procedure

A 10% dilution of each specimen was made by adding 1g of solid stool (pea-sized portion) or approximately 1ml of liquid stool to 9 ml distilled water in sterile and labeled screw-cap bottles. The suspension was mixed thoroughly with a vortex mixer for 30 seconds and allowed to settle for about 30 minutes prior to testing. Ninety-six well microtitre plates pre-coated with VP6 group A rotavirus specific rabbit polyclonal antibodies and ready–to-use reagents required for the test supplied by the manufacturer were brought to room temperature. A microtitre pipette was used to dispense 100µl of each diluted specimen, a positive (10^5 pfu/ml inactivated bovine rotavirus strain 3209175 in buffer containing antimicrobial agents) and a negative control (distilled water) into each separate wells of each plate carefully to avoid cross contamination. Then 100µ1 of rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxidase was added to each of plate and the plates were incubated for 1 hour at 37^{o}C in a memmert incubator (Lasec, Johannesburg, South Africa).

After the period of incubation, the plates were washed twice by hand and then 4 times on an El_x50 Auto strip washer (Bio-Tek instrument Inc., Winooski, USA) with washing buffer (tris buffered solution containing detergent and antimicrobial agent supplied with the ELISA Kit). The plates were blot-dried using tissue paper and 50 µ1 each of substrate part A (substrate buffer + tetramethylbenzidine (TMB) + antimicrobial agent**)** and B (hydrogen peroxide) was added into each well. The plates were then incubated in the dark for 10 minutes before 50µ1 of stopping solution (0.46 mol/litre sulphuric acid) was added to each well. The test was read both visually and spectrophotometrically at a wavelength of 450nm on an El_x800 Universal Microplate Reader (Bio-Tek Instrument, Inc., USA). The 10% stool suspensions were kept at 4°C for any repeat assays. Rotavirus infection was diagnosed if rotavirus antigen was present in a stool specimen.

Any specimen well that showed a blue colour visually at the end of the test period was interpreted as positive provided the negative control well was colourless. Any specimen well with no visible colour was considered negative. However if the negative control well showed any visible blue colour, the test was repeated. In cases where after the addition of substrate the content of a well turned dark blue and formed a blue-black precipitate, the specimen was interpreted as positive. The negative control value, or the mean of negative control values, should be less than 0.150 absorbance units (A_{450}). The cut-off value was

calculated by adding 0.100 absorbance units to the negative control value or mean value when more than one negative control was included. Any clinical specimen with absorbance value greater than the cut-off value was considered positive while any with absorbance value less than the cut-off was considered negative.

3.6.2 Negative Staining for Detection of Rotavirus by EM

A droplet of fresh solid or 10 µl of watery faecal specimens and a drop or 10 µl of distilled water was placed onto a piece of parafilm and mixed thoroughly. Equal volumes of the faecal suspension and 3% phosphotungstic acid (PTA) (Appendix 2) at a pH of 6.3 was mixed and incubated for 2-3 minutes at room temperature. A drop of the PTA: faecal suspension was placed on top of formvar-carbon coated grids and drain-blot-dry slightly on the side of a filter paper. The grids were examined using a Phillips 301 transmission electron microscope (TEM) at a magnification of x45 000 as described by Steele *et al*. (1998a). Any specimen that showed a distinctive appearance of a well-defined rim or wheel with short spikes radiating from a wide hub against a dark background was regarded as positive.

3.6.3 Rotavirus Strains Characterization

All the specimens that tested positive in the rotavirus group A ELISA were subjected to further analysis by polyacrylamide gel electrophoresis (PAGE) according to the method of Herring *et al*. (1982) followed by silver nitrate staining after extraction of the dsRNA. Polyacrylamide gel electrophoresis was carried out to detect the different patterns of rotavirus genomic RNA present in the specimens thus identifying the strains and to screen for any possible non-group A rotaviruses.

3.6.3.1 Extraction of Double Stranded RNA from Stool Specimens

The viral RNA genomes from all rotavirus positive specimens were extracted according to previously published methods (Steele and Alexander 1987).

Previously prepared 10% dilution of each stool suspension was vortexed in a CSV-90 Auto Vortex Mixer (Multi Vortex Scientific Industries, U.S.A), allowed to stand for 30 minutes and the supernatant used for extraction. About 450µ l of supernatant of each specimen was placed in a labelled eppendorf tube and 50µ l of pre-warmed 1M sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS) at pH 5.0 (Appendix 3) was added to the

suspension and mixed thoroughly. The NaAc was pre-warmed to prevent SDS from precipitating out. The mixture was incubated in a 37^{o} C water bath for 15 minutes. After incubation, 500µ l of phenol/chloroform (1:1) (Appendix 3) mixture was added to each eppendorf tube and the mixture vortexed for 1 minute. In order to reduce the air pressure within the tube, the tube was opened and immediately resealed prior to vortexing for 1 minute. The tube was again incubated for a further 15 minutes in a 56^{o}C water bath. The contents of the tubes were then centrifuged for 3 minutes at 12 000 rpm in a Sorvall MC-12V centrifuge (Dupont, Newton, USA). The upper aqueous phase containing the dsRNA was carefully pipetted into a new labeled sterile eppendorf tube while avoiding the interphase layer. In very rare situation, 250µ1 of phenol/chloroform mixture was again added to the content of the old eppendorf tube from which the aqueous phase was pipetted out and incubated for another 15 minutes at 56^{o} C, centrifuged at 12 000 rpm for 3 minutes and the upper aqueous phase removed and added to the content of the new eppendorf tube. A 1/10 volume (40µ l) of 3M NaAc, followed by 2 volume (1ml) ice-cold absolute ethanol (Appendix 3) was added to the aqueous solution and the solution was mixed gently by turning the tube over 4-6 times. The aqueous extract was incubated at -20^{o}C overnight in order to precipitate the dsRNA.

After overnight incubation, the extract was centrifuged at 4^{o}C for 15 minutes at 12 000 rpm in a centrifuge, eppendorf 5415R (Eppendorf, Hamburg, Germany) to pellet the dsRNA. The supernatant was immediately and carefully poured off and the pellet at the bottom of the tube allowed to air-dry (to remove excess alcohol) and re-suspended in 30µl PAGE sample dye before loading on a PAGE gel.

3.6.3.2 Polyacrylamide Gel Electrophoresis

Electrophoresis of the dsRNA extract was performed in 10% polyacrylamide vertical slab gel using the discontinuous buffer system described by Laemmli (1970). Electrophoresis was performed at a constant voltage of 100V for 18-20 hours at room temperature. A 30% stacking gel cast on a removable pair of glass plates was employed to enhance the resolution of the RNA bands. The plates were cleaned with alcohol and a 1.5mm spacer inserted between them on the left and right sides. This set up was clamped upright into a gel stand on an even surface to ensure the plates and spacers were leveled to prevent leakage. A comb was inserted between the plates and the position of the tip of its teeth marked and it was removed.

Preparation of 10% Resolving gel: A 1.5mm 10% resolving gel was prepared by pipetting 15.8ml distilled water, followed by 10ml 30% acrylamide stock and 3.75ml resolving buffer into a beaker. The beaker was swirled gently and 15µ l of N, N, N', N' tetra methyl ethylene diamine (TEMED) and 450µ l of 10% ammonium persulphate was added respectively. The content of the beaker was mixed thoroughly and poured immediately and continuously into the space between the pair of glass plates up to the marked level ensuring no air bubble was trapped. Approximately 2ml of distilled water was pipetted on top of the gel layer and left to polymerize at room temperature for 2 hour. The distilled water was poured off before the addition of stacking gel.

Preparation of 3% Spacer Gel (Stacking Gel): A 3% stacking gel was prepared by pipetting 1.6ml of 30% acrylamide stock (Appendix 4), 6.8ml of distilled water and 1.25ml of spacer buffer into a beaker and swirled gently. This was followed by the addition of 5µl of TEMED and 150µl of 10% ammonium persulphate (Appendix 4). The content of the beaker was mixed thoroughly and poured immediately on top of the 10% resolving gel. A 15-teeth comb was immediately inserted through the stacking gel and more gel was poured to fill up space to the brim. Any overflow was sucked up with a 1ml pipette. The gel was left to polymerize for 1 hour at room temperature before loading. After setting for 1hour, the combs were gently and carefully removed. Wells were formed in the gel by the teeth of the comb. The gel slab was carefully clamped into a SE600 Electrophoresis Unit (Hoefer, San Francisco, USA) and 1 X Tris-glycine running buffer (Appendix 4) was added to the bottom and top buffer troughs. The wells were filled with 1 X Tris-glycine running buffer to rinse them and 30µl of each of the previously prepared extracted dsRNA re-suspended in PAGE sample dye was carefully loaded into each respective well using microtitre pipette. The gel was run at a constant voltage of 100V for 18-20 hours at room temperature. The gel was silver stained at the end of the run and visualized according to the method described by Herring *et al*. (1982) with slight modification.

Silver Staining and Photographing of Gel: After the period of electrophoresis, the apparatus was switched off, the glass plates carefully taken out of the tank unit and unclamped. The gel was removed from the glass plates

using plastic spacer and cut at the bottom right hand corner for orientation. The gel was carefully hand lifted and placed in a square baking glass dish containing fixing solution 1 and incubated for 30 minutes while swirling on a Red Rotor orbital shaker (Hoefer, San Francisco, USA). After 30 minutes, fixing solution 1 was drained off using suction pump and replaced by fixing solution 2. The gel was incubated for another 30 minutes on the orbital shaker. Fixing solution 2 was drained off and silver nitrate (AgNO3) staining solution was immediately added. The gel was again incubated for a further 30 minutes on the orbital shaker. The $AgNO_3$ staining solution was drained off and the gel was carefully rinsed twice with distilled water for 2 minutes each time. After each washing, the distilled water was drained off and fresh distilled water added. After the last wash, 50ml of developing solution was added to the gel and swirled gently by hand for 30 seconds. The solution was drained off, to remove black silver precipitate. About 200ml of remaining developing solution was then added and the gel was incubated on an orbital shaker for approximately 5 minutes or until RNA bands could be seen. The development solution was drained off and stopping solution was added to stop the bands on gel from developing further. The gel was incubated for another 2 minutes, rinsed in distilled water and dried overnight. The reagents for silver staining were prepared as described in Apendix 5.

The gel was prepared for drying by carefully handlifing and placing it on a wet 31 X 31 cm cellophane sheet (The Gel Company, San Francisco, USA) spread on a platform. The gel was labelled, superimposed with another wet cellophane sheet and the edges were secured with gel drying screws. The gel was fitted into a gel dryer and dried overnight at low breeze. When it was dried, the edges were trimmed off and the protected gel photographed and /or stored away.

3.6.4 Determination of VP6 Subgroup Specificity

The VP6 subgroup specificity was determined by ELISA technique previously described by Steele and Alexander (1988) using group antigen (A3M4) (Beards *et al*., 1984) and Subgroup I (Mouse # 255/60/125/14) and Subgroup II (Mouse # 631/9/104/56) monoclonal antibodies (Greenberg *et al*., 1983) kindly donated by Harry Greenberg, Stanford Medical School, California, USA. Briefly, coating antibody, rabbit anti-human rotavirus #0903 (DAKO, Cambridge shire, United Kingdom) was diluted in a ratio of 1:50 with 0.05M-

carbonate/bicarbonate buffers. To each well of 96-well costar® flat bottom microtiter plates (Corning Incorporated, New York, USA), 100µl of the diluted antibody solution was added. The plates were incubated overnight at 4˚C.

The coating solution was drained off and the plates were washed twice by hand and four times on an El_x50 Auto Strip Washer (Bio-Tek Instruments, Inc., Winooski, USA) with phosphate buffered saline/Tween (PBS/T). Hundred microlitre of PBS/T/ethylene diamine tetra acetic acid (EDTA) (Appendix 6) was added to each well and this was followed by the addition of 50µl of each 10% suspension of specimen to three consecutive wells. A negative control (distilled water) and positive control (known rotavirus positive stool specimen for group antigen, Subgroup I and subgroup II respectively) were added to the plates and incubated overnight at 4˚C. The plates were washed twice by hand and four times on an Auto Strip Washer with PBS/T.

After the period of incubation, 100µl of monoclonal antibodies, Rotavirus Group Antigen (A3M4) (Beards *et al*., 1984), Subgroup I (Mouse # 255/60/125/14) and Subgroup II (Mouse # 631/104/56) (Greenberg *et al*., 1983) each diluted 1: 5000 in PBS/T/BSA (Appendix 6) were transferred into correct microtiter wells (i.e. every third row for each monoclonal antibody) and the plates were incubated at 37˚C for 3 hours. The plates were then drained and washed 4-6 times (twice by hand and 4 times on an Auto Strip washer) with PBS/T.

Horseradish peroxidase conjugated goat anti-mouse IgG (Zymed, San Francisco, USA) was diluted 1:1000 with PBS/T/BSA and 100µ l was dispensed into each well. The plates were incubated at 37˚C for 2 hours after which the conjugated antibody was discarded and the plates were washed 4-6 times in PBS/T. After washing, 100µ l of substrate, comprising of hydrogen peroxide and 3, 3', 5, 5'- tetramethyibenzidine, TMB (TMB Enzymatic Kit, Roche) was dispensed into each well and incubated for 10 minutes in the dark after which the plates were read visually. Stopping solution (5% sulphuric acid) was added and the plates were read spectrophotometrically at 450nm on an EL_X800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, USA).

For each specimen, the ratio between the optical density (OD) readings obtained for the two subgroups monoclonal antibodies was determined. A ratio of >1.7 (SGI: SGII) was used as the criterion for subgroup I specificity and a ratio of >2.0 (SGII: SGI) was used as the criterion for subgroup II specificity. The OD cut-off value for the group specific monoclonal antibody was >1.0 for a positive result.

3.6.5 VP7 and VP4 Genotyping of Rotaviruses

Genotyping was carried out by reverse transcription-polymerase chain reaction (RT-PCR) using purified rotavirus dsRNA. The viral genomic dsRNA of all the ELISA positive group A rotaviruses were purified by extraction from each specimen using the TRIzol® (Life Technologies, New York, USA) extraction method.

3.6.5.1 Extraction of Rotavirus dsRNA by TRIzol® Method

Previously prepared 10% stool suspension of each rotavirus EIA positive specimen was vortexed, mixed thoroughly and allowed to stand for about 30 minutes. Two hundred and fifty microlitre of the supernatant was transferred into a sterile eppendorf tube and 750 µ l of TRIzol® or Tri® reagent (MRC Molecular Research Centre Inc.) was added to each tube. The content of the tube was vortexed for 30 seconds and incubated at room temperature for 5 minutes after which 200µ l of chloroform was added to each tube. The mixture was again vortexed for 1 minute, incubated at room temperature for 3 minutes and then centrifuged at 4^{o}C at 12 000 rpm in a centrifuge, eppendorf model 5415R (Eppendorf, Hamburg, Germany) for 15 minutes.

Approximately 450µl of the clear aqueous phase was pipetted out and transferred into a sterile eppendorf tube and 700µl of ice-cold isopropyl alcohol was added and the mixture was gently mixed prior to incubation at room temperature for 20 minutes. The tubes were centrifuged at 4^{o}C at 12 000 rpm in a centrifuge, eppendorf 2415R (Eppendorf, Hamburg, Germany) for 15 minutes to pellet the dsRNA. The supernatant was immediately and carefully poured off and the dsRNA pellet allowed to air dry. The dsRNA was re-suspended in 15µl DEPC treated water (BIO 101, Carlsbad, USA) and stored at -20^{o}C until used for RT-PCR reaction.

3.6.5.2 VP7 First Round RT-PCR Amplification

Portions of previously extracted rotavirus dsRNA were used as templates in reverse transcription reaction to generate complementary DNA (cDNA) copies that were amplified by PCR using VP7 consensus primers pair (Gouvea *et al*., 1990b).

In order to synthesize full-length VP7 cDNA copies of the previously extracted dsRNA, 1 µl of the specific primer pair (sBeg9/End9) was added to 8 µl of the extracted viral RNA in a 500 µl PCR tube. The primer pair sBeg9 and End9 (Table 3.1) were used at concentrations of 190nM and 200nM respectively. The mixture was heated at 95°C for 5 minutes to denature the dsRNA and immediately chilled on ice for 2 minutes. This was followed by the addition of 3.2 µl of the reverse transcriptase master mix (RT-MM) consisting of 0.2 µ l of 10mM dATP, 0.2 µ l of 10mM dCTP, 0.2 µ l of 10mM dGTP, 0.2 µ l of 10mM dTTP, 2.0 µ l of 5x avian myeloblastosis virus (AMV) buffer, and 0.2 µ l AMV reverse transcriptase enzyme (Promega). The mixture was centrifuged and incubated at 42˚C in a heating block for 30 minutes for reverse transcription to occur.

After the generation of the VP7 cDNA by reverse transcription, the tubes were removed from the heating block, placed on ice and 40 µ l of PCR master mix (PCR-MM) was added to each tube and they were subjected to PCR cycles to amplify both viral strands. The PCR-MM consisted of 1 µ l of 10mM dATP, 1 µ l of 10mM dCTP, 1 µ l of 10mM dGTP, 1 µ l of 10mM dTTP, 4 µ l of 10x Taq buffer without M_gCl_2, 2.4 µ l of 25mM M_gC1_2, Taq polymerase enzyme (Promega) and 30 µ l sterile Baxter water. A negative control containing only the PCR-MM was included in each PCR assay. The amplification was performed in a GeneAMP PCR System 2400 (PERKIN ELMER, NORWALK, U.S.A) programmed to run 30 cycles. Each cycle comprised 1 minute denaturing at 94°C, 2 minutes annealing at 42°C, and 3 minutes extending at 72°C followed by a final extension cycle at 72°C for 7 minutes.

At the end of the 30 cycles, the amplified product was mixed with bromophenol blue (BPB) tracking dye and run by gel electrophoresis in 1.2% agarose gels (Seakem, Flowgen) containing 4µ l /ml of ethidium bromide (Appendix 7). The specimens were run alongside a known molecular weight marker (Promega) to determine the sizes of the different bands obtained. The cDNA amplification product appeared as a thick band when viewed under UV light. Products were stored at -20^{o}C.

Table 3:1 Oligonucleotide primers used for RT-PCR and genotyping

Primer	Sequence (5'-3')	Polarity	Position (nt)
For VP7 RT-PCR and Typing			
SBeg9	GGCTTTAAAAGAGAGAATTTC	+	1-21
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	+	1-28
End9	GGTCACATCATACAATTCTAATCTAAG	-	1062-1036
EndA	ATAGTATAAAATACTTGCCACCA	-	942-919
RVG9	GGTCACATCATACAATTCT	-	
aAT8	GTCACACCATTTGTAAATTCG	+	
aBT1	CAAGTACTCAAATCAATGTGG	+	
aCT2	CAATGATATTAACACATTTTCTGTG	+	
aDT4	CGTTTCTGGTGAGGAGTTG	+	
aET3	CGTTTGAAGAAGTTGCAACAG	+	
aFT9	CTAGATGTAACTACAACTAC	+	
For VP4 RT-PCR and Typing			
Con3	TGGCTTCGCCATTTTATATAGACA	+	11-32
Con2	ATTTCGGACCATTTATAACC	-	887-868
IT-1	ACTTGGATAACGTGC	-	356-339
2T-1	CTATTGTTAGAGGTTAGAGTC	-	494-474
3T-1	TGTTGATTAGTTGGATTCAA	-	278-259
4T-1	TGAGACATGCAATTGGAC	-	402-385
5T-1	ATCATAGTTAGTAGTCGG	-	594-575

VP7 human primers by Gouvea *et al*. (1990b) and VP4 primers by Gentsch *et al*. (1992)

3.6.5.3 VP7 Second-Round Nested-PCR Amplification

The re-amplification of cDNA was carried out using a cocktail (genotype-specific) of primers (Table 3.1) so as to determine the specific genotypes depending on their relative migration on agarose gel. The full-length VP7 cDNA obtained from the first round amplification served as the template for the second typing amplification. Between 0.5 to 6 µ l of the full-length VP7 cDNA was transferred to 500 µ l PCR tube and 40µ l of PCR-MM was added to each specimen tube. The PCR-MM was similar to that described above except that 1 µ l of each of the six serotype-specific primers and common primer RVG9 or sBeg9 or 9Con1 with concentration of approximately 200nM was added to the mix (Table 3.1). The samples were amplified on the same PCR program described above and the amplified products were examined on 2% agarose gels.

3.6.5.4 Running Agarose Gel

To 40ml 1 X Tris acetate EDTA (TAE) buffer (pH 7.9) in a small glass bottle was added 0.6g agarose and boiled in a microwave oven for 3 minutes. After boiling, the volume was made up to 50mls with distilled water and cooled to about 50^{o}C. Two µl of ethidium bromide was added, swirled and the gel poured into gel cassette with a comb in place. The gel was allowed to solidify at room temperature for 30minutes and the comb removed. The cassette tray was submerged beneath TAE running buffer in an electrophoresis apparatus, Submarine Agarose Gel Unit, HE33 (Hoefer, San Francisco, USA). About 7 µl of each product and a 100-1500 base-pair (bp) molecular size marker was mixed with BPB and loaded into separate wells of the gel. The first and last wells of the gels were loaded with the molecular size marker. The gel was electrophoresed at 90V for 30-45 minutes at room temperature until the BPB reached the bottom of the vertical gel. The gel was removed,

viewed under UV light and photographed (UVP Gel documentation system, England). The various genotypes separated out as different bands on the gels and were determined based on their sizes.

3.6.5.5 VP4 Genotyping by PCR

3.6.5.6 VP4 First Round Amplification

In the case of P-typing, the method of Gentsch *et al*. (1992) was used to generate full length VP8* gene products using consensus primers Con2 and Con3 (Table 3.1) at concentration of 200mM.

3.6.5.7 VP4 Second-Round Nested-PCR Amplification

The second round VP4 genotyping PCR amplification was carried out with primer, Con3 and a cocktail of genotype-specific primers described by Gentsch *et al*. (1992) The PCR products were examined on agarose gels and the genotypes assigned according to the genome sizes.

3.7 Astroviruses and Adenoviruses

Because of limitation of reagents and resources, 282 specimens were randomly selected from the 1063 stool specimen previously analyzed for rotaviruses and tested for enteric adenoviruses (EAds) and human astroviruses (HAstVs) using commercially available EIA kits. Sample size (282) was determined using the sample size for adequate power formula (Appendix 8) in conjunction with Epi Info Stat Cal Utilities 2002.

3.7.1 Detection of Astrovirus Antigen in Stool Specimens by ELISA

Astrovirus antigen was detected using Astrovirus IDEIA^{TM} DAKO, UK kit code Number K6040. The test was carried out according to the manufacturer's instructions.

The test procedure used here was essentially the same with that described for rotavirus above, except for the following:

1. Specimens were diluted in sample diluent (tris buffered saline containing antimicrobial agent and green dye) provided in the kit instead of distilled water.
2. Antibody reagent was added to the wells after the addition of test specimens and incubated for 30 minutes at 37°C before the addition of conjugate, which was also incubated for 30 minutes instead of 60 minutes.

Any specimen well that showed a blue colour visually at the end of the test period was interpreted as positive provided the negative control well was colourless. Any specimen well with no visible colour was considered negative. However if the negative control well showed any visible blue colour, the test was repeated. In cases where after the addition of substrate the content of a well turned dark blue and formed a blue-black precipitate, the specimen was interpreted as positive.

A cut-off value of 0.150 absorbance units was used. Any clinical specimen with absorbance value greater than the cut-off value was considered positive while any with absorbance value less than the cut-off was considered negative. Astrovirus infection was diagnosed if astrovirus antigen was present in a stool specimen.

3.7.2 RT-PCR Analysis of Astroviruses

Analysis of the EIA positive astrovirus specimens by RT-PCR was successfully performed after clarification of the specimens using Genetron and extraction of the ssRNA using QIAamp^{R} Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. RT-PCR was carried out to confirm astrovirus in the EIA positive specimens

3.7.2.1 Stool Clarification

A 10% dilution of each specimen was made in sterile labeled specimen bottles using distilled water. The suspension was mixed thoroughly with a vortex mixer for 30 seconds and allowed to settle for about 30 minutes prior to testing. Two hundred microliters (200 µl) of each stool suspension (10-50% in water) was mixed with an equal volume of Genetron (1,1,2-trichlorotrifluoroethane: Sigma T-5271) or Freon; the suspension was mixed thoroughly for 30 seconds; and centrifuged at 12000 rpm for 5 minutes at room temperature. One hundred and forty microlitre of supernatant was withdrawn for viral RNA extraction.

3.7.2.2 QIAamp® Extraction of Astrovirus ssRNA

Fifteen microliters (15 µl) of QIAGEN® Proteinase K were pipetted into the bottom of 1.5ml microfuge tubes before 140 µl of each stool suspension supernatant was added. Following the addition of 140 µl of buffer, all the samples were mixed by pulse-vortexing

for 15 seconds and incubated at 56°C for 10 min. One hundred and forty microlitres (140 µl) of 96% ethanol were added to the specimens before mixing by pulse-vortexing for 15 seconds. The mixtures were subsequently applied to the QIAamp® spin columns (in a 2ml collection tube) and centrifuged at 8000 rpm for 1 min in a centrifuge, eppendorf model 5415R (Eppendorf, Hamburg, Germany). The spin columns containing the viral RNA were washed with 400 µl of Buffer AW1 and centrifuged at 8000 rpm for 1 min. A second wash step was performed with 400 µl of Buffer AW2 followed by centrifugation at 13 000 rpm for 3 min. The viral RNA was eluted from the spin column by the addition of 60 µl of nuclease-free water (Promega Corp., Madison, WI) followed by incubation at room temperature for 1 min and centrifugation at 8 000 rpm for 1 min. An extraction control, nuclease-free water (Promega Corp.) was included for each extraction. Isolated viral RNA was stored at -20°C until further use.

3.7.2.3 Amplification of Astrovirus Gene by RT-PCR

Group-Specific Oligonucleotide Primers: Mon2/Mon67 (Mon2 reverse primer: 5'-GCT TCT GAT TAA ATC AAT TTT-3'; Mon67 forward primer: 5'-CGA GTA GGA TCG AGG GTA-3'; Mitchell *et al*., 1995) which amplifies a 89 bp region of the capsid gene (nt 6909-6797; HAstV-2 [L13745.1]) was used for the screening of the EIA positive specimens for HAstVs.

Mon348/Mon340 (Mon348 reverse primer: 5'-ACA TGT GCT GCT GTT ACT ATG-3'; Mon340 forward primer: 5'-CGT CAT TAT TTG TTG TCA TAC-3'; Belliot *et al*., 1997) which amplifes a 289 bp within ORF1a region (nt 1470-1182; HAstV-2 [L137451]) was used for the detection of HAstV RNA in antigen positive samples and specimens found to be HAstV RNA negative using the Mon2/Mon67 primer pair. Mon2/prBEG: this primer pair (Mon2 reverse primer: prBEG forward primer: 5'-ACC GTG TAA CCC TCC TCT C-3'; Saito *et al*., 1995) amplifies a 319 bp region at the 3' end of ORF2 (nt 6453-6771; HAstV-1 [L23513.1]) of all HAstVs except type 4. The primers used in this study were synthesised and purified by Sigma-Genosys Ltd., Pampisford, UK.

A positive control (HAstV-1 positive stool), negative control (nuclease-free water) and negative extraction control were included in all RT-PCR reactions. Unless stated to the contrary, RT-PCR reagents were from Promega Corp.

Mon2/Mon67 RT was performed in a 48 μ l reaction cocktail. The cocktail contained 10 μl extracted RNA, 5 U of AMV reverse transcriptase, 1 X PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$) (1:1 Opti-Prime™ 10X PCR buffers 1 and 2, Stratagene, La Jolla, CA), 1 mM dithiothreitol (DTT), 20 pmol reverse primer and 4 U ribonuclease inhibitor (rRNasin®) and 0.5 mM dNTPs (PCR grade: Roche Molecular Biochemicals, Mannheim, Germany). The reaction mix was incubated for 1 h at 42°C. After completion of the RT reaction, 20 pmol of the forward primer and 2.5U Taq DNA polymerase was added. The PCR cycles included: denaturation for 2 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 45°C, elongation for 1 min at 72°C and final extension for 10 min at 72°C.
Mon348/Mon340 RT was performed in a 48 μ l reaction cocktail. The cocktail contained 10 μl extracted RNA, 5 U AMV reverse transcriptase, 1 X PCR buffer (10 mM Tris-HCl [pH 8.8], 25 mM KCl, 3.5 mM $MgCl_2$) (Opti-Prime™ 10X PCR buffer 7, Stratagene, La Jolla, CA), 1 mM DTT, 20 pmol reverse primer and 4 U rRNasin® and 0.5 mM dNTPs (Roche). The reaction mix was incubated for 1 h at 42°C. After completion of the RT reaction, 20 pmol of the forward primer and 2.5 U Taq DNA polymerase was added. The PCR cycles included: denaturation for 2 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50°C, elongation for 1 min at 72°C and final extension for 10 min at 72°C.

Mon2/prBEG nucleic acid (RNA) amplification was essentially the same as for Mon2/Mon67 primer pair except for 1X PCR buffer (10mM Tris-HCl [pH 9.0],

50 mM KCl, 0.1 % Triton® X-100, and 1.5 mM $MgCl_2$). The steps of the RT-PCR were the same as for Mon2/Mon67 primer pair. The amplified products were electrophoresed and examined under UV light as described previously.

Negative staining EM was performed on the positive HAstV specimens to further confirm the presence of viral particles. The procedure was essentially the same with that described previously for some of the ELISA positive rotavirus specimens.

3.7.3 Detection of Adenovirus Antigen in Stool Specimens by ELISA

Adenovirus antigen was detected using RIDASCREEN^R Adenovirus r-biopharm, Germany. The test was carried out according to the manufacturer's instructions. The test procedure used here was essentially the same with that described for rotavirus except for the use of distilled water as wash buffer instead of PBS/T in all the washing steps.

Any specimen well that showed a blue colour visually at the end of the test period was interpreted as positive provided the negative control well was colourless. Any specimen well with no visible colour was considered negative. However if the negative control well showed any visible blue colour, the test was repeated. In cases where after the addition of substrate the content of a well turned dark blue and formed a blue-black precipitate, the specimen was interpreted as positive.

The negative control value, or the mean of negative control values, should be less than 0.150 absorbance units (A_{450}). The cut-off value was calculated by adding 0.150 absorbance units to the negative control value or mean value when more than one negative control was included. Any clinical specimen with absorbance value greater than the cut-off value was considered positive while any with absorbance value less than the cut-off was considered negative.

3.7.4 Detection of Enteric Adenovirus Serotypes 40 and 41

The enteric adenovirus positive specimens were further examined for adenovirus serotypes 40 and 41 using Premier Adenoclone^R -Type 40/41 EIA kit, USA, Catalog No. 696006. The test was carried out according to the manufacturer's instructions and was used essentially for the qualitative detection of adenovirus serotypes 40 and 41.

The test procedure used here was essentially the same with that described for rotavirus. Here, a transfer or precision pipette provided in the kit was used for addition of specimens to each well; distilled water was used as wash buffer instead of PBS/T in all the washing steps and all incubations were at room temperature.

3.7.5 Data Analysis

Data generated from questionnaires and the results obtained from laboratory analysis were entered into excel and reduced to percentages by descriptive statistics. SPSS 11.0 data analysis programme was used to analyse the data. Chi Square and Odds ratio were calculated to determine association between viral infection and the various parameters studied. The ratio to moving average method was used to determine seasonal variation.

CHAPTER FOUR

4.0 RESULTS

4.1 Analysis of the Total Study Population

Analysis of the study population showed that about 14.6% (155/170) of the children were aged 0-6 months, while 23.3% (248/170) and 10.2% (109/170) were aged 7-12 months and 13-18 months respectively. Those aged 19-24 months, 25-36 months, 37-48 months and 49-60 months made up 18.8% (200/170), 16.1% (171/170), 8.6% (91/170) and 8.4% (89/170) of the study population respectively.

A history of diarrhoea could be recalled in 66.7% of the 869 diarrhoeic children enrolled in the study, it could not be recalled in 3.3% and 30% had not experienced it. More than half (55.5%) of the specimens were collected within the first four days of the symptom while 5.74% were collected after a week (>7 days) of onset of symptoms. There was no statistically significant difference between when diarrhoea started and the detection of rotavirus antigen ($\chi^2 = 40.093$, df = 24, $p > 0.05$).

The frequency of stool passed per day varied among the children. About 42% of the children passed stool 1-3 times a day, 25.2%, 4-6 times a day, 7.1%, 7-10 times a day, 1.8% more than 10 times a day and 5.6% passed stool uncountable times. The four types of stool observed in this study were, bloody (0.2%: 2/1063), mucoid (4.9%: 52/1063), normal (16.6%: 176/1063) and watery (59.1%: 628/1063). Combinations of these four types of stool were also observed with the watery and mucoid (WM) combination occurring more frequently (13.2%: 140/1063), followed by the watery, mucoid and bloody (WMB) (4.1%: 44/1063), the Mucoid and bloody (MB) (1.0%: 11/1063) and the watery and bloody (WB) (0.9%: 10/1063) combinations. The most common stool type among children in the study population was watery stool.

About 40.4% of the children had not been treated with any antibiotics before stool specimens were collected, however, 54.6% had received one or more types of antibiotics and there was no response from 5% of them. The most widely used antibiotic by the children was flagyl (metronidazole) followed by septrin (co-trimaxazole). Majority (87.7%) of the children had nobody in the household with diarrheoa at the time of sample collection. However, 10.9% and 1.4% did have a member of the family and a neighbor respectively, with the symptom of diarrheoa. About 45.7% of the children sampled showed no sign of dehydration. However, 39.3%, 7.7%, and 7.2%, showed sign of mild, moderate and severe dehydration respectively. About 8.4% of the children looked very ill, 40.4% looked ill and 51.2% looked normal. Tears were seen in 12.1% of the children studied and 10.3% had rashes on various parts of their body.

Oral rehydration salts (ORS) were used by only 30.9% of the children while 69.1% did not use them. Analysis of the feeding regimens showed that 4.1% of the children studied were exclusively breastfed, 34.3% fed on both breast milk and solid food, 4.1% were given water in addition to breast milk and 57.4% ate solid food. The child's food was mainly prepared by the mother (91.6%) but sometimes by the nanny (1.4%) or just anybody (7.0%). Drinking boiled water was found to be uncommon, as 88% did not drink boiled water. About 25.4% of the children came from homes with various domestic animals and about 17.6% of them had previous contact with these animals.

About 60.7% of the children belonged to a low socio-economic background, while 15.2% and 24.1% had a high and medium socio economic background respectively. About 13.4% (142/1063) of the children studied were either attending a day care (72.5%: 103/142) or an Islamic school (27.5%: 39/142) while 86.6% (921/1063) did not.

4.2 Rotaviruses

4.2.1 Detection by ELISA

Of the 1063 stool specimens tested for group A rotavirus antigen, 170 (16%) were positive. Rotavirus antigen was detected in 18% (156/869) of the diarrhoeic stools and in 7.2% (14/194) of the non-diarrhoeic stools.

The children with diarrhoea were two and a half times more likely to have been infected with rotavirus than those without diarrhoea (odds ratio = 2.50 at 95% CI). There was a significant association between diarrhoea and rotavirus infection ($\chi^2 = 13.604$, df = 1, $p < 0.01$). There was significant relationship between diarrhoea duration and the presence of rotavirus antigen in stool ($\chi^2 = 43$, df = 25, $p < 0.05$). Detection of rotavirus antigen in stool specimen was possible from the first day to over seven days of diarrhoea and even in diarrhoea that has persisted for months. The frequency of diarrhoea passed per day was significantly associated with infection with rotavirus ($\chi^2 = 16.707$, df = 5, $p < 0.01$). Analysis of rotavirus infection by state showed the highest prevalence (19.1%) in Kaduna State (Table 4.1). However, there was no significant difference in the prevalence by State ($\chi^2 = 4.248$, df = 3, $p > 0.05$).

There was no significant difference ($\chi^2 = 0.219$, df = 1, $p > 0.05$) in the prevalence of rotavirus among males and females although viral shedding was slightly higher in males (16.4%) than females (15.4%). This was also true for the diarrhoeic population where the prevalence of rotavirus infection was 17.8% in the males and 18.2% in the females ($\chi^2 = 0.017$, df = 1, $p > 0.05$) (Table 4.2).

Table 4.1: Distribution of rotavirus infection by state in children 0-5 years old in Northwestern Nigeria.

State	Rotavirus Positive (%)	Rotavirus Negative (%)	Total (%)
Kaduna	**70 (19.1)**	296 (80.9)	366 (34.4)
Kebbi	14 (14.6)	82 (85.4)	96 (9.0)
Sokoto	31 (13.5)	198 (86.5)	229 (21.5)

Zamfara	55 (14.8)	317 (85.2)	372 (35.0)
Total	**170 (16.0)**	**893 (84.0)**	**1063 (100.0)**

Table 4.2: Distribution of rotavirus infection by sex in children 0-5 years old in Northwestern Nigeria.

Sex	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
	Positive	Negative	Total	Positive	Negative	Total

Female	**70 (15.4)**	385 (84.6)	455 (42.8)	**65 (18.2)**	293 (81.8)	358 (41.2)
Male	**100 (16.4)**	508 (83.6)	608 (57.2)	**91 (17.8)**	420 (82.2)	511 (58.8)
Total	**170 (16.0)**	**893 (84.0)**	**1063 (100.0)**	**156 (18.0)**	**713 (82.0)**	**869 (100.0)**

Figure in parentheses represent percentages.

Rotavirus was most prevalent among children aged 0-6 months with a significant association between age group and rotavirus infection (χ^2 = 24.777, df = 6, $p < 0.01$). Generally, children under the age of 2 years were most vulnerable to rotavirus infection (Table 4.3).

The prevalence of rotavirus when examined by year of specimen collection was 13.3% (46/346) and 17.3% (124/717) for the first year of study (July 2002-July 2003) and the second year of study (August 2003-July 2004) respectively. There was however no significant relationship between year of specimen collection and rotavirus infection ($\chi^2 = 2.779$, df = 1, $p > 0.05$).

Rotavirus was detected throughout the study period with slightly higher peaks in the drier months between October and March (Figure 4.1). In order to determine seasonal variation of rotavirus infection in the region, the results were further analysed using the ratio-to-moving average method. There was a seasonal variation over time (Figure 4.2) with the frequency being higher in the drier season.

Table 4.3: Age distribution of rotavirus infection in children 0-5 years old in Northwestern Nigeria.

	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
Age Group (Months)	**Negative**	**Positive (%)**	**Total**	**Negative**	**Positive (%)**	**Total**
0-6	115	40(25.8)	155	91	39(30.0)	130
7-12	197	51(20.6)	248	156	47(23.1)	203
13-18	92	17(15.6)	109	79	15(16.0)	94
19-24	175	25(12.5)	200	144	24(14.3)	168
25-36	152	19(11.1)	171	127	16(11.2)	143
37-48	81	10(11.0)	91	60	8(11.8)	68
49-60	81	8(9.0)	89	56	7(10.3)	63
Total	**893**	**170 (16.0)**	**1063**	**713**	**156 (18.0)**	**869**

Figures in parentheses represent percent of total.

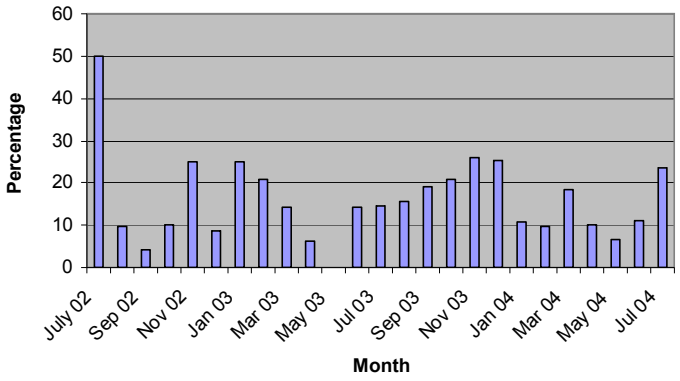


Figure 4.1: Monthly distribution of rotavirus infection in children in Northwestern Nigeria.

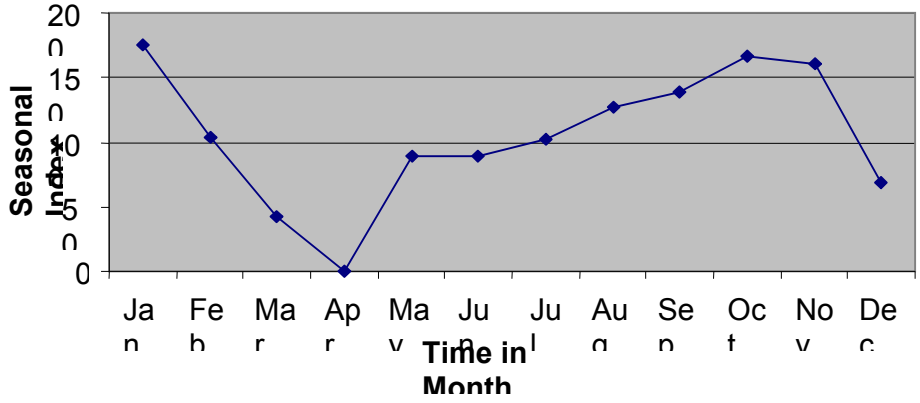

Seasonal Index
20
15
0
5
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Time in Month

Figure 4.2: Seasonal variation of rotavirus infection in children 0-5 years old in Northwestern Nigeria.

Among the diarrhoeic children positive for rotavirus, the most common stool type was the combination of watery, mucoid and bloody (WMB) stool (20.5%), followed by watery stool (19.2%) and mucoid stool in 16.0% of the children (Table 4.4). There was no statistically significant relationship between the presence of rotavirus antigen and stool type in both population.

The prevalence of rotavirus infection was highest (22.1%: 75/340) in children who passed stool continuously compared to those who did not (13.1%: 95/723). The association between continuous diarrhoea and rotavirus infection was highly significant ($\chi^2 = 13.693$, df = 1, $p < 0.01$). There was also a statistically significant relationship between the frequency of stool passed per day and shedding of rotavirus among the children ($\chi^2 = 16.842$, df = 5, $p < 0.05$). About 76.5% (130/170) of the children infected with rotavirus passed stool 1-7

times a day, 15.3% (26/170) passed stool more than 8 times a day and 8.2% (14/170) passed stool 0-1 time a day.

The most common clinical features associated with rotavirus diarrhoea in this study were, fever (71.2%: 121/170), vomiting (64.1%: 109/170) and symptoms of upper respiratory tract (URT) infection (13.5%: 23/170). Combination of two or three of these symptoms were also observed with fever and vomiting being the most frequently occurring (48.2%: 82/170), followed by symptoms of URT infection and vomiting (9.41%: 16/170) and symptoms of URT infection and fever (8.82%: 15/170). Although there were differences in the prevalences obtained for the clinical features, they were not statistically significant except in the case of vomiting where a strong association was observed ($\chi^2 = 12.793$, df = 1, $p < 0.01$). Other symptoms accompanying rotavirus diarrhoea in this study were being irritable (16/170: 9.4%), drowsiness (13/170: 7.6%), skin turgor (2/170: 1.2%) and eye infection (2/170: 1.2%). About 26.5% (45/170) and 10.6% (18/170) of the children had sunken eyes and fontanel respectively while 40% (68/170) presented with dry mouth.
Dehydration among children infected with rotavirus in the study population was found to be absent, mild, moderate or severe in 39.4%, 40.0%, 13.0% and 7.64% of cases respectively (Table 4:5). Viral infection was found to be significantly associated with dehydration ($\chi^2 = 8.930$, df = 3, $p < 0.05$).

Table 4.4: Stool type and rotavirus infection in children 0-5 years old in Northwestern Nigeria.

Stool Type	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
	Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Bloody	0 (0.00)	2 (100.0)	2 (0.2)	0 (0.0)	2 (100.0)	2 (0.23)
Mucoid	8 (15.4)	44 (84.6)	52 (4.9)	8 (16.0)	42 (84.0)	50 (5.75)
MB	1 (9.0)	10 (90.9)	11 (1.0)	0 (0.00)	9 (100.0)	9 (1.03)
Normal	12 (6.82)	164 (93.2)	176 (16.6)	3 (11.54)	23 (88.46)	26 (3.0)
Watery	118 (18.8)	510 (81.2)	628 (59.1)	114 (19.2)	480 (80.8)	594 (68.35)

WB	1 (10.0)	9 (90.0)	10 (0.9)	1 (10.0)	9 (90.0)	10 (1.15)
WM	21 (15.0)	119 (85.0)	140 (13.2)	21 (15.7)	113 (84.3)	134 (15.4)
WMB	9 (20.5)	35 (79.5)	44 (4.1)	9 (20.5)	35 (79.5)	44 (5.1)
Total	**170 (16.0)**	**893 (84.0)**	**1063 (100.0)**	**156(18.0)**	**713 (82.0)**	**869 (100.0)**

M = Mucoid **MB = Mucoid and Bloody** **WMB = Watery, Mucoid and Bloody**

W = Watery **WB = Water and Bloody**

B = Bloody **WM = Watery and Mucoid**

Figures in parentheses represent percent of total.

Table 4.5: Degree of dehydration in rotavirus infected children in Northwestern Nigeria.

Degree of Dehydration	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
	Negative	Positive	Total	Negative	Positive	Total

Absent	418	67 (39.4)	485	266	55 (17.1)	321
Mild	350	68 (40.0)	418	325	67 (17.1)	392
Moderate	60	22 (13.0)	82	58	21 (26.6)	79
Severe	65	13 (7.64)	78	64	13 (16.9)	77
Total	**893**	**170 (16.0)**	**1063**	**713**	**56 (18.0)**	**869**

Figures in parentheses represent percent of total.

Over one quarter (36.0%: 61/170) of the children infected with rotavirus had used ORS while 64.0% (109/170) had not. Almost a half (48.8%: 83/170) of them had also used antibiotics while 46.5% (79/170) had not. However this could not be ascertained in some (4.7%: 8/170) of the children. There were no statistically significant associations between ORS or antibiotic usage and the frequency of rotavirus infection ($p > 0.05$).

Rotavirus antigen was detected in 31 (22.8%) of the 136 children who were admitted for diarrhoea on hospital visit while 139 (15.0%) of the 927 children who merely required rehydration were shedding rotavirus. However, 127 of the 927 children who required

rehydration were sent to the laboratory for stool analysis and then subsequently treated. Of the 127 children sent to laboratory for stool analysis, 14 (11.0%) were shedding rotavirus. There was a statistically significant association between the outcome of hospital visit and rotavirus infection ($\chi^2 = 5.370$, df = 1, $p < 0.05$).

Analysis of the feeding regimen in the rotavirus infected children showed that the rate of viral infection varied with the food type. The prevalence of infection in the study population was highest among those exclusively breast-fed (Table 4.6). There was a significant association between food type and rotavirus infection ($\chi^2 = 8.930$, df = 3, $p < 0.05$).

Table 4.6: Prevalence of rotavirus infection among children under different feeding regimens.

Food Type	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
	Negative	Positive	Total	Negative	Positive	Total
BS	284 (77.8)	81 (22.2)	365 (34.4)	227 (75.0)	76 (25.0)	303 (34.9)

BW	36 (81.8)	8 (18.2)	44 (4.1)	28 (80.0)	7 (20.0)	35 (4.0)
EBF	34 (77.3)	10 (22.7)	44 (4.1)	28 (73.7)	10 (26.3)	38 (4.4)
Solid	539 (88.4)	71 (11.6)	610 (57.4)	430 (87.2)	63 (12.8)	493 (56.7)
Total	**893 (84.0)**	**170 (16.0)**	**1063 (100.0)**	**713 (82.0)**	**156 (18.0)**	**869 (100.0)**

EBF = Exclusive Breast Feeding

BW = Breast and Water

BS = Breast and Solid

Figures in parentheses represent percent of total.

Analysis of child's food preparation showed that there was no association between rotavirus infection and the person that prepared the child's food. In the study population, the prevalence of rotavirus was 24.3% amongs those children whom anybody could prepare their food while a prevalence of 15.4% and 3.3% were found among those whose mothers and the housemaid could prepare their food respectively (χ^2 = 4.299, df = 3, p > 0.05). Among the diarrhoeic children, 27.6%, 17.3% and 13.3 % had their food prepared by anybody, their mother and the maid respectively (χ^2 = 4.206, df = 3, p > 0.05). Even though there were differences in the prevalence, there was no statistically significant relationship between the person who prepared the child's food and the development of rotavirus diarrhoea.

The prevalence of rotavirus was 14.5% (19/131) amongst the diarrhoeic children infected with rotavirus who had another person in the household with the symptom of diarrhoea. About 20.0% of the children whose parents had diarrhoea at the time of stool collection were shedding rotavirus while infection rate was 17.4%, 13.3% and 10.53% among those whose siblings, neighbours and housemaid had diarrhoea respectively.

Of the 1063 children enrolled in this study only 12.0% (128/1063) drank boiled water as indicated by the parents while 88.0% (935/1063) did not drink boiled water (χ^2 = 2.331, df = 2, p > 0.05). Similarly of the 869 diarrhoeic children enrolled in the study, only 13.3% agreed they boil their drinking water while majority (86.7%) do not boil their drinking water. The prevalence of rotavirus infection was 20.7% and 17.53% among the children that drank boiled water and those who did not respectively (χ^2 = 0.975, df = 2, p > 0.05). There was no statistically significant association between viral infection and whether drinking water was boiled or not.

Having domesticated animals in homes where child lived had no association with rotavirus infection. About 24.7% (42/170) and 22.9% (35/156) of the children in the study and diarrhoeic population respectively whose parents owned animals were infected with rotavirus. There was no significance difference in the prevalence observed. This was also true for child contact with animal where 18.24% (31/170) and 15.4 (24/156) of the children in the study and diarrhoeic population respectively were infected with rotavirus.

The level of education of a child's parent had no relationship with viral infection in this study. Even though there was no significant relationship observed between mothers' level of education and viral infection ($\chi^2 = 8.717$, df = 5, $p > 0.05$), there were differences in the prevalence rates. The highest rate of infection in children in the study population was 19.1% among children from mothers with a secondary school education, closely followed by 18.4% in children from illiterate mothers and then 16.4%, 13.4% and 8.75% in children from mothers with an Islamic, primary and tertiary school education respectively (Table 4.7). In the diarrhoeic population, the highest rate (20.7%) of infection was recorded for children among illiterate mothers closely followed by children from mothers with a secondary school education (20.0%). In children from mothers with an Islamic, primary and tertiary school education, the rates were 17.8%, 15.3% and 10.2% respectively.

Table 4.7: Percentage distribution of rotavirus infection in relation to mother's education in Northwestern Nigerian children.

Level of Mother's Education	Study Population Rotavirus			Diarrhoeic Population Rotavirus		
	Negative	Positive	Total	Negative	Positive	Total
Illiterate	231	52 (18.4)	283	188	49 (20.7)	237
Islamic	173	34 (16.4)	207	145	33 (18.5)	178
Primary	264	41 (13.4)	305	199	36 (15.3)	235
Secondary	152	36 (19.1)	188	128	32 (20.0)	160
Tertiary	73	7 (8.75)	80	53	6 (10.2)	59
Total	**893**	**170 (16.0)**	**1063**	**713**	**156 (18.0)**	**869**

Figures in parentheses represent percent of total.

There was no relationship between socio-economic background and viral infection. Similar trend in prevalence rate in both the study ($\chi^2 = 2.936$, df = 2, $p > 0.05$) and diarrhoeic ($\chi^2 = 0.486$, df = 2, $p > 0.05$) population was observed. The highest prevalences (17.5% and 18.6% for study and diarrhoeic population respectively) were observed among children from low socio-economic background while the lowest (13.0% and 16.7% for study and diarrhoeic population respectively) was observed among those from high socio economic background.

There was no relationship between a child schooling and the rate of rotavirus infection. The prevalence of rotavirus infection among the 142 children who either attended a day care or an Islamic school in the study population was 14.1% (20/142) against 16.3% (150/921) among the 921 children who were not schooling ($\chi^2 = 2.079$, df = 2, $p > 0.05$). In the diarrhoeic population, the prevalence rate was 16.8% (18/107) and 18.1% (138/762) among children who were schooling and those not schooling respectively ($\chi^2 = 2.767$, df = 2, $p > 0.05$).

4.2.2 Electron Microscopy

All the rotavirus EIA-positive specimens examined by EM were positive. Distinctive appearances of a well-defined rim or wheel with short spikes radiating from a wide hub against a dark background were observed. Some empty particles were also observed (Figure 4.3). All the specimens examined were those that were strong ELISA positive hence with high concentration of viral particles.

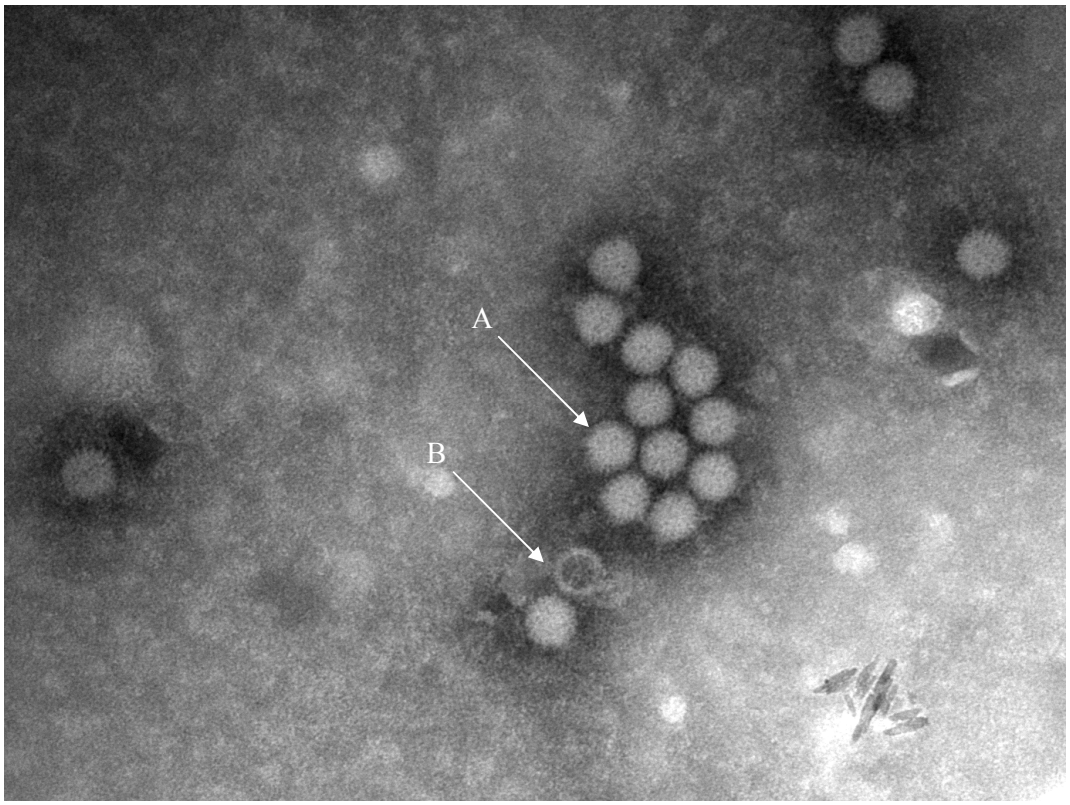


Figure 4.3: Typical rotavirus complete and empty particles observed by electro microscope in the study.

A = a complete rotavirus particle.

B = an empty rotavirus particle.

4.2.3 Electropherotypes

Of the 170 group A rotavirus positive specimens whose viral nucleic acid were extracted and subjected to PAGE, 54% (92/170) had visible profile. The long (L) electropherotypes predominated (74/92: 80.4%) with 19.6% (18/92) being the classical short (S) profiles while 2 strains showed RNA profiles typical of mixed infection. The 2 strains that exhibited mixed infection profiles were of the Long profile. Various types of RNA profiles were observed among the long and short electropherotypes

The long profile exhibited 10 distinct genomic variants that were labeled L1-L10 (Figure 4.4) while 6 genomic variants labeled S1-S6 were observed among the short profiles (Figure 4.5). Among the virus particles with the long electropherotypes, L5 and L2 were the most common, occurring with a prevalence of 28.3% and 20.3% respectively. The most predominant short profile observed was S2 with a prevalence of 33.3% (6/18) followed by S3, S5 and S1 (Table 4.8).

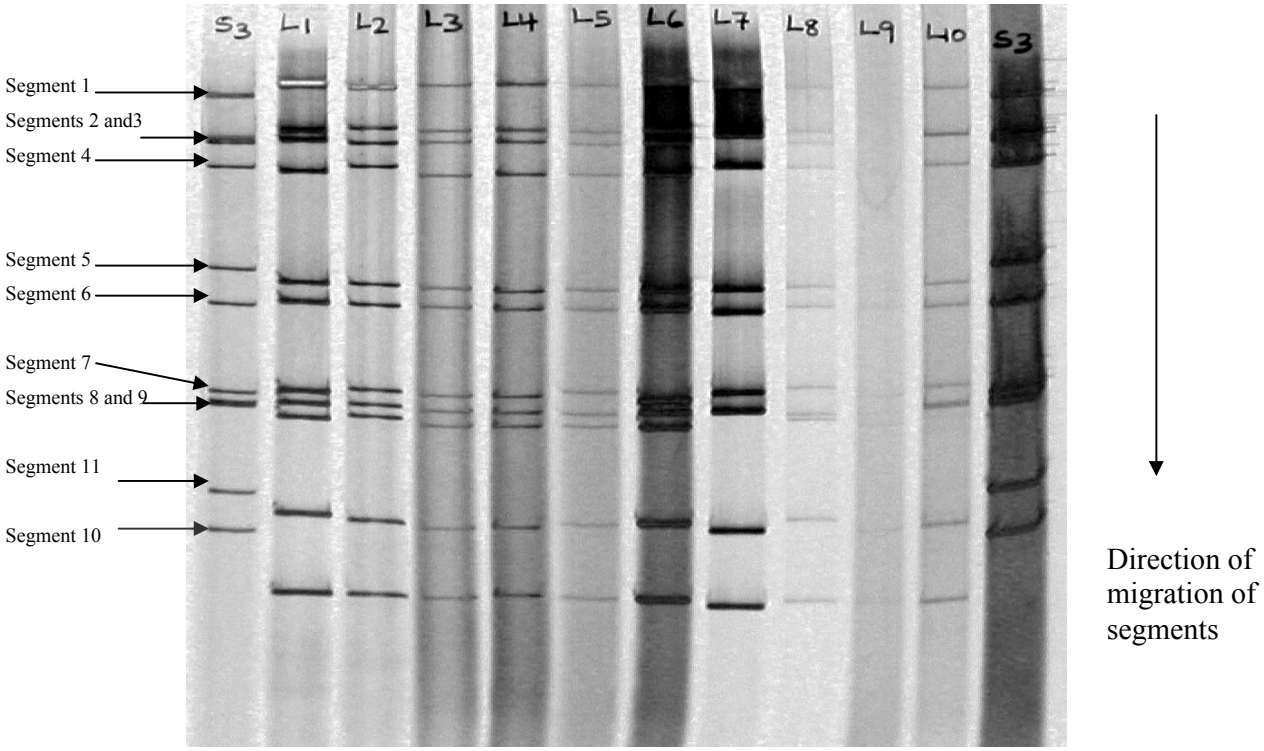


Figure 4.4: Rotavirus dsRNA long (L1-L10) electropherotypes flanked by short (S3) electropherotypes.

NB: For short electrophoretic pattern, gene segment 11 move slower than segment 10 on PAGE gel hence gel pictures are labbeled as above.

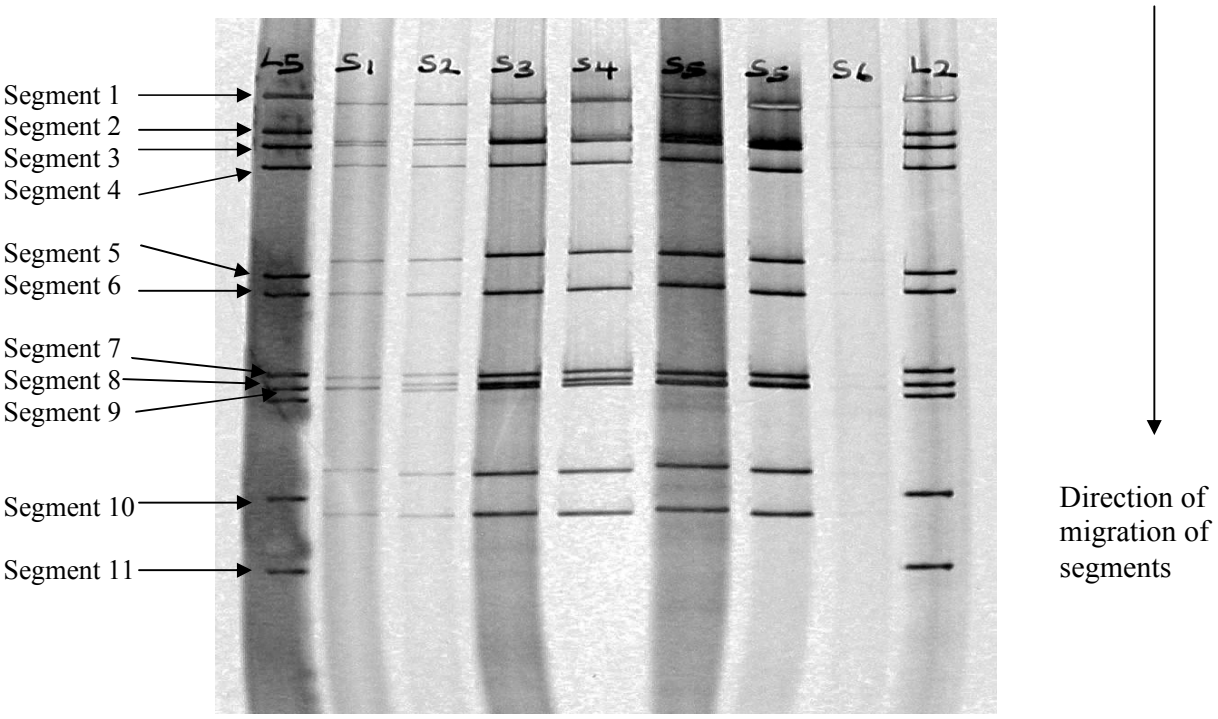


Figure 4.5: Rotavirus dsRNA short (S1-S6) electropherotypes flanked by long (L2 and L5) electropherotypes.

Table 4.8: Distribution of long electrophoretic variants of rotavirus in children 0-5 years old in Northwestern Nigeria.

Type of Long Profile	Frequency	Percentage (%)
L1	3	4.0
L2	15	20.3
L3	2	2.7
L4	6	8.0
L5	**21**	**28.3**
L6	11	14.9
L7	1	1.4
L8	4	5.4
L9	2	2.7
L10	1	1.4
L faint	5	6.8
L missing bands	1	1.4
L mix	2	2.7
Total	**74**	**100.0**

Further analysis of PAGE results showed a significant variation in the electropherotypes in the various States ($\chi^2 = 21.856$, df = 6, $p < 0.001$). The long profile predominated in Sokoto State (92.3%: 12/13), while the short profile was more common in Zamfara State (34.6%: 9/26) during the study period (Table 4.9).

The electropherotypes varied significantly in the two-year study period ($\chi^2 = 13.065$, df = 2, $p < 0.001$). In the first year of the study (July 2002-July 2003), the long profile occurred at a slightly higher rate (56%) than the short profile (44.0%). However, in the second year of study the long profile predominated, occurring with a high prevalence of 89.5% (60/67) (Table 4.10).

In the first year of the study (July 2002-July 2003), L2 and S2 were the most prevalent electropherotypes (20.0%: 5/25) followed by L5 (16.0%: 4/25). S5 and those without a visible profile circulated with a prevalence of 12.0% (3/25) each while S3 circulated with a prevalence of 8% (2/25). Each of the others that circulated within the study period occurred with a prevalence of less than 5%. In the second year of the study (August 2003-July 2004), S5 could not be traced in the population, but L1, L3, L7, L8, L9, L10, S4 and S6 emerged. In addition, those with incomplete profiles or missing bands and those with RNA profiles typical of mixed infection were also seen.

Table 4.9: Distribution of electropherotypes by state in children 0-5 years old in Northwestern Nigeria.

Type of Electropherotype	State				Total
	Kaduna	**Kebbi**	**Sokoto**	**Zamfara**	
Long	42 (85.7)	3 (75.0)	12 (92.3)	17 (65.4)	**74 (80.4)**
Short	7 (14.3)	1(25.0)	1 (7.7)	**9 (34.6)**	**18 (19.6)**
Total	**49 (100.0)**	**4 (100.0)**	**13 (100.0)**	**26 (100.0)**	**92 (100.0)**

Table 4.10: Prevalence of electropherotypes of rotavirus in children 0-5 years old by year of specimen collection.

Year	PAGE Positive		
	Long Profile (%)	Short Profile (%)	Total (%)
July 02-July 03	14 (56.0)	11 (44.0)	25 (27.2)
August 03-July 04	60 (89.5)	7 (10.5)	67 (72.8)
Total	**74 (80.4)**	**18 (19.6)**	**92 (100.0)**

Long electropherotypes were more prevalent in the age group 7-12 months (Figure 4.6). There was a statistically significant association between age group and distribution of electropherotypes ($\chi^2 = 24.777$, df = 6, $p < 0.01$).

Of the 14 rotavirus positive specimens from non-diarrhoeic population, 64.3% (9/14) had no visible RNA profile while 35.7% (5/14) had long electrophoretic profiles. The diarrhoeic specimens had at least a representative of each of the electropherotypes (except the L10 variant), including those with incomplete electrophoretic profiles and those with RNA profiles typical of mixed infection (Table 4.11). L5 was the most prevalent (12.8%: 20/156) electrophoretic profile.

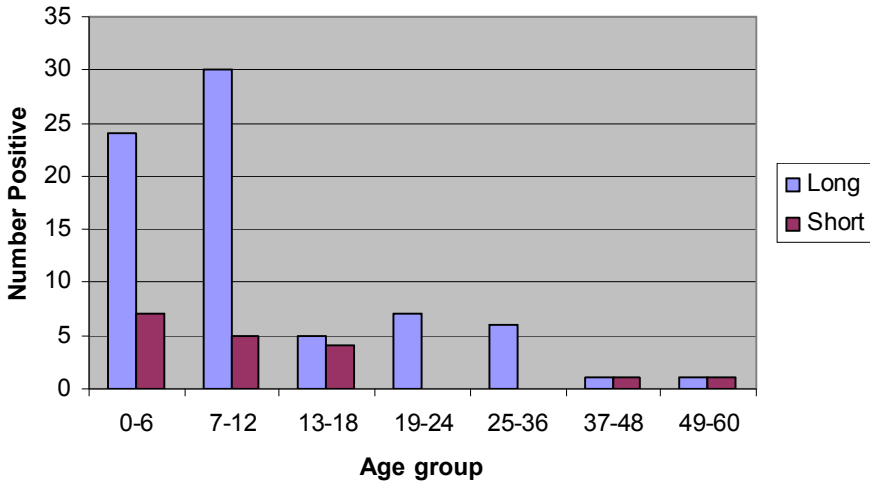


Figure 4.6: Distribution of electropherotypes of rotavirus in children 0-5 years old by age group in Northwestern Nigeria.

Table 4.11: Distribution of electropherotypes of rotavirus among diarrhoeic and non-diarrhoeic children 0-5 years old in Northwestern Nigeria.

Electropherotype Variant	Diarrhoeic Population		Non-diarrhoeic Population	
	Frequency	Percent (%)	Frequency	Percent (%)
L1	3	1.9	0	0.00
L2	15	9.6	0	0.00
L3	2	1.3	0	0.00
L4	5	3.2	1	7.14
L5	20	12.8	2	14.28
L6	10	6.4	1	7.14
L7	1	0.6	0	0.00
L8	4	2.6	0	0.00
L9	2	1.3	0	0.00
L10	0	0.0	1	7.14
L faint	5	3.2	0	0.00
L missing bands	1	0.6	0	0.00
L mix	1	0.6	0	0.00
S1	2	1.3	0	0.00

S2	6	3.8	0	0.00
S3	5	3.2	0	0.00
S4	1	0.6	0	0.00
S5	3	1.9	0	0.00
S6	1	0.6	0	0.00
No band	69	42.3	9	64.30
Total	**156**	**100.0**	**14**	**100.00**

L = Long
S = Short

Of the 35 PAGE positive specimens from hospitalized children, 77% (27/35) were of the long electropherotype while 23% (8/35) were of the short electropherotype. Similarly, of the 57 PAGE positive specimens from outpatients, 82.5% (47/57) were of long profile while 17.5(10/57) were short profile. There was an association between electropherotypes and severity of infection ($\chi^2 = 141.010$, df = 2, $p < 0.01$). The long pattern was strongly associated with severe infection.

4.2.4 VP6 Subgroup Specificity

One hundred and sixty eight (168) out of the 170 EIA positive specimens were subjected to VP6 sub-group ELISA to determine the VP6 subgroups. The remaining 2 specimens had no sufficient stool for the sub-grouping assay. Of the 168 specimens analyzed, 87% (146/168) showed reaction to the specific monoclonal antibodies. Forty-one of 146 (28%) showed subgroup I reactivity while 52 of 146 (36%) were of subgroup I + II. Sixteen of 146 (11%) were neither subgroup I nor II.

Statewise, subgroup I + II strains were the most prevalent in Kaduna, Sokoto and Zamfara States (Table 4.12). The highest prevalence (30%: 51/170) was recorded for children in the age group 7-12 months old (Table 4.13). There was an association between rotavirus subgroup and age group ($\chi^2 = 58.252$, df = 42, $p < 0.05$).

In the first year of the study (July 2002-July 2003), subgroup I predominated but during the second year of study (August 2003-July 2004), subgroup I + II became more prevalent

(Table 4.14). There was also an increase in the circulation of subgroup II isolates to 25% (31/124) and a decrease in subgroup I prevalence to 16.1% (20/124) in the second year.

Subgroup I + II strains were equally observed among diarrhoeic and non-diarrhoeic study groups and there was no statistically significant association between subgroup antigen and diarrhoea ($\chi^2 = 2.675$, df = 7, $p > 0.05$).

Table 4.12: Distribution of VP6 subgroup antigens of rotavirus by state in children 0-5 years old in Northwestern Nigeria.

State	SG I	SG II	SG I + SG II	Non SG I or SG II	Negative	ND	Total
Kaduna	10 (14.3)	17 (24.3)	27 (38.6)	6 (8.6)	8 (11.4)	2 (2.8)	70 (41.2)
Kebbi	3 (21.4)	4 (28.6)	4 (28.6)	1 (7.1)	2 (14.3)	0 (0.0)	14 (8.2)
Sokoto	5 (16.1)	11 (35.5)	8 (25.8)	3 (9.7)	4 (12.9)	0(0.0)	31 (18.2)
Zamfara	19 (34.5)	9 (16.4)	13 (23.6)	6 (10.9)	8 (14.5)	0(0.0)	55 (32.4)
Total	37 (21.8)	41 (24.1)	52 (30.6)	16 (9.4)	22 (12.9)	2 (1.2)	170 (100.0)

ND = Not determined

SG = Subgroup

Figures in parentheses represent percent of total.

Table 4.13: Distribution of VP6 subgroup antigens among different age groups of children 0-5 years old in Northwestern Nigeria.

	Age Group in Months							
Subgroup	**0-6**	**7-12**	**13-18**	**19-24**	**25-36**	**37-48**	**49-60**	Total
I	8	11	7	5	4	0	2	37
II	13	14	2	4	2	4	2	41
I + II	14	11	4	9	7	4	3	52
Non I or II	2	8	2	3	0	1	0	16
Negative	3	7	2	3	5	1	1	22
ND	0	0	0	1	1	0	0	2
Total	**40**	**51**	**17**	**25**	**19**	**10**	**8**	**170**

ND = Not determined

Table 4.14: Distribution of rotavirus subgroup antigens by year in children 0-5 years old in Northwestern Nigeria (July 2002-July 2004).

Subgroup	July 2002-July 2003		August 2003-July 2004		Total	
	Frequency	Percent (%)	Frequency	Percent (%)	Frequency	Percent (%)
I	17	37.0	20	16.1	37	21.8
II	10	21.7	31	25.0	41	24.1
I + II	10	21.7	42	33.9	52	30.6
Non I or II	5	10.9	11	8.9	16	9.4
Negative	4	8.7	18	14.5	22	12.9
Not Determined	0	0.0	2	1.6	2	1.2
Total	**46**	**100.0**	**124**	**100.0**	**170**	**100.0**

4.2.5 Electropherotypes and Subgroups

The correlation between electropherotype and subgroup antigen is as shown in Table 4.15. Three (3) strains exhibited the unusual combination of a VP6 Subgroup I specificity with a long (L) electropherotype.

Table 4.15: Electropherotypes and subgroups of rotavirus in children 0-5 years old in Northwestern Nigeria (2002-2004).

Electropherotype	SG I	SG II	SG I + SG II	Non SG I or SG II	Total
Long	3 (4.1)	31 (41.9)	28 (37.8)	12 (16.2)	**74 (80.4)**
Short	17 (94.4)	0 (0.0)	0 (0.0)	1 (5.6)	**18 (19.6)**
Total	**20 (21.7)**	**31 (33.7)**	**28 (30.4)**	**13 (14.1)**	**92 (100.0)**

SG = Subgroup

Figures in parentheses represent percent of total.

4.2.6 VP7 and VP4 Genotyping (RT-PCR)

4.2.6.1 Rotavirus VP7 Genotypes

First round RT-PCR cDNA amplification products were obtained from 40% (68/170) of rotavirus positive EIA specimens analysed. Bands indicating full-length product (1062bp) of VP7 RT-PCR as seen on agarose gel under UV light is shown on Figure 4.8. All the 68 strains with a first round PCR product could be assigned a VP7 genotype. An additional 51 strains from the positive specimens with no visible first round VP7 products could be assigned a VP7 genotype. Of the 170 EIA positive specimens, 119 (70%) were assigned VP7 genotype while 30% were untypeable.

Six different rotavirus VP7 genotypes were detected. The predominant genotype was G1 (27.0%: 46/170), followed by G8 and G2 each with a prevalence of 7.6% (13/170) and then G3 (1.8%: 3/170). Mixed infections were detected and consisted of 19 different combinations of genotype specificity accounting for 26.0% of cases. Genotypes G4 and G9 occurred as mixed infections with G9 occuring more commonly (7.0%: 12/170) than G4 (5.3%: 9/170). The predominant mix infection combination was G1G8 with a prevalence of 7.0% (12/170) followed by the G1G2G8 combination (3.5%: 6/170) (Table 4.16). Figure 4.8 shows a typical mixed VP7 infections observed as seen on agarose gel under UV light

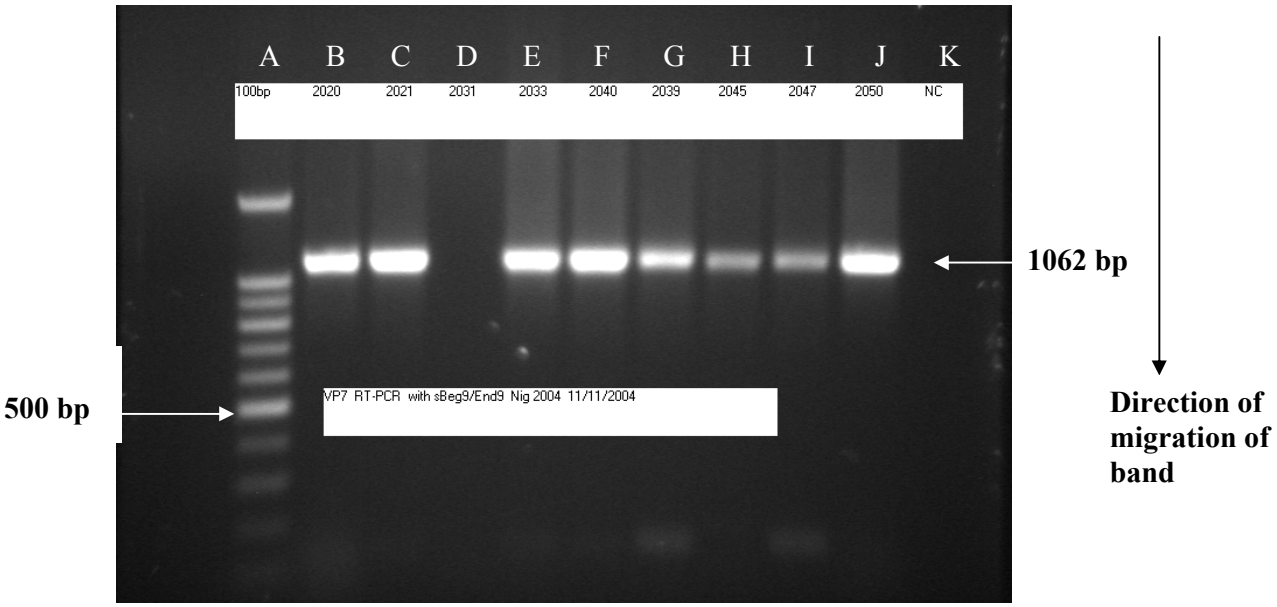


Figure 4.7: First round VP7 RT-PCR product (1062 bp) on agarose gel seen under the UV light.

Lane A: 100 bp molecular weight marker.

Lanes B-J: different specimens.

Lane K: represents the negative control.

Table 4.16: Percentage distribution of VP7 G-genotypes of rotavirus in children 0-5 years old in Northwestern Nigeria (2002-2004).

G-Genotype	Frequency	Percent (%)
G1	46	27.0
G1G2	2	1.18
G1G2G4	1	0.6
G1G2G8	6	3.5
G1G2G8G9	2	1.18
G1G2G9	1	0.6
G1G3	2	1.18
G1G3G8	1	0.6
G1G3G9	1	0.6
G1G8	12	7.0
G1G9	2	1.18
G2	13	7.6
G2G3	1	0.6
G2G4	3	1.8
G2G4G8G9	1	0.6
G2G4G9	1	0.6
G2G8	3	1.8
G2G9	1	0.6
G3	3	1.8
G3G9	1	0.6
G4G8	1	0.6
G4G9	2	1.18
G8	13	7.6
Non-typeable	51	30.0
Total	**170**	**100.0**

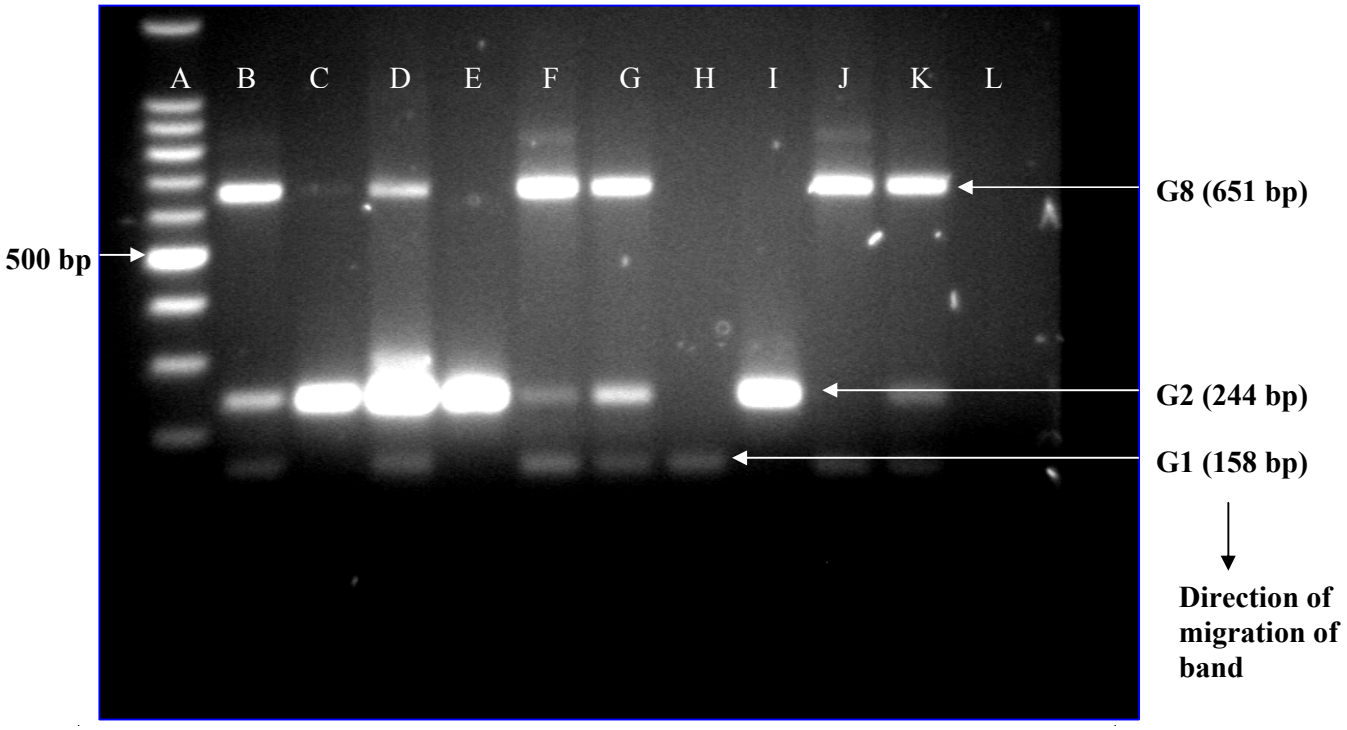


Figure 4.8: Agarose gel under UV light indicating mixed VP7 rotavirus genotype amplicons.

Lane A: 100 bp molecular weight marker.

Lanes B-K: different specimens.

Lane L represents the negative control.

Analysis of the epidemiological characteristics of the VP7 genotypes showed genotype G1 and G8 to circulate in all the states studied. G2 and G9 were not detected in Kebbi State while G3 and G4 circulated in Kaduna and Sokoto States. The highest prevalence of each genotype was recorded for Kaduna State. More than half (57.1%:8/14) of the EIA positive specimens from Kebbi state were nontypeable just as a third (35.5%: 11/31) of the EIA positive specimens from Sokoto state, 40% (22/55) from Zamfara State and 14.3% (10/70) from Kaduna State could not be typed. There was no significant association between genotypes and State ($\chi^2 = 80.518$, df = 69, $p > 0.05$).

Genotypes G1, G3 and G8 occurred more commonly in male children (60.9%, 66.7% and 61.5% respectively) than female children (39.1%, 33.3% and 38.5% respectively) while genotype G2 was more prevalent in females (61.5%) than males (38.5%). The non-typeable isolates were also more prevalent in males (34%: 34/100) than females (24.3%: 17/70). There was however no significant association between the genotypes and the sex of the children ($\chi^2 = 24.356$, df = 23, $p > 0.05$). The distribution of the VP7 genotypes among the different age groups is as presented in Table 4.17. There was no association between the age groups and the distribution of genotypes circulating in the study ($p > 0.05$).

All the genotypes circulated throughout the study period with a significant association between their distribution and the year of sample collection ($\chi^2 = 43.395$, df = 23, $p < 0.01$). In the first year of study (July 2002-July 2003), only 5 of the 6 genotypes were in circulation in single and mixed infections with G2 predominating (17.4%: 8/46) followed by G1 and G8 8.75% (4/46) each. During the subsequent period (August 2003-July 2004), genotype G1 circulated with the highest prevalence (33.9%: 42/124) followed by G1G8 (8.1%: 10/124) (Table 4.18). Furthermore, in the first year of study, more of the isolates (43.5%: 20/46) could not be genotyped compared to the second year (25.0%: 31/124).

Table 4.17: Distribution of VP7 genotypes among children in the various age groups in Northwestern Nigeria.

Genotype	Age Group in Months							
	0- 6	**7-12**	**13-18**	**19-24**	**25-36**	**37- 48**	**49- 60**	**Total**
G1	17	20	3	3	2	0	1	46
G1G2	0	0	0	1	1	0	0	2
G1G2G4	1	0	0	0	0	0	0	1
G1G2G8	2	1	0	2	1	0	0	6
G1G2G8G9	1	1	0	0	0	0	0	2
G1G2G9	0	1	0	0	0	0	0	1
G1G3	0	0	0	0	0	1	1	2
G1G3G8	0	0	0	0	0	1	0	1
G1G3G9	0	1	0	0	0	0	0	1
G1G8	1	5	2	1	3	0	0	12
G1G9	0	2	0	0	0	0	0	2
G2	5	4	3	0	0	0	1	13
G2G3	1	0	0	0	0	0	0	1
G2G4	1	1	1	0	0	0	0	3
G2G4G8G9	1	0	0	0	0	0	0	1
G2G4G9	0	1	0	0	0	0	0	1
G2G8	2	0	1	0	0	0	0	3
G2G9	1	0	0	0	0	0	0	1
G3	2	0	0	0	1	0	0	3
G3G9	1	0	0	0	0	0	0	1
G4G8	0	1	0	0	0	0	0	1
G4G9	0	0	1	1	0	0	0	2
G8	1	3	1	5	2	1	0	13
Non-typeable	3	10	5	12	9	7	5	51
Total	**40**	**51**	**17**	**25**	**19**	**10**	**8**	**170**

Table 4.18: Distribution of VP7 G-genotypes of rotavirus by year of study in children 0-5 years old in Northwestern Nigeria (2002-2004).

G-Genotype	Study Period		Total (%)
	July 2002-July 2003	August 2003-July 2004	
	Frequency (%)	Frequency (%)	
G1	4 (8.7)	42 (33.9)	46 (27.0)
G1G2	1 (2.2)	1 (0.8)	2 (1.2)
G1G2G4	0 (0.0)	1 (0.8)	1 (0.6)
G1G2G8	0 (0.0)	6 (4.8)	6 (3.5)
G1G2G8G9	1 (2.2)	1 (0.8)	2 (1.2)
G1G2G9	0 (0.0)	1 (0.8)	1 (0.6)
G1G3	0 (0.0)	2 (1.6)	2 (1.2)
G1G3G8	0 (0.0)	1 (0.8)	1 (0.6)
G1G3G9	0 (0.0)	1 (0.8)	1 (0.6)
G1G8	2 (4.3)	10 (8.1)	12 (7.0)
G1G9	2 (4.3)	0 (0.00	2 (1.2)
G2	8 (17.4)	5 (4.0)	13 (7.6)
G2G3	1 (2.2)	0 (0.0)	1 (0.6)
G2G4	0 (0.0)	3 (2.4)	3 (1.8)
G2G4G8G9	1 (2.2)	0 (0.0)	1 (0.6)
G2G4G9	0 (0.0)	1 (0.8)	1 (0.6)
G2G8	2 (4.3)	1 (0.8)	3 (1.8)
G2G9	0 (0.0)	1 (0.8)	1 (0.6)
G3	0 (0.0)	3 (2.4)	3 (1.8)
G3G9	0 (0.0)	1 (0.8)	1 (0.6)
G4G8	0 (0.0)	1 (0.8)	1 (0.6)
G4G9	0 (0.0)	2 (1.6)	2 (1.2)
G8	4 (8.7)	9 (7.3)	13 (7.6)
Non-typeable	20 (43.5)	31 (25.0)	51 (30.0)
Total	**46 (100.0)**	**124 (100.0)**	**170 (100.0)**

Among the 46 inpatients enrolled in the study, G1 was the most prevalent genotype occurring in more than half of the children (52.2%: 24/46), followed by G2 (15.2%: 7/46) and G8 (6.5%: 3/46). Among the outpatients, G1 was the most predominant genotype (17.7%: 22/124), followed by G1G8 (8.9%: 11/124). Only 6.5% (3/46) of the EIA positive specimens from the inpatients could not be typed while more than one quarter (38.7%: 48/124) of the EIA positive specimens from the outpatient were non-typeable (Table 4.19).

Most of the EIA positive specimens from diarrhoeic population were non-typeable (29.5%: 46/156). Of those typed G1 predominated (28.8%: 45/156) (Table 4.20). Among the asymptomatic (non-diarrhoeic) children, genotype G8 and G1G8 occurred more often than the other genotypes (14.3%: 2/14) each. G3 was not detected among the asymptomatic children while 35.7% (5/14) of the EIA positive specimens were non-typeable. There was no statistically significant association between the G-genotypes and the presence of diarrhoea ($\chi^2 = 28.671$, df = 23, $p > 0.05$).

Table 4.19: Distribution of VP7 genotypes of rotavirus among inpatients and outpatients in Northwestern Nigeria.

G-Genotype	Inpatient		Outpatient	
	Frequency	Percent (%)	Frequency	Percent (%)
G1	24	52.2	22	17.7
G1G2	1	2.2	1	0.8
G1G2G4	0	0.0	1	0.8
G1G2G8	2	4.3	4	3.2
G1G2G8G9	0	0.0	2	1.6
G1G2G9	0	0.0	1	0.8
G1G3	0	0.0	2	1.6
G1G3G8	0	0.0	1	0.8
G1G3G9	0	0.0	1	0.8
G1G8	1	2.2	11	8.9
G1G9	1	2.2	1	0.8
G2	7	15.2	6	4.8
G2G3	1	2.2	0	0.0
G2G4	1	2.2	2	1.6
G2G4G8G9	0	0.0	1	0.8
G2G4G9	0	0.0	1	0.8
G2G8	0	0.0	3	2.4
G2G9	0	0.0	1	0.8
G3	1	2.2	2	1.6
G3G9	0	0.0	1	0.8
G4G8	1	2.2	0	0.0
G4G9	0	0.0	2	1.6
G8	3	6.5	10	8.1
Non-typeable	3	6.5	48	38.7
Total	**46**	**100.0**	**124**	**100.0**

Table 4.20: Distribution of G-genotypes of rotavirus among diarrhoeic and non-diarrhoeic children in Northwestern Nigeria.

G-Genotype	Diarrhoeic Population		Non-diarrhoeic Population	
	Frequency	Percent (%)	Frequency	Percent (%)
G1	45	28.8	1	7.1
G1G2	2	1.3	0	0.0
G1G2G4	1	0.6	0	0.0
G1G2G8	5	3.2	1	7.1
G1G2G8G9	2	1.3	0	0.0
G1G2G9	1	0.6	0	0.0
G1G3	2	1.3	0	0.0
G1G3G8	1	0.6	0	0.0
G1G3G9	1	0.6	0	0.0
G1G8	10	6.4	2	14.3
G1G9	1	0.6	1	7.1
G2	13	8.3	0	0.0
G2G3	1	0.6	0	0.0
G2G4	3	1.9	0	0.0
G2G4G8G9	1	0.6	0	0.0
G2G4G9	0	0.0	1	7.1
G2G8	3	1.9	0	0.0
G2G9	1	0.6	0	0.0
G3	3	1.9	0	0.0
G3G9	1	0.6	0	0.0
G4G8	1	0.6	0	0.0
G4G9	1	0.6	1	7.1
G8	11	7.1	2	14.3
Non typeable	46	29.5	5	35.7
Total	**156**	**100.0**	**14**	**100.0**

4.2.6.2 Rotavirus VP4 Genotypes

First round RT-PCR cDNA amplification products were obtained from 66 (38.8%) of the 170 EIA positive specimens analysed. Bands indicating full-length product (876 bp) of VP4 RT-PCR as seen on agarose is shown on Figure 4.9. All the 66 strains with a first round PCR product could be assigned a VP4 genotype. An additional 48 strains from the EIA positive specimens with no visible first round VP7 products could be assigned a VP4 genotype. Of the 170 EIA positive specimens, 114 (67%) were assigned VP4 genotype while 33% were untypeable.

The 5 recognized human rotavirus VP4 gene alleles were detected. The most common was P[6] (22.3%: 38/170), followed by P[8] (17.1%: 29/170) and P[4] (5.9%: 10/170). Also detected were 8 different combinations of mixed infection genotypes accounting for 21.8% (37/170) of cases. The predominant mixed combinations were P[4][6] and P[6][10] (5.9%: 10/170 each), followed by P[6][8] (4.1%: 7/170) (Table 4.21). The novel human rotavirus genotypes, P[10] and P[14], detected for the first time in Nigeria as mixed infections were found in 8.8% and 2.4% of cases respectively. Figure 4.10 show agarose gel indicating mixed VP4 infections observed in the study.

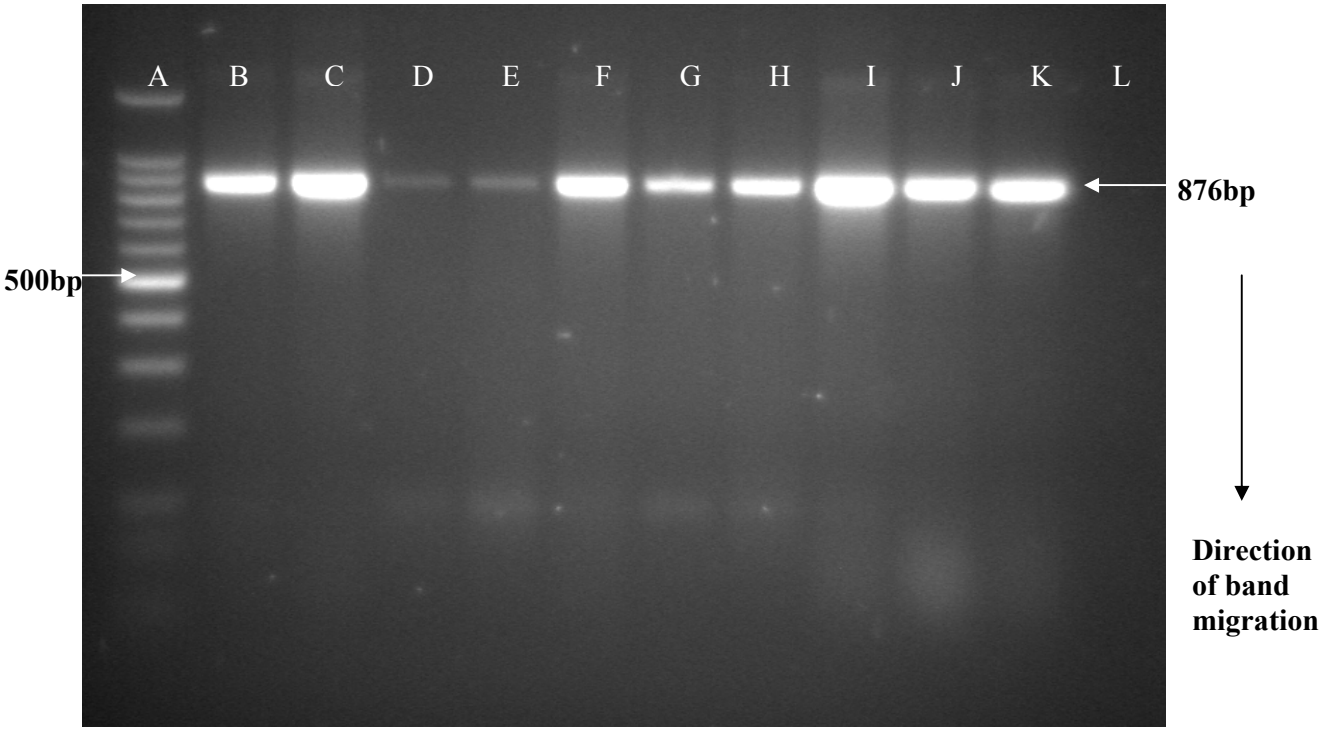


Figure 4.9: First round VP4 RT-PCR product (876 bp) on agarose gel seen under the UV light.

Lane A: 100bp molecular weight marker.

Lanes B-K: different specimens.

Lane L: represents the negative control.

Table 4.21: Distribution of VP4 P-genotypes of rotavirus circulating in children 0-5 years old in Northwestern Nigeria.

P-Genotype	Frequency	Percentage (%)
P[4]	10	5.9
P[4][6]	10	5.9
P[4][6][14]	2	1.2
P[4][P8]	1	0.6
P[6]	38	**22.3**
P[6][10]	10	5.9
P[6][14]	1	0.6
P[6][8]	7	4.1
P[6][8][10]	5	2.9
P[8]	29	17.1
P[8][14]	1	0.6
Non-typeable	56	33.0
Total	**170**	**100.0**

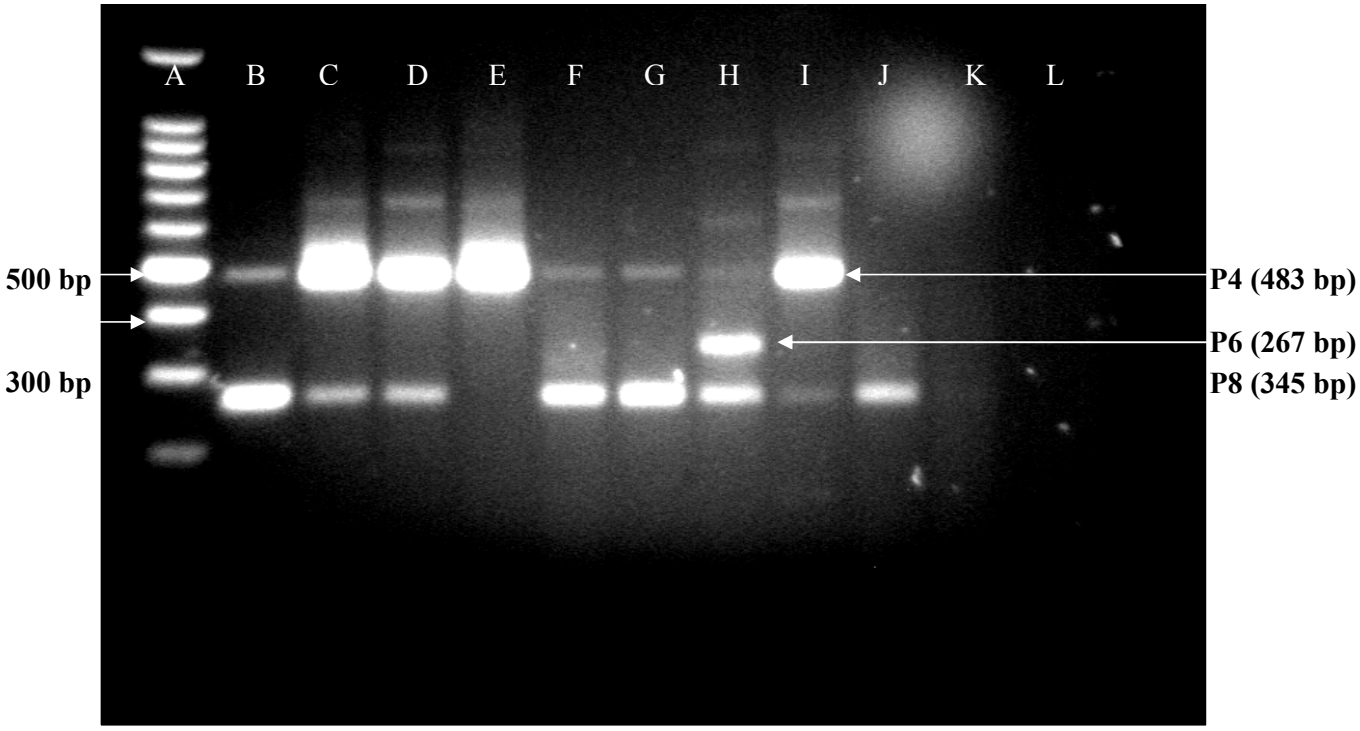


Figure 4.10: Agarose gel showing mixed rotavirus VP4 genotypes amplicons.

Lane A: 100 bp molecular weight marker.

Lanes B-K: different specimens.

Lane L: represents the negative control.

Direction of band migration

Genotype P[6] was detected in all the states. Genotype P[6] was predominant in Kaduna and Kebbi States while P[8] was the commonest P-type in Zamfara State. A high percentage (71.4%: 10/14) of the EIA positive specimens from Kebbi State were non-typeable (Table 4.22). The association between the genotypes and their distribution in the states was highly significant ($\chi^2 = 68.131$, df = 33, $p < 0.01$).

There was an equal distribution of genotypes among male children and female children with genotypes P[6] predominating followed by P[8]. The predominant mixed P-genotypes in male children and female children were P[4][6] and P[6][10] respectively.

The distribution of the VP4 genotypes among the different age groups is as presented in Table 4.23. Of the 4 novel P[14] strains, 3 were detected in children < 12 months old while all the 15 novel P[10] strains were detected in children < 18 months of age.

All the 5 detected genotypes occurred throughout the study period with a statistically significant relationship between their distribution and the year of specimen collection ($\chi^2 = 20.982$, df = 11, $p < 0.05$). Genotype P[6] predominated throughout the study period, while the non-typeable strains were detected more frequently in the first year of study (Table 4.24).

Table 4.22: Distribution of P-genotypes of rotavirus by state in children 0-5 years old in Northwestern Nigeria (2002-2004).

P-Genotype	State				
	Kaduna	Kebbi	Sokoto	Zamfara	Total
P[4]	3	0	1	6	10
P[4][6]	8	0	0	2	10
P[4][6][14]	0	0	0	2	2
P[4][P8]	1	0	0	0	1
P[6]	15	3	14	6	38
P[6][10]	6	0	2	2	10
P[6][14]	0	0	1	0	1
P[6][8]	7	0	0	0	7
P[6][8][10]	2	1	1	1	5
P[8]	14	0	1	14	29
P[8][14]	0	0	1	0	1
NT	14	10	10	22	56
Total	**70**	**14**	**31**	**55**	**170**

NT = Non-typeable

Table 4.23: Distribution of P-genotypes of rotavirus among different age group of children 0-5 years old in Northwestern Nigeria (2002-2004).

P-Genotype	Age Group in Months							
	0-6	7-12	13-18	19-24	25-36	37-48	49-60	Total
P[4]	2	5	2	0	0	0	1	10
P[4][6]	6	1	1	1	1	0	0	10
P[4][6][14]	1	0	0	1	0	0	0	2
P[4][P8]	1	0	0	0	0	0	0	1
P[6]	8	12	3	8	5	1	1	38
P[6][10]	4	4	2	0	0	0	0	10
P[6][14]	1	0	0	0	0	0	0	1
P[6][8]	5	1	0	0	1	0	0	7
P[6][8][10]	3	1	1	0	0	0	0	5
P[8]	6	15	3	2	2	1	0	29
P[8][14]	0	1	0	0	0	0	0	1
NT	3	11	5	13	10	8	6	56
Total	**40**	**51**	**17**	**25**	**19**	**10**	**8**	**170**

NT = Non-typeable

Table 4.24: Distribution of P-genotypes of rotavirus by year of study in children 0-5 years old in Northwestern Nigeria (2002-2004).

P-Genotype	Study Period		
	July 2002-July 2003 Frequency	August 2003-July 2004 Frequency	Total
P[4]	6	4	10
P[4][6]	2	8	10
P[4][6][14]	1	1	2
P[4][P8]	0	1	1
P[6]	9	29	38
P[6][10]	2	8	10
P[6][14]	1	0	1
P[6][8]	0	7	7
P[6][8][10]	2	3	5
P[8]	3	26	29
P[8][14]	1	0	1
NT	19	37	56
Total	**46**	**124**	**170**

NT = Non-typeable

4.2.6.3 VP7 (G) and VP4 (P) Genotype Combination Analysis

Only 32% (54/170) of the rotavirus strains could be assigned a single sero- and genotype. Seven single sero- and genotype were identified. The predominant strains were G1 P[8] (25/54), followed by G8 P[6] (10/54) and G2 P[4] (9/54) (Table 4.25). A further 6 strains could be assigned a single G or P type, i.e. either had a G but no P type (GNT) or a P but no G type (PNT). Of the remaining 110 rotavirus strains, 47 could not be typed at all and 63 were mixed infections consisting of 37 different strain combinations. The most prevalent combinations were G1 P[X] and G2 P[X] variants, i.e. G1G8 P[6] (10/63), G1 P[6][8] (5/63), G1 P[6][10] (5/63), G2 P[4][6] (3/63) and 2 each of G2G8 P[4][6] and G2G4 P[6][10]. Genotypes G9 and G4 occurred only as mixed infections, 7.0% and 5.3% respectively. Unusual single sero- and genotype (G1 P[4], G2 P[6] and G3 P[6]) were also identified (Table 4.26).

In the first year of study (July 2002-July 2003), genotype G2 P[4] predominated (13.0%: 6/46), followed by G1 P[6][10], G1G8 P[6] and G8 P[6] that each occurred with a prevalence of 4.3% (2/46). During August 2003-July 2004, the predominant genotype was G1 P[8] (20.2%: 25/124). Some of the strains (G2 P[4], G1 P[6][10], G1G8 P[6] etc) were prevalent throughout the study period while others (G1 P[4], G3 P[6], G1 P[4][8], G1 P[6][8] e.t.c.) occurred only in August 2003-July 2004. Most of the GNT were observed in the second year of study while more of the PNT were observed in the first year of study (Table 4.27).

Table 4.25: Distribution of G and P rotavirus strains in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Total
Dual Genotype	
G1 P[8]	26
G1 P[6]	5
G1 P[4]	1
G2 P[4]	9
G2 P[6]	1
G3 P[6]	2
G8 P[6]	10
Sub-Total	**54**
Single genotype	
G1 NT	2
G8NT	1
Sub-total	**3**
Single genotype	
NT P[6]	2
NT P[8]	1
Sub-total	**3**
Mixed genotypes	
Sub-total	**63**
Non Typed	**47**
Total	**170**

Table 4.26: Frequency of single, dual and mixed rotavirus genotypes in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Frequency
G1 NT	2
G1G2G4 P[6][8][10]	1
G1G2G8G9 P[4][6]	1
G1G2G8G9 P[6]	1
G1G2G8 P[4][6]	4
G1G2G8 P[4][6][14]	1
G1G2G8 P[6]	1
G1G2G9 P[6]	1
G1G2 P[6]	1
G1G2 P[8]	1
G1G3 NT	2
G1G3G8 NT	1
G1G3G9 P[6]	1
G1G8 NT	2
G1G8 P[6]	10
G1G9 NT	1
G1G9 P[8]	1
G1 P[4]	1
G1 P[4][8]	1
G1 P[6]	5
G1 P[6][10]	5
G1 P[6][8]	5
G1 P[6][8][10]	1
G1 P[8]	**26**
G2G3 P[4][6][14]	1
G2G4G8G9 P[6][8][10]	1
G2G4G9 P[6][8][10]	1
G2G4 P[6]	1
G2G4 P[6][10]	2
G2G8 P[4][6]	2
G2G8 P[6][8][10]	1
G2G9 P[6]	1
G2 P[4]	9
G2 P[4][6]	3
G2 P[6]	1
G3G9 P[6][8]	1
G3 P[6]	2
G3 P[6][8]	1
G4G8 P[6][10]	1
G4G9 P[6]	1
G4G9 P[6][10]	1
G8NT	1
G8 P[6]	10
G8 P[6][10]	1
G8 P[6][14]	1
NT P[6]	2
NT P[8]	1
NT P[8][14]	1
NT	47
Total	**170**

NT = Non-typeable

Table 4.27: Distribution G and P genotypes of rotavirus by year of study in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Study Period		
	Jul 2002-July 2003	August 2003-July 2004	Total
G1 NT	0	2	2
G1G2G4 P[6][8][10]	0	1	1
G1G2G8G9 P[4][6]	0	1	1
G1G2G8G9 P[6]	1	0	1
G1G2G8 P[4][6]	0	4	4
G1G2G8 P[4][6][14]	0	1	1
G1G2G8 P[6]	0	1	1
G1G2G9 P[6]	0	1	1
G1G2 P[6]	1	0	1
G1G2 P[8]	0	1	1
G1G3 NT	0	2	2
G1G3G8 NT	0	1	1
G1G3G9 P[6]	0	1	1
G1G8 NT	0	2	2
G1G8 P[6]	2	8	10
G1G9 NT	1	0	1
G1G9 P[8]	1	0	1
G1 P[4]	0	1	1
G1 P[4][8]	0	1	1
G1 P[6]	1	4	5
G1 P[6][10]	2	3	5
G1 P[6][8]	0	5	5
G1 P[6][8][10]	0	1	1
G1 P[8]	1	25	26
G2G3 P[4][6][14]	1	0	1
G2G4G8G9 P[6][8][10]	1	0	1
G2G4G9 P[6][8][10]	0	1	1
G2G4 P[6]	0	1	1
G2G4 P[6][10]	0	2	2
G2G8 P[4][6]	1	1	2
G2G8 P[6][8][10]	1	0	1
G2G9 P[6]	0	1	1
G2 P[4]	6	3	9
G2 P[4][6]	1	2	3
G2 P[6]	1	0	1
G3G9 P[6][8]	0	1	1
G3 P[6]	0	2	2
G3 P[6][8]	0	1	1
G4G8 P[6][10]	0	1	1
G4G9 P[6]	0	1	1
G4G9 P[6][10]	0	1	1
G8NT	1	0	1
G8 P[6]	2	8	10
G8 P[6][10]	0	1	1
G8 P[6][14]	1	0	1
NT P[6]	1	1	2
NT P[8]	1	0	1
NT P[8][14]	1	0	1
NT	17	30	47
Total	**46**	**124**	**170**

NT = Non-typeable

In Kaduna State, strain G1 P[8] predominated (18.6%: 13/70), followed by G1 P[6][8] (7.1%: 5/70). In Kebbi State, all the strains detected occurred with same frequency (7.1%: 1/14) while Sokoto State had strain G1G8 P[6] predominating (16.2%: 5/31). The G1 P[8] strain predominated in Zamfara State (21.8%: 12/55). Strains G1G8 P[6] and G8 P[6] were detected in all the States. Seventy five percent (3/4) of the PNT were detected in Sokoto State. Three quarter of the unusual strains were detected in Kaduna State while the NT strains predominated in Kebbi State (Table 4.28).

The G1 P[8] strain was prevalent in the 7-12 months old children (27.5%: 14/51). However, G1 P[8] was not identified in the age groups 37-48 and 49-60 months. A common strain, G8 P[6], predominated in children 19-24 months old (20%: 5/25) while G2 P[4] was detected mainly in children 7-12 months old. The unusual strains, G1 P[4], G2 P[6] and G3 P[6] were found in children <12 months of age, the GNT and PNT were evenly distributed in the various age groups while the NT strains increased in prevalence with age (Table 4.29).

In the diarrhoeic population, all the distinct strains and most of the mixed infection strains except G1G2G8 P[6], G1G9 P[8], G2G4G9 P[6][8][10], G4G9 P[6][10] and G8 P[6][10] were detected (Table 4.30). All the PNT and the unusual strains were detected in diarrhoeic population while G8 P[6] was the only strain detected in the asymptomatic children.

Table 4.28: Distribution of P and G strains of rotavirus by state in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	State				
	Kaduna	Kebbi	Sokoto	Zamfara	Total
G1 NT	1	0	0	1	2
G1G2G4 P[6][8][10]	1	0	0	0	1
G1G2G8G9 P[4][6]	1	0	0	0	1
G1G2G8G9 P[6]	0	0	1	0	1
G1G2G8 P[4][6]	3	0	0	1	4
G1G2G8 P[4][6][14]	0	0	0	1	1
G1G2G8 P[6]	1	0	0	0	1
G1G2G9 P[6]	1	0	0	0	1
G1G2 P[6]	0	0	0	1	1
G1G2 P[8]	0	0	0	1	1
G1G3 NT	1	1	0	0	2
G1G3G8 NT	0	0	1	0	1
G1G3G9 P[6]	1	0	0	0	1
G1G8 NT	2	0	0	0	2
G1G8 P[6]	**2**	**1**	**5**	**2**	**10**
G1G9 NT	0	1	0	0	1
G1G9 P[8]	1	0	0	0	1
G1 P[4]	1	0	0	0	1
G1 P[4][8]	1	0	0	0	1
G1 P[6]	3	1	1	0	5
G1 P[6][10]	2	0	2	1	5
G1 P[6][8]	5	0	0	0	5
G1 P[6][8][10]	0	1	0	0	1
G1 P[8]	**13**	**0**	**1**	**12**	**26**
G2G3 P[4][6][14]	0	0	0	1	1
G2G4G8G9 P[6][8][10]	0	0	1	0	1
G2G4G9 P[6][8][10]	1	0	0	0	1
G2G4 P[6]	1	0	0	0	1
G2G4 P[6][10]	2	0	0	0	2
G2G8 P[4][6]	1	0	0	1	2
G2G8 P[6][8][10]	0	0	0	1	1
G2G9 P[6]	1	0	0	0	1
G2 P[4]	2	0	1	**6**	9
G2 P[4][6]	3	0	0	0	3
G2 P[6]	0	0	1	0	1
G3G9 P[6][8]	1	0	0	0	1
G3 P[6]	2	0	0	0	2
G3 P[6][8]	1	0	0	0	1
G4G8 P[6][10]	1	0	0	0	1
G4G9 P[6]	0	0	1	0	1
G4G9 P[6][10]	1	0	0	0	1
G8NT	0	0	1	0	1
G8 P[6]	**3**	**1**	**3**	**3**	**10**
G8 P[6][10]	0	0	0	1	1
G8 P[6][14]	0	0	1	0	1
NT P[6]	0	0	2	0	2
NT P[8]	0	0	0	1	1
NT P[8][14]	0	0	1	0	1
NT	10	8	8	21	47
Total	**70**	**14**	**31**	**55**	**170**

NT = Non-typeable

Table 4.29: Age distribution of P and G rotavirus strains in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Age group in Months							
	0-6	7-12	13-18	19-24	25-36	37-48	49-60	Total
G1 NT	1	0	0	1	0	0	0	2
G1G2G4 P[6][8][10]	1	0	0	0	0	0	0	1
G1G2G8G9 P[4][6]	1	0	0	0	0	0	0	1
G1G2G8G9 P[6]	0	1	0	0	0	0	0	1
G1G2G8 P[4][6]	1	1	0	1	1	0	0	4
G1G2G8 P[4][6][14]	0	0	0	1	0	0	0	1
G1G2G8 P[6]	1	0	0	0	0	0	0	1
G1G2G9 P[6]	0	1	0	0	0	0	0	1
G1G2 P[6]	0	0	0	1	0	0	0	1
G1G2 P[8]	0	0	0	0	1	0	0	1
G1G3 NT	0	0	0	0	0	1	1	2
G1G3G8 NT	0	0	0	0	0	1	0	1
G1G3G9 P[6]	0	1	0	0	0	0	0	1
G1G8 NT	0	1	0	0	1	0	0	2
G1G8 P[6]	1	4	2	1	2	0	0	10
G1G9 NT	0	1	0	0	0	0	0	1
G1G9 P[8]	0	1	0	0	0	0	0	1
G1 P[4]	0	1	0	0	0	0	0	1
G1 P[4][8]	1	0	0	0	0	0	0	1
G1 P[6]	1	2	0	0	1	0	1	5
G1 P[6][10]	3	2	0	0	0	0	0	5
G1 P[6][8]	4	1	0	0	0	0	0	5
G1 P[6][8][10]	1	0	0	0	0	0	0	1
G1 P[8]	6	14	3	2	1	0	0	26
G2G3 P[4][6][14]	1	0	0	0	0	0	0	1
G2G4G8G9 P[6][8][10]	1	0	0	0	0	0	0	1
G2G4G9 P[6][8][10]	0	1	0	0	0	0	0	1
G2G4 P[6]	0	1	0	0	0	0	0	1
G2G4 P[6][10]	1	0	1	0	0	0	0	2
G2G8 P[4][6]	2	0	0	0	0	0	0	2
G2G8 P[6][8][10]	0	0	1	0	0	0	0	1
G2G9 P[6]	1	0	0	0	0	0	0	1
G2 P[4]	2	4	2	0	0	0	1	9
G2 P[4][6]	2	0	1	0	0	0	0	3
G2 P[6]	1	0	0	0	0	0	0	1
G3G9 P[6][8]	1	0	0	0	0	0	0	1
G3 P[6]	2	0	0	0	0	0	0	2
G3 P[6][8]	0	0	0	0	1	0	0	1
G4G8 P[6][10]	0	1	0	0	0	0	0	1
G4G9 P[6]	0	0	0	1	0	0	0	1
G4G9 P[6][10]	0	0	1	0	0	0	0	1
G8NT	0	1	0	0	0	0	0	1
G8 P[6]	0	1	1	5	2	1	0	10
G8 P[6][10]	0	1	0	0	0	0	0	1
G8 P[6][14]	1	0	0	0	0	0	0	1
NT P[6]	1	1	0	0	0	0	0	2
NT P[8]	0	0	0	0	0	1	0	1
NT P[8][14]	0	1	0	0	0	0	0	1
NT	2	8	5	12	9	6	5	47
Total	**40**	**51**	**17**	**25**	**19**	**10**	**8**	**170**

NT = Non-typeable

Table 4.30: Distribution of G and P rotavirus strains amongst diarrhoeic and non-diarrhoeic children in Northwestern Nigeria (2002-2004).

Genotype	Population		
	Diarrhoeic	Non-diarrhoeic	Total
G1 NT	1	1	2
G1G2G4 P[6][8][10]	1	0	1
G1G2G8G9 P[4][6]	1	0	1
G1G2G8G9 P[6]	1	0	1
G1G2G8 P[4][6]	4	0	4
G1G2G8 P[4][6][14]	1	0	1
G1G2G8 P[6]	0	1	1
G1G2G9 P[6]	1	0	1
G1G2 P[6]	1	0	1
G1G2 P[8]	1	0	1
G1G3 NT	2	0	2
G1G3G8 NT	1	0	1
G1G3G9 P[6]	1	0	1
G1G8 NT	1	1	2
G1G8 P[6]	9	1	10
G1G9 NT	1	0	1
G1G9 P[8]	0	1	1
G1 P[4]	1	0	1
G1 P[4][8]	1	0	1
G1 P[6]	5	0	5
G1 P[6][10]	5	0	5
G1 P[6][8]	5	0	5
G1 P[6][8][10]	1	0	1
G1 P[8]	26	0	26
G2G3 P[4][6][14]	1	0	1
G2G4G8G9 P[6][8][10]	1	0	1
G2G4G9 P[6][8][10]	0	1	1
G2G4 P[6]	1	0	1
G2G4 P[6][10]	2	0	2
G2G8 P[4][6]	2	0	2
G2G8 P[6][8][10]	1	0	1
G2G9 P[6]	1	0	1
G2 P[4]	9	0	9
G2 P[4][6]	3	0	3
G2 P[6]	1	0	1
G3G9 P[6][8]	1	0	1
G3 P[6]	2	0	2
G3 P[6][8]	1	0	1
G4G8 P[6][10]	1	0	1
G4G9 P[6]	1	0	1
G4G9 P[6][10]	0	1	1
G8NT	1	0	1
G8 P[6]	9	1	10
G8 P[6][10]	0	1	1
G8 P[6][14]	1	0	1
NT P[6]	2	0	2
NT P[8]	1	0	1
NT P[8][14]	1	0	1
NT	42	5	47
Total	**156**	**14**	**170**

NT = Non-typeable

The distribution of the G/P strain combinations among the electropherotypes is as presented in Table 4.31. More than half (52.6%: 41/78) of the rotavirus EIA positive specimens that were PAGE negative could not be typed. The ones that could be typed were characterized by mixed infection strains with G1G8 P[6] predominating (10.3%: 8/78), followed by G1G2G8 P[4][6] (5.1%: 4/78). Of the 18 short electropherotypes noted in this study, 16 were associated with rotavirus strains exhibiting the G2 genotype. The mixed infection strains and the unusual strains were associated with long pattern and with no visible bands (Table 4.32).

The distribution of the G/P strains among the electropherotic variants is as presented in Table 4.33. The predominant long variant associated with the G1 P[8] strains was L6 (34.6%: 9/26) while the predominant short variant associated with the G2 P[4] strain was S2 (44.4%: 4/9). The variant with missing top band (incomplete profile) was a G1 P[8] strain, the variant with faint bands (not very visible band) was a G1G8 P[6] strain and the variant with a single extra band was a G1 P[6][10] strain.

The distribution of the G/P strain among the VP6 subgroup strains is as presented in Table 4.34. Interestingly, G1 P[8] and G8 P[6] were associated with all the four subgroups including subgroup I, a finding which is unusual. The unusual strains G2 P[6] and G3 P[6] were detected among the subgroup I + II strains while the G1 P[4] were associated with strains with no bands (NB). Most of the GNT (G1 NT, G1G9 NT, G8 NT), and the NT strains were detected among the subgroup I rotaviruses.

Table 4.31: Distribution of G and P rotavirus strains among electropherotypes circulating in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Electrophoretic Patterns			
	No band	Long	Short	Total
Dual Genotype				
G1 P[8]	1	25	0	26
G1 P[6]	2	3	0	5
G1 P[4]	1	0	0	1
G2 P[4]	0	0	9	9
G2 P[6]	0	1	0	1
G3 P[6]	1	1	0	2
G8 P[6]	6	4	0	10
Sub-Total	11	34	9	**54**
Single serotype				
G1 NT	2	0	0	2
G8NT	1	0	0	1
Sub-total	3	0	0	**3**
Single genotype				
NT P[6]	1	1	0	2
NT P[8]	0	1	0	1
Sub-total	1	2	0	**3**
Mixed genotypes				
Sub-total	22	33	8	**63**
Non Typed	41	5	1	**47**
Total	**78**	**74**	**18**	**170**

Table 4.32: Distribution of mixed rotavirus strains among the electropherotypes circulating in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Electrophoretic Patterns			
	No band (NB)	Long	Short	Total
G1 NT	2	0	0	2
G1G2G4 P[6][8][10]	0	1	0	1
G1G2G8G9 P[4][6]	0	0	1	1
G1G2G8G9 P[6]	1	0	0	1
G1G2G8 P[4][6]	4	0	0	4
G1G2G8 P[4][6][14]	1	0	0	1
G1G2G8 P[6]	1	0	0	1
G1G2G9 P[6]	0	1	0	1
G1G2 P[6]	0	1	0	1
G1G2 P[8]	1	0	0	1
G1G3 NT	2	0	0	2
G1G3G8 NT	1	0	0	1
G1G3G9 P[6]	0	1	0	1
G1G8 NT	1	1	0	2
G1G8 P[6]	8	2	0	10
G1G9 NT	0	0	1	1
G1G9 P[8]	0	1	0	1
G1 P[4]	1	0	0	1
G1 P[4][8]	0	1	0	1
G1 P[6]	2	3	0	5
G1 P[6][10]	0	5	0	5
G1 P[6][8]	0	5	0	5
G1 P[6][8][10]	0	1	0	1
G1 P[8]	1	25	0	26
G2G3 P[4][6][14]	0	0	1	1
G2G4G8G9 P[6][8][10]	0	1	0	1
G2G4G9 P[6][8][10]	0	1	0	1
G2G4 P[6]	0	1	0	1
G2G4 P[6][10]	0	2	0	2
G2G8 P[4][6]	1	0	1	2
G2G8 P[6][8][10]	0	0	1	1
G2G9 P[6]	0	1	0	1
G2 P[4]	0	0	9	9
G2 P[4][6]	0	0	3	3
G2 P[6]	0	1	0	1
G3G9 P[6][8]	0	1	0	1
G3 P[6]	1	1	0	2
G3 P[6][8]	0	1	0	1
G4G8 P[6][10]	0	1	0	1
G4G9 P[6]	0	1	0	1
G4G9 P[6][10]	0	1	0	1
G8NT	1	0	0	1
G8 P[6]	6	4	0	10
G8 P[6][10]	0	1	0	1
G8 P[6][14]	0	1	0	1
NT P[6]	1	1	0	2
NT P[8]	0	1	0	1
NT P[8][14]	1	0	0	1
NT	41	5	1	47
Total	**78**	**74**	**18**	**170**

NT = Non-typeable

Table 4.33: Distribution of G and P strains of rotavirus among the electrophoretic variants detected in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	Electrophoretic Variants																						
	NB	LF	LMB	LM	L1	L10	L2	L3	L3EB	L4	L5	L5M	L6	L7	L8	L9	S1	S2	S3	S4	S5	S6	Total
G1 NT	2																						2
G1G2G4 P[6][8][10]											1												1
G1G2G8G9 P[4][6]																	1						1
G1G2G8G9 P[6]	1																						1
G1G2G8 P[4][6]	4																						4
G1G2G8 P[4][6][14]	1																						1
G1G2G8 P[6]	1																						1
G1G2G9 P[6]											1												1
G1G2 P[6]							1																1
G1G2 P[8]	1																						1
G1G3 NT	2																						2
G1G3G8 NT	1																						1
G1G3G9 P[6]										1													1
G1G8 NT	1												1										2
G1G8 P[6]	8	1					1																10
G1G9 NT																		1					1
G1G9 P[8]										1													1
G1 P[4]	1																						1
G1 P[4][8]										1													1
G1 P[6]	2										1				2								5
G1 P[6][10]									1		3	1											5
G1 P[6][8]				1						2	1		1										5
G1 P[6][8][10]							1																1
G1 P[8]	1		1		3		5	1		1	3		9			2							26
G2G3 P[4][6][14]																			1				1
G2G4G8G9 P[6][8][10]											1												1
G2G4G9 P[6][8][10]											1												1
G2G4 P[6]							1																1
G2G4 P[6][10]							1				1												2
G2G8 P[4][6]	1																	1					2
G2G8 P[6][8][10]																					1		1
G2G9 P[6]											1												1
G2 P[4]																		4	3	1	1		9
G2 P[4][6]																	1		1			1	3
G2 P[6]											1												1
G3G9 P[6][8]							1																1
G3 P[6]	1										1												2
G3 P[6][8]		1																					1
G4G8 P[6][10]														1									1
G4G9 P[6]											1												1
G4G9 P[6][10]											1												1
G8NT	1																						1
G8 P[6]	6						1				2				1								10
G8 P[6][10]						1																	1
G8 P[6][14]		1																					1
NT P[6]	1						1																2
NT P[8]		1																					1
NT P[8][14]	1																						1
NT	41	1					2				1				1						1		47
Total	78	5	1	1	3	1	15	1	1	6	21	1	11	1	4	2	2	6	5	1	3	1	170

NB = No band, FB = Faint Band, MB = Missing Band, MB = Mixed Band, EB = Extra Band, M = Mixed, L = Long, NT = Non typeable, LF = Long Faint

Table 4.34: Distribution of G and P rotavirus strains among the VP6 subgroups circulating in children 0-5 years old in Northwestern Nigeria (2002-2004).

Genotype	VP6 Subgroup						
	NR	Non I or II	I	I+II	II	ND	Total
G1 NT	1	0	1	0	0	0	2
G1G2G4 P[6][8][10]	0	0	0	1	0	0	1
G1G2G8G9 P[4][6]	0	0	1	0	0	0	1
G1G2G8G9 P[6]	0	0	0	0	1	0	1
G1G2G8 P[4][6]	2	0	0	1	0	1	4
G1G2G8 P[4][6][14]	0	0	0	1	0	0	1
G1G2G8 P[6]	0	0	0	1	0	0	1
G1G2G9 P[6]	0	0	0	0	1	0	1
G1G2 P[6]	0	0	0	0	1	0	1
G1G2 P[8]	0	0	0	1	0	0	1
G1G3 NT	0	0	0	2	0	0	2
G1G3G8 NT	0	0	0	0	1	0	1
G1G3G9 P[6]	0	0	0	0	1	0	1
G1G8 NT	1	0	0	1	0	0	2
G1G8 P[6]	5	0	0	4	1	0	10
G1G9 NT	0	0	1	0	0	0	1
G1G9 P[8]	0	0	1	0	0	0	1
G1 P[4]	1	0	0	0	0	0	1
G1 P[4][8]	0	0	0	0	1	0	1
G1 P[6]	1	1	0	0	3	0	5
G1 P[6][10]	0	1	0	0	4	0	5
G1 P[6][8]	0	2	0	1	2	0	5
G1 P[6][8][10]	0	0	0	0	1	0	1
G1 P[8]	0	7	1	12	6	0	26
G2G3 P[4][6][14]	0	0	1	0	0	0	1
G2G4G8G9 P[6][8][10]	0	0	0	1	0	0	1
G2G4G9 P[6][8][10]	0	0	0	0	1	0	1
G2G4 P[6]	0	0	0	0	1	0	1
G2G4 P[6][10]	0	1	0	0	1	0	2
G2G8 P[4][6]	0	0	2	0	0	0	2
G2G8 P[6][8][10]	0	0	1	0	0	0	1
G2G9 P[6]	0	0	0	1	0	0	1
G2 P[4]	0	0	9	0	0	0	9
G2 P[4][6]	0	0	3	0	0	0	3
G2 P[6]	0	0	0	1	0	0	1
G3G9 P[6][8]	0	0	0	0	1	0	1
G3 P[6]	0	0	0	2	0	0	2
G3 P[6][8]	0	0	0	1	0	0	1
G4G8 P[6][10]	0	0	1	0	0	0	1
G4G9 P[6]	0	0	0	1	0	0	1
G4G9 P[6][10]	0	0	0	0	1	0	1
G8NT	0	0	1	0	0	0	1
G8 P[6]	1	3	1	4	1	0	10
G8 P[6][10]	0	0	0	0	1	0	1
G8 P[6][14]	0	0	0	0	1	0	1
NT P[6]	1	0	0	0	1	0	2
NT P[8]	0	0	0	1	0	0	1
NT P[8][14]	0	0	0	1	0	0	1
NT	9	1	13	14	9	1	47
Total	**22**	**16**	**37**	**52**	**41**	**2**	**170**

ND = Not Determined, NR = Non Reactive (Negative)

4.3 Detection of Human Astroviruses

Two hundred and eighty two (282) stool specimens (including 34 non-diarrhoeic and 6 intussusception) previously analysed for group A rotaviruses were tested for human astrovirus (HAstV) by commercial EIA. Astrovirus antigen was detected in 19.1% (54/282) of all stool specimens. This compares with an incidence of 23.4% (66/282) of rotavirus in same cohort. Astrovirus antigen was detection in 19.4% (48/248) of the diarrhoeic and in 17.6% (6/34) of the non-diarrhoeic specimens. Of the 6 intussusception specimens included in the study 3 were positve for astrovirus. Rotavirus was, however, detected in only one intussusception specimen.

The prevalence of astrovirus infection was marginally higher in outpatients (19.5%: 46/236) than inpatients (17.4%: 8/46), as against rotavirus infection that was significantly associated with hospitalization (χ^2= 33.629, df = 1, $p < 0.01$) (Table 4.35).

Astrovirus infection was more prevalent in Kaduna State (21.2%: 33/156) than Zamfara State (18.6%: 13/70) (Table 4.36). Astrovirus infection occurred more frequently in females 21.5% (26/121) than males 17.4% (28/161) although statistically insignificant ($p>0.05$).

Astrovirus infection was more frequent among children 0-18 months of age with the highest frequency in the age group 0-6 months (27.0%: 20/74) (Figure 4.11).

Table 4.35: Prevalence of astrovirus and rotavirus infections in inpatients and outpatients in Northwestern Nigeria.

Inpatient/Outpatient	Astrovirus Antigen (%)	Rotavirus Antigen (%)	Total (%)
Inpatient	8 (17.4)	**26 (56.5)**	46 (16.3)
Outpatient	46 (19.5)	40 (17.0)	236 (83.7)
Total	**54 (19.1)**	**66 (23.4)**	**282 (100.0)**

Table 4.36: Prevalence of astrovirus and rotavirus infection by state in children 0-5 years old in Northwestern Nigeria.

State	No of Specimen Examined	Astrovirus	Rotavirus
		Frequency (%)	Frequency (%)
Kaduna	156	33 (21.2)	42 (26.9)
Kebbi	16	1 (6.3)	6 (37.5)
Sokoto	40	7 (17.5)	5 (12.5)
Zamfara	70	13 (18.6)	13 (18.6)
Total	**282**	**54 (19.1)**	**66 (23.4)**

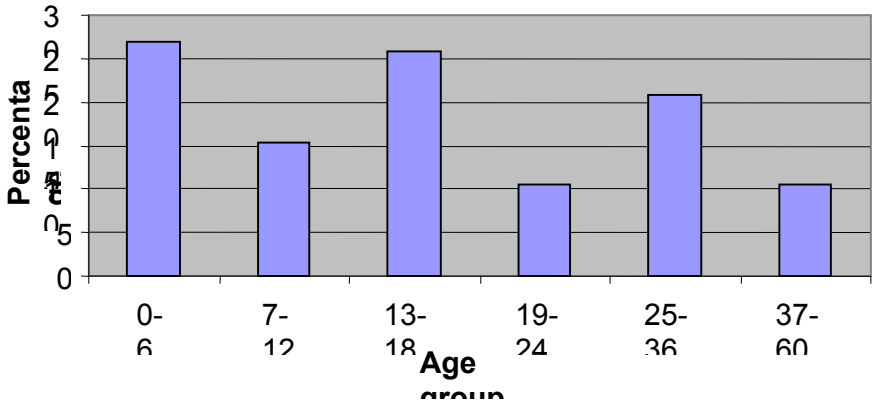


Figure 4.11: Percentage distribution of astrovirus infection by age group in Northwestern Nigeria.

There was no major variation in the monthly prevalence of astrovirus observed in this study with no virus isolated in the first quarter of the study (Figure 4.12). However, viral shedding occurred more frequently in the cool drier months between October and March as shown by the calculation of seasonal index over time (Figure 4.13).

A total of 48 out of 54 astrovirus positive specimens were from the diarrhoeic patients. Faecal specimens were watery in 72.2% (39/54) of patients, contained mucus in 15.0% of patients and mucus and blood in 5.5% (Table 4.37). Detection of astrovirus antigen in stool specimen was possible from the first day to over seven days of diarrhoea and even in diarrhoea that has persisted for a month. More than half (53.7%: 29/54) of patients had diarrhoea duration of 1-3 days, while 20.4% (11/54) had diarrhoea duration lasting for 7 days and above. No astrovirus antigen was detected in all the 8 children that had diarrhoea lasting for 6 days (Table 4.38). The prevalence of astrovirus infection was highest (64.8%: 35/54) in children who did not pass stool continuously than those who did (35.2%: 19/54). The association between continuous diarrhoea and astrovirus infection was not significant ($p > 0.05$).

The clinical symptoms associated with HAstV infection were similar to those of rotavirus infection and included fever (66.7%: 36/54) and vomiting (57.4%: 31/54) in more than half of the patients. Mild dehydration (37.0%: 20/54) and symptoms of upper respiratory tract (URT) infection (18.5%: 10/54) were also common symptoms. More than half (28/54) of the astrovirus infected children appeared normal, 38.8% (21/54) appeared ill and 9.3% (5/54) of them appeared very ill. There was, however, no significant difference in general condition of child and astrovirus infection ($p>0.05$).

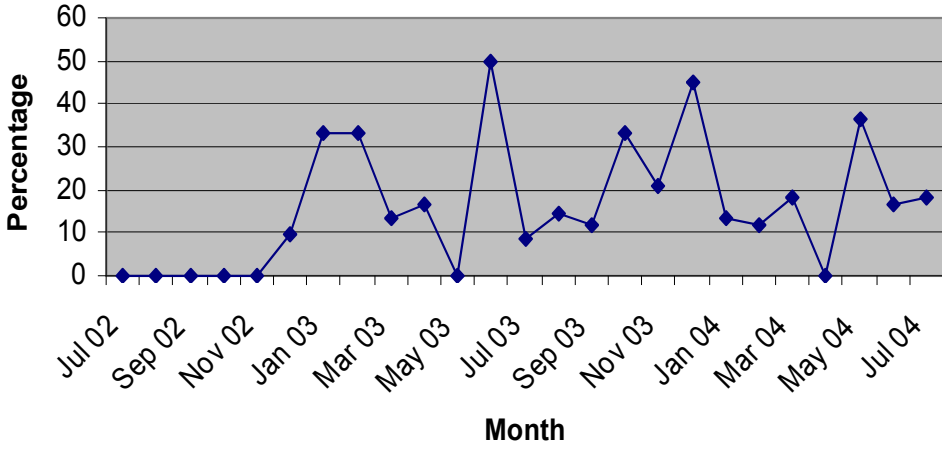


Figure 4.12: Monthly distribution of astrovirus infection in children 0-5 years old in Northwestern Nigeria.

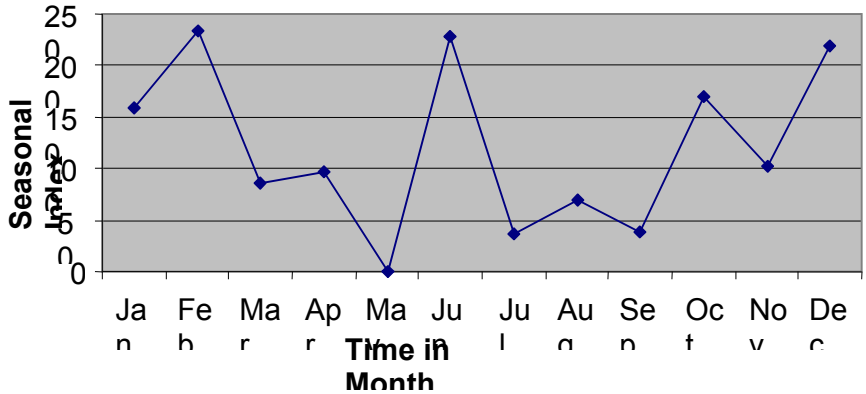


Figure 4.13: Seasonal variation of astrovirus infection in Northwestern Nigeria.

Table 4.37: Stool type and astrovirus infection in children 0-5 years old in Northwestern Nigeria.

Stool Type	Astrovirus Antigen		
	Positive	Negative	Total
Bloody	0	1	1
Mucoid	1	12	13
Mucoid & Bloody	2	1	3
Normal	4	26	30
Watery	39	147	186
Watery & Bloody	0	1	1
Watery & Mucoid	7	32	39
WMB	1	8	9
Total	**54**	**228**	**282**

W M B = Watery, Mucoid and Bloody

Table 4.38: Duration of diarrhoea in astrovirus infected children in Northwestern Nigeria.

Diarrhoea Duration in Days	Astrovirus Antigen		
	Positive	Negative	Total
1	2	13	15
10	0	1	1
14	1	1	2
16	0	1	1
2	16	54	70
21	0	1	1
3	11	45	56
30	1	0	1
366	0	1	1
4	4	23	27
5	5	13	18
6	**0**	8	8
60	0	1	1
7	9	37	46
90	0	1	1
Nil	5	28	33
Total	**54**	**228**	**282**

Analysis of the outcome of hospital/clinic visit showed that 19.9% (4/211) of astrovirus infected children required rehydration, 18.0% (9/50) required hospitalization on visit, and 15.0% (3/20) were sent to the laboratory for stool analysis and then subsequently treated while the only referred child was not shedding astrovirus.

Analysis of the feeding regimen in the astrovirus infected children showed that the rate of viral infection varied with the food type. The prevalence of infection was highest among those fed on breast milk and water (33.3%: 6/18), followed by those on breast milk and solid food (21.7%: 28/129). Those on solid food or adult diet had a prevalence of 15.8% (18/114) while those on only breast milk had a rate of 14.3% (3/21). There was no statistically significant association between food type and astrovirus infection ($\chi^2 = 4.041$, $df = 4$, $p > 0.05$).

Of the 282 children enrolled in this study only 14.5% (41/282) drank boiled water as indicated by the parents in the questionnaires while 85.5% (241/282) did not drink boiled water. The prevalence of astrovirus infection was 17.1% (7/41) and 19.5% (47/241) among the children that drank boiled water and unboiled water respectively. There was no significant association between viral infection and type of drinking water despite the difference in prevalence ($\chi^2 = 0.134$, $df = 1$, $p > 0.05$).

There was no statistically significant relationship between socioeconomic background and infection with astrovirus ($\chi^2 = 0.208$, $df = 2$, $p > 0.05$). The highest prevalence (20.0%: 8/40) was observed among children from high socio economic background, followed by those from low background (19.7%: 35/178) and the lowest was in those from medium background (17.2%: 11/64). There was also no relationship between a child schooling and the rate of viral infection. The prevalence of astrovirus infection among the 26 children who either attended a day care or an Islamic school was 15.4% (4/26) against 19.5% (50/256) among the 256 children who were not schooling ($\chi^2 = 0.544$, $df = 2$, $p > 0.05$).

Dual infection with rotaviruses was observed in one third (33.3%: 18/54) of astrovirus infected patients. There was, however, no significant relationship between the frequencies of clinical symptoms observed in these children and co-infection.

Of the 20 astrovirus EIA-positive specimens examined by EM, HAstV particles were observed in 45.0% (9/20) of the specimen due to insufficient materials and insufficient virus in prepared specimens (Figures 4.14). The virus was mostly observed in specimens with high concentration of viruses that were strong ELISA positive.

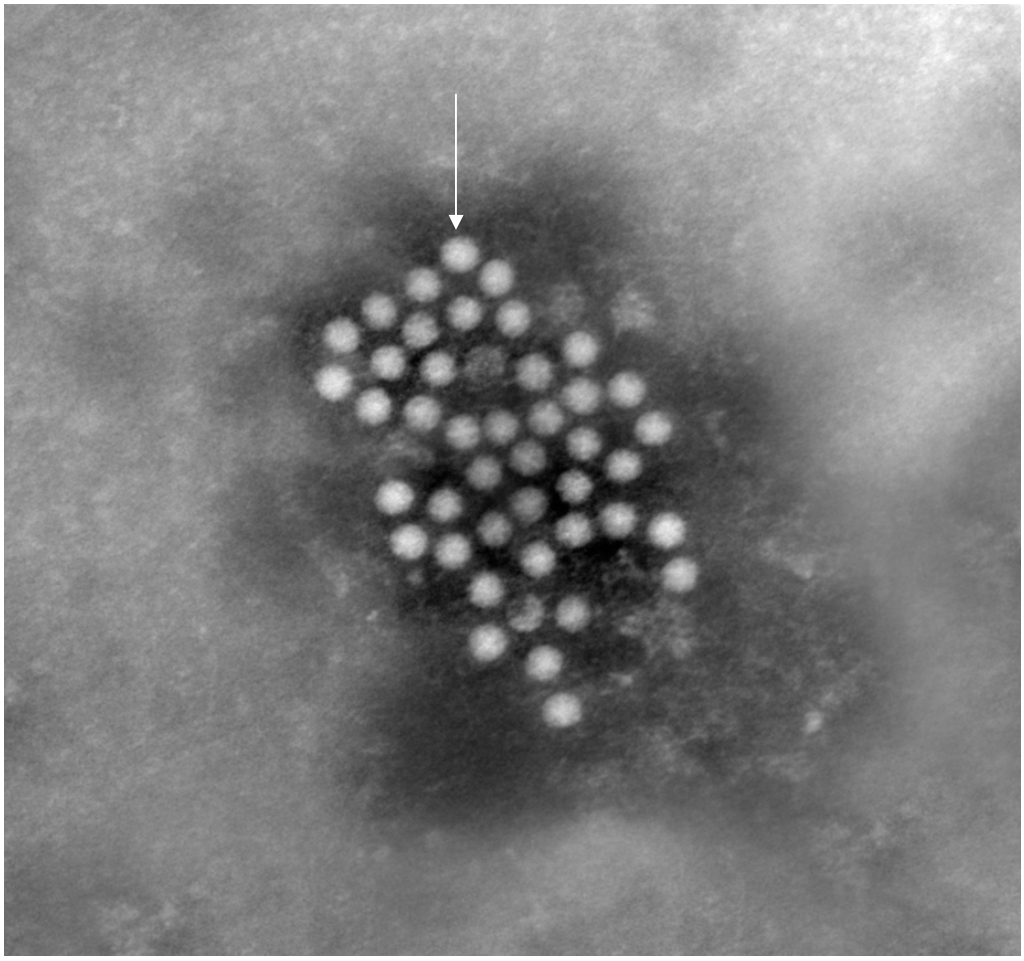


Figure 4.14: Astrovirus particles observed by EM in the study (arrow indicates a single astrovirus particle).

4.4 Detection of Enteric Adenoviruses

Adenoviruses were detected in 22.3% (63/282) of the specimens examined by EIA. Further analysis detected human enteric adenovirus 40/41 in 8% (5/63) of the EIA positive specimens. Adenovirus antigen was detected in 23.0% (57/248) of the diarrhoeic, 17.6% (6/34) of the non-diarrhoeic and in 16.7% (1/6) of the intussusception specimens. The prevalence of adenovirus infection was higher in the outpatients (24.2%: 57/236) than inpatients (13.0%: 6/46). Furthermore, the outpatients`` were two times more likely to have been infected with adenovirus than the inpatients (OR = 2.123, 95% CI = 0.856-5.265).

Analysis of adenovirus infection by state showed the highest prevalence in Sokoto State (32.5%: 13/40), followed by Kebbi State (25.0%: 4/16), Kaduna State (20.5%: 32/156) and Zamfara State (20.0%: 14/70) (Table 4.39). Although Sokoto State had a high prevalence as seen in Figure 4.15, compared to other States, the differences observed in prevalence in the state was not significant ($p>0.05$). Viral shedding occurred more frequently in males 26.7% (43/161) than in females 16.5% (20/121) in a ratio of 1:6. The difference observed in the sex prevalence was significant ($\chi^2 = 4.126$, df = 1, $p< 0.05$).

Table 4.39: Prevalence of adenovirus and rotavirus infection by state in children 0-5 years old in Northwestern Nigeria.

State	Total	Adenovirus	Rotavirus
		Frequency (%)	Frequency (%)
Kaduna	156	32 (20.5)	42 (26.9)
Kebbi	16	4 (25.0)	6 (37.5)
Sokoto	40	13 (32.5)	5 (12.5)
Zamfara	70	14 (20.0)	13 (18.6)
Total	**282**	**63 (22.3)**	**66 (23.4)**

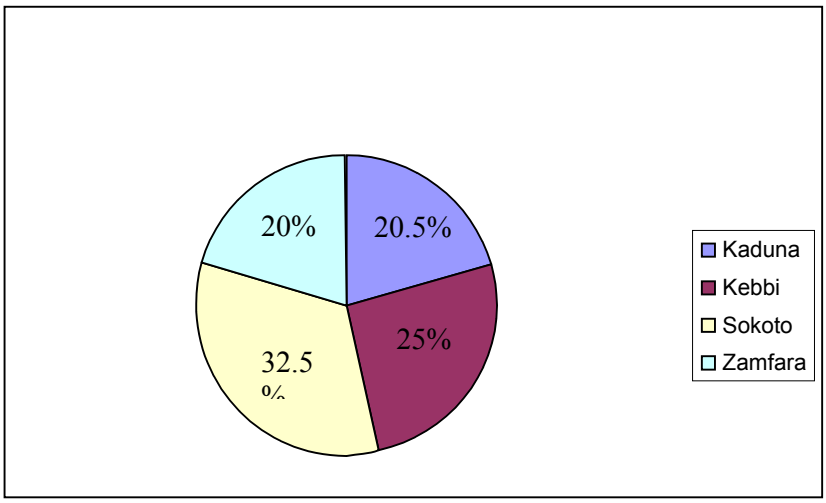


Figure 4.15: Percentage distribution of adenovirus infection by state in children 0-5 years old in Northwestern Nigeria.

Adenovirus infection was more prevalent in the older children with almost a third of patients (31.0%: 9/29) infected in the age group 25-36 months. Children in the age group 19-24 (15.8%: 6/38) had the lowest prevalence (Figure 4.16). Adenoviruses were detected throughout the year except in the month of June, demonstrating no major seasonal variation (Figure 4.17). The prevalence of adenovirus when examined by year of specimen collection was 22.7% (15/66) and 22.2% (48/216) for the first and the second year of study respectively.

A total of 57 (90.5%) out of 63 episodes of adenovirus infections were accompanied by diarrhoea. Analysis of stool specimen showed watery diarrhoea in 87.3% (55/63), diarrhoea with mucus in 19.0% (12/63) and diarrhoea with mucus and blood in 3.2% (2/63). There was no statistically significant association between infection with adenovirus and stool type in this study ($\chi^2 = 4.198$, df = 7, $p > 0.05$). Detection of adenovirus antigen in stool specimen was possible from the first day to seven days of diarrhoea and even in diarrhoea that has persisted for 3 months. About 47.6% (30/63) of patients had diarrhoea duration lasting 1-3 days, while 17.5% (11/63) had diarrhoea duration lasting for 7 days (Table 4.40).

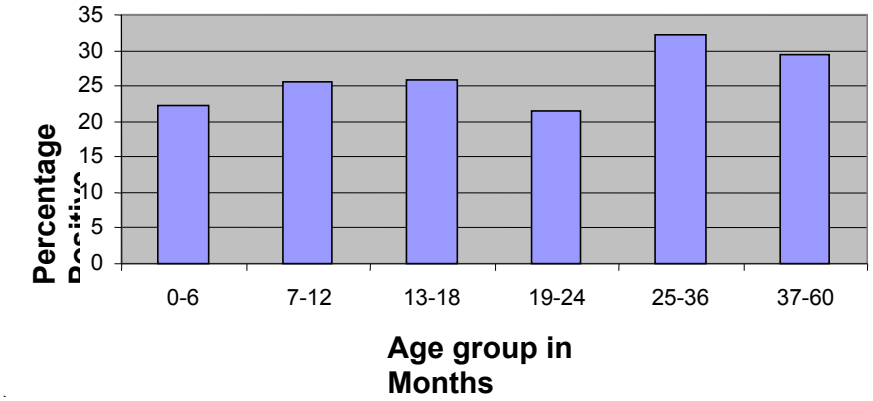


Figure 4.16: Percentage distribution of adenovirus infection among different age group of children 0-5 years old in Northwestern Nigeria.

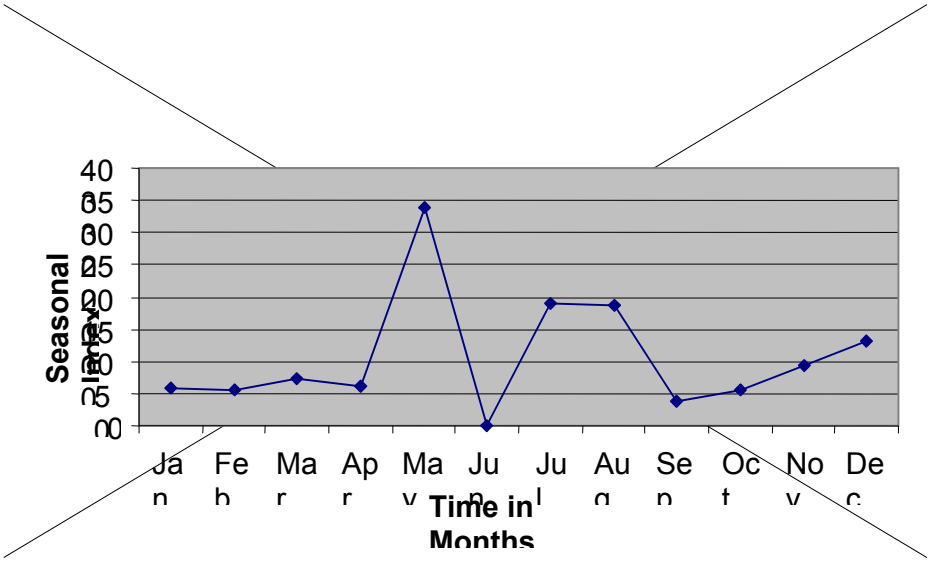


Figure 4.17: Seasonal variation of adenovirus infection in Northwestern Nigeria.

Table 4.40: Duration of diarrhoea in adenovirus infected children in Northwestern Nigeria.

Diarrhoea Duration	Adenovirus Antigen		
	Positive	Negative	Total
1	2	13	15
10	0	1	1
14	0	2	2
16	0	1	1
2	15	55	70
21	0	1	1
3	13	43	56
30	0	1	1
366	0	1	1
4	9	18	27
5	6	12	18
6	1	7	8
60	0	1	1
7	11	35	46
90	1	0	1
Negative	5	28	33
Total	**63**	**219**	**282**

The prevalence of adenovirus infection was highest (63.5%: 40/63) in children who did not pass stool continuously than those who did (36.5%: 23/63). The association between continuous diarrhoea and adenovirus infection was not significant ($p > 0.05$). More than half (50.8%: 32/63) of the adenovirus infected children appeared normal, while 42.9% (27/63) appeared ill and 6.3% (4/63) appeared very ill. There was however no significant difference in general condition of child and adenovirus infection ($\chi^2 = 1.792$, df = 2, $p > 0.05$).

Clinical features associated with adenovirus infection included fever (60.3%: 38/63), vomiting (55.6%: 35/63), fever and vomiting (42.9: 27/63), mild dehydration (49.2%: 31/63), symptoms of URT infection (12.7%:8/63), abdominal pain (4.8%: 3/63) and eye infection (1.6%: 1/63) (Table 4.41).

Twenty five percent (58/232) of adenovirus infected children required treatment while 10.0% (5/50) required hospitalization on visit. There was a significant association between outcome of visit and adenovirus infection ($\chi^2 = 5.335$, df = 1, $p < 0.05$). The treated children were three times more likely to have been infected with adenovirus (OR = 3.000, 95% CI = 1.137-7.918).

The rate of viral infection varied with the food type. The prevalence of infection was highest among those fed on solid food or adult diet (26.3%: 30/114), followed by those fed on breast milk and water (22.2%: 4/18), those fed on breast milk and solid food (20.2%: 26/129), and those that were fed on only breast milk (14.3%: 3/21). There was no significant association between food type and adenovirus infection ($\chi^2 = 2.179$, df = 3, $p > 0.05$).

Of the 282 children enrolled in this study only 14.5% (41/282) drank boiled water as indicated by parents in the questionnaires while 85.5% (241/282) did not drink boiled water. The prevalence of adenovirus infection was 29.3% (12/41) and 21.2% (51/241) among the children that drank boiled water and those who did not drink boiled water respectively. There was no significant association between type of drinking water and viral infection despite the difference in prevalence ($p > 0.05$).

Table 4.41: Clinical manifestation of enteric adenovirus infection in children 0-5 years old in Northwestern Nigeria (n = 63).

Symptom/Sign	Frequency (%)
Diarrhoea	57 (90.5)
Watery stool	55 (87.3)
Stool with mucus	12 (19.0)
Stool with mucus and blood	2 (3.2)
Fever	38 (60.3)
Vomiting	35 (55.6)
Fever and Vomiting	27 (42.9)
Dehydration (Mild)	31 (49.2)
Symptoms of URT[a] infection	8 (12.7)
Abdominal pain	3 (4.8)
Eye infection	1 (1.6)

[a]URT = upper respiratory tract

There was no statistically significant relationship between socioeconomic background and viral infection ($\chi^2 = 3.912$, df = 2, $p > 0.05$). The highest prevalence (25.8%: 46/178) was observed among children from low socioeconomic background, followed by those from high background (20.0%: 8/40) and the lowest was observed in those from medium background (14.1%: 9/64). There was also no relationship between a child schooling and the rate of viral infection ($\chi^2 = 2.180$, df = 2, $p > 0.05$). The prevalence of adenovirus infection among the 26 children who either attended a day care or an Islamic school was 27.3% (6/22) and 50% (2/4) respectively while the prevalence among the children not schooling was 21.5% (55/256).

Co-infection with rotaviruses was observed in 14.3% (9/63) of adenovirus infected patients. There were no significant relationships between co-infection and the prevalence of clinical symptoms observed in these children despite the differences in prevalence. Almost all (89%:8/9) of the co-infected children were from the diarrhoeic population and merely required rehydration and treatment while only one was admitted. About 44.4% (4/9) of the co-infected children appeared ill, one (11.1%) was very ill while 44.4% (4/9) appeared normal. About 77.8% (7/9) of the co-infected children were mildly dehydrated, while 89% (8/9) experienced fever as well as vomiting. Children with fever were three and half times more likely to have been infected with both viruses than those without fever (OR = 3.679, 95% CI = 0.453-29.879), while those who experienced vomiting were five and half times more likely to have been infected than those who did not (OR = 5.565, 95% CI = 0.686-45.120).

Two third (66.7%: 6/9) of these children passed stool continuously and were three and half times more likely to have been infected with both viruses than those who did not (OR = 3.515, 95% CI = 0.860-14.365).

4.5 Co-Infection with Adenoviruses, Astroviruses and Rotaviruses

Co-infection with adenoviruses, astroviruses and rotaviruses was observed in 1.8% (5/282) of cases. Co-infection was more prevalent in males (2.5%: 4/161) than females (0.8%: 1/121) and occurred only in children less than 12 months old with the highest prevalence (60.0%: 3/5) in children in the age group 7-12 months old (Table 4.42). Four out of the 5 multiple infections were detected in Kaduna State while the remaining one was detected in Sokoto State. Multiple infections were more common in the non-diarrhoeic population (2.9%: 1/34) than the diarrhoeic population (1.6%: 4/248).

Eighty percent (4/5) of the triple co-infected patients had diarrhoea duration lasting 2-5 days and watery stool type. The remaining patient (1/5) had stool with mucus and blood. Three children passed stool continuously and were two times more likely to have been infected than those who did not (OR = 2.042, 95% CI = 1.167-3.575). All the triple infected children experienced both fever and vomiting, one had symptoms of URT infection and 3 were moderately dehydrated. Two of the 5 triple infected children were ill while 1 was very ill.

Only a child with triple infection was admitted while the remaining 4 were treated. Analysis of the feeding regimen in the multiple infected children showed that the rate of viral infection varied with the food type. The prevalence was highest among those fed on breast milk and solid food (23.3%: 3/129) and lowest among those fed on breast milk and water (11.1%: 2/18). Triple infection was not detected among other food types (Table 4.43).

Table 4.42: Prevalence of adenovirus, astrovirus and rotavirus (multiple) infection by age group in Northwestern Nigeria.

Age group	Adenovirus, Astrovirus and Rotavirus Infection		
	Positive (%)	Negative	Total
0-6	2 (40.0)	72	74
7-12	3 (60.0)	88	91
13-18	0	31	31
19-24	0	38	38
25-36	0	29	29
37-48	0	10	10
49-60	0	9	9
Total	**5**	**277**	**282**

Table 4.43: Prevalence of adenovirus, astrovirus and rotavirus infection by feeding regimen of children in Northwestern Nigeria.

Food Type	Adenovirus, Astrovirus and Rotavirus Infection		
	Negative	Positive (%)	Total
BS	126	3 (23.3)	129
EBF	21	0	21
Solid	114	0	114
WB	16	2 (11.1)	18
Total	**277**	**5**	**282**

EBF = Exclusive Breast Feeding

BW = Breast and Water

BS = Breast and Solid

WB =Watery and Bloody

All the 5 children with triple infection were detected among the children enrolled in this study that did not drink boiled water. The prevalence of multiple viral infections among the 26 children who either attended a day care or an Islamic school was 3.8% (1/26) while the prevalence among the children not schooling was 1.7% (4/256).

Further analysis of multiple infection and electropherotypes show that all the detected triple infection isolates had the long pattern with RNA profiles L2, L4, L9, L (Faint) and one without visible profile occurring singly. VP6 analysis also showed the long pattern to be associated mainly with subgroup II and subgroup I + II strains and did not occur in other subgroups.

The most frequent VP7 genotypes detected among the triple infected children were the G1G2G8 combination and the G8 that each occurred with a prevalence of 33.3% (1/3), followed by G1 that occurred with a prevalence of 10.0% (3/30). The VP4 genotypes were distributed among the multiple infected children with only one mixed strain P[6][14] detected among them. The commonly detected P[8] strain occurred with a prevalence of 17.6% (3/17) while P[6] was only detected in one multiple infected child (8.3%: 1/12).

In multiple infected children only one single G/P strain combination (G1 P[8]) that occurred with a rate of 17.6% (3/17), and two of the mixed infection strains (G1G2G8 P[6] and G8 P[6][14]) that each occurred with a rate of 100% (1/1) were detected.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Rotaviruses

In this study, rotavirus was shown to be an important cause of acute infantile diarrhoea requiring a hospital visit or hospitalization, as has been observed in other developing countries such as Gambia, Central African Republic, Ghana, Papua New Guinea and Cameroon (Rowland *et al*., 1985; Georges-Courbot *et al*., 1988a; Armah *et al*., 1994; Howard *et al*., 2000; Esona *et al*., 2003).

The 18% rate seen in the present study constrast earlier reports conducted in Lagos State, Southern Nigeria where higher rates of 22.3%, 37.5%, and 45.3% were recorded (Ogunsanya *et al*., 1994; Alabi *et al*., 1998; Audu *et al*., 2002b). The rate was, however, higher than those previously reported in other parts of the country, such as 13.8% in Ife, 13.0% in Ibadan, 11.9% in Maiduguri, 11.0% in Jos, 16.3% in Zaria and 16.2% in Ilorin (Paul and Erinle, 1982a; Ojeh *et al*., 1995; Adah *et al*., 1997a; Nimzing *et al*., 2000; Pennap *et al*., 2000; Audu *et al*., 2002b).

The observed difference in prevalence could be due to difference in climatic conditions, environmental and socio-economic factors or cultural practices. Overcrowding, poverty and poor general sanitation, which are all factors enhancing the transmission of human rotavirus infection in the tropics may account for the high level of rotavirus infection seen in Lagos State. The lower prevalence observed particularly in Jos (11.0%) was attributed to the short period (February to May 1998) of specimen collection by the authors. The period also encompassed the wet season when circulation of rotavirus might be lower (Paul and Erinle, 1982b; Gomwalk *et al*., 1993).

The detection of rotavirus in 18.0% of diarrhoeic cases in this study is consistent with the report of Steele *et al*. (2003), which summarizes results obtained from three African rotavirus workshops organized between 1998 and 2000. The report showed that between 10-20% of diarrhoeic young African children and between 2% and 4% of non-diarrhoeic children attending emergency outpatient department at the hospitals or clinics were excreting rotavirus. The prevalence (7.2%) obtained for the non-diarrhoeic children in this study was, however, higher than that of the above report. Similarly, the result was consistent with a very recent epidemiological profile report of rotavirus infection in India

where the rates of rotavirus detection was 16.2%-35.4% with a median of 23.4% (Kang *et al*., 2005a). The prevalence is also consistent with the rates of 21.9% from neighboring Cameroon (Esona *et al*., 2003), 23.0% from Venezuela (Salinas *et al*., 2004), 22.3% from Denmark (Olesen *et al*., 2005) and 20.0% from China (Wang *et al*., 2005). Prevalence as high as 26.6% from Ethiopia (Bizuneh *et al*., 2004), 43% from Taiwan (Chen *et al*., 2005) and 55% from Vietnam (Van Man *et al*., 2005) have, however, been reported.

The lower prevalence observed in the present study might also be due to the long period (2 years) of specimen storage, which might have led to disintegration of viral particles hence, insufficient antigen in the specimen. This situation may have been compounded by the fact that these specimens were packed in ice and transported from Nigeria to South Africa for analysis leading to further disintegration during transport.

The detection of rotavirus in just a case of intussusception is comparable to the findings of Raul-Valazquez *et al*. (2004) in Mexico, where rotavirus was not associated with intussuception but a significant association between adenovirus and intussusception was observed. This result is reassuring given the fact that rotaviruses have been implicated in intussusception cases and several studies have provided evidence of causal association between the tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) and intussusception (Murphy *et al*., 2001).

The prevalence of rotavirus was highest in Kaduna State and lowest in Sokoto State. Similar variability in area of study has been observed in studies conducted in Italy (Ruggeri and Declich, 1999) and Vietnam (Van-Man *et al*., 2005). This result could be due to the cooler, less dry climate and the more dense population of Kaduna State compared to other states.

Although there was no significant difference, rotavirus was slightly more common in males than females in the study population. The result is similar to previous reports in Nigeria where rotavirus was detected in slightly higher rates in males than females (Paul and Erinle, 1982a; Abiodun *et al*., 1985; Pennap *et al*., 2002c). Similarly, studies in Ethiopia (Abebe *et al*., 1995), Netherlands (Koopmans and Asperen, 1999), Argentina (Giordano *et al*., 2001), Cameroon (Esona *et al*., 2003), Vietnam (Nguyen *et al*., 2004) and Venezuela (Salinas *et al*., 2004) reported higher detection rates of rotavirus infection in males than females. The

reasons for the difference in detection rate between males and females were, however, not known. In this study, the slight difference observed might be due to sex susceptibility or chance. Alternatively, may probably be due to lack of difference in the life styles between boys and girls at age 0-5 years.

Similar to previous studies in Nigeria, rotavirus was detected throughout the study period with slightly higher peaks in the drier months (Adah *et al*., 1997; Pennap *et al*., 2000; Steele *et al*., 2002c). In sub-Saharan Africa (Central Africa Republic, Ethiopia, Ghana, Kenya, Nigeria and Somalia), rotaviruses detection are 6 times more likely to peak during the dry season, but the seasonality of virus detection is not clearly defined in the dry season (Cunliffe *et al*., 1998). The same pattern was seen in the present study where there was no clear peak in rotavirus infection observed during the cool dry months as has been observed in some studies in Nigeria (Paul and Erinle, 1982b; Gomwalk *et al*, 1990), Ghana (Armah *et al*., 1994), and Gambia (Rowland *et al*., 1985). The higher prevalence of rotavirus infection in the dry season may be attributable to geographical and environmental factors. Low relative humidity might be the most important environmental factor for rotavirus survival and spread during the dry season in this study as determined by Paul and Erinle (1982b) in their study in South Western Nigeria.

In this study, the outcome of rotavirus infection was significantly related to the age of the child. The detection of rotavirus infection more often in children under the age of 2 years is consistent with a number of similar studies conducted in Nigeria (Fagbami *et al*., 1985; Gomwalk *et al*., 1990; Audu *et al*., 2002a) and most parts of the world (Abebe *et al*., 1995; Nguyen *et al*., 2004; Chen *et al*., 2005). Rotavirus detection peaked in children in the age group 0-6 months old in this study. Similar peaks of infection had also been reported in Nigeria (Oyejide and Fagbami 1988; Adah *et al*., 1997; Pennap, 2002c), Guinea Bissau (Molbalk *et al*., 1994) and Zambia (Mpabalwani *et al*., 1995). Similarly, studies in Ghana (Armah *et al*., 1994), South Africa (Steele *et al*., 1988, 2003a; Griffiths *et al*., 1992; Wolfaardt *et al*., 1997), Ethiopia (Bizuneh *et al*., 2004) and India (Bahl *et al*., 2005) have shown that rotavirus infection predominantly occur in infants less than 12 months of age. This trend, which has been observed in many African countries, supports Steele (2002a) notion that rotavirus infections occur early in life. It is also in accordance with the assumption in developed areas that the early peak of rotavirus diarrhoea may

result from early exposure to contaminated materials as well as to overcrowded homes (Flores *et al*., 1986). Some of the children in this age group can move around, poking all sorts of things they can lay hands on into their mouths, and the virus is known to survive on surfaces and formites for long period of time. The early peak of infection might again probably be as a result of the absence of maternally acquired antibody or the passive immunity acquired was not to the infecting genotype (Haffejee *et al*., 1991).

Rotaviruses have however been detected in older children in some studies (Marie-Cardine *et al*., 2002; Esona *et al*., 2003; Van Man *et al*., 2005). The decrease in prevalence of rotavirus infection after 24 months in this study might imply that by age 2, most of the older children in the studied area would have been infection and are protected against subsequent symptomatic re-infection. Natural immunity has also been suggested as a reason for decreased incidence with age (Parashar *et al*., 1998).

About 60.6% of the infected children were dehydrated. Viral infection rate was slightly higher among children with mild dehydration than children with severe dehydration. Pennap *et al*. (2002c) and Oyejide and Fagbami (1988) have previously reported higher dehydration rates of 78.4% and 91.0% in Zaria and Ibadan respectively. The association of rotavirus infection with dehydration in this study as similarly reported by other studies worldwide (El Assouli *et al*., 1995; Unicomb *et al*., 1997; Marie-Cardine *et al*., 2002; Salinas *et al*., 2004; Rivest *et al*., 2004) supports the notion that rotavirus diarrhoea is dehydrating diarrhoea.

The administering of ORS to about 36.0% of the diarrhoeic children before presentation at clinic or hospital as seen might be responsible for the lower prevalence of dehydration observed. This is also an indication that the recommendation of ORS, as the first line of action in the management of diarrhoea might be popular among mothers in this region. However, a previous study reported only 8.0% of mothers in the riverine areas to have seen a sachet of ORS before, when shown (Iyun and Oke, 2000).

About 40.4% of the children had not been treated with any antibiotics before stool specimens were collected. However, 54.6% had received one or more types of antibiotics,

antidiarrhoeal and antiparasitics. Antimalarial were also used to treat associated symptoms like fever and vomiting. The most widely used antibiotic by the children was flagyl (metronidazole), followed by septrin (co-trimaxazole) and thalazole. These drugs, which are formulated to stop diarrhoea, did not affect viral detection since they do not have antiviral properties. This indiscriminate use of drugs for the treatment of diarrhoea observed had earlier been reported in Nigeria (Omotade *et al*., 1995; Pennap, 2002c; Aminu *et al*., 2005).

Antibiotics, anti-protozoans, anti-diarrhoeal and herbal remedies have been used mainly in the developing countries for the treatment of diarrhoea (Kasi *et al*., 1995; Singh *et al*., 1995). However, this is contrary to the recommendation of the Global CDD Programme in paediatrics practice. This is because anti-diarrhoeal interfere with efficacy of other drugs, misuse of antibiotics leads to resistance and anti-motility drugs cause adverse drug reaction (Singh *et al*., 1995: Okoro and Jones, 1995).

Although higher prevalence of clinical features were observed in rotavirus-infected children, they were not statistically significant except in the case of vomiting where a strong association was observed ($p < 0.01$). Similar results have been noted in previous studies in Nigeria where fever and vomiting were the common clinical symptoms non-significantly associated with rotavirus diarrhoea in children (Omotade *et al*., 1995; Pennap, 2002c). This result is consistent with a report from Malawi (Pavone *et al*., 1990) and contrasts the significant association of these features reported in Ghana (Armah *et al*., 1995) and Venezuela (Salinas *et al*., 2004). However, similar to reports from Canada (Rivest *et al*., 2004) and France (Salinas *et al*., 2004), vomiting was significantly associated with rotavirus diarrhoea in the present study. The symptoms of URT infection sometimes associated with rotavirus infection as observed might be by chance, as some samples might have been collected during the peak of respiratory infection (Mangia *et al*., 1993; Dodet *et al*., 1997).

Analysis of the feeding regimen in the rotavirus infected children showed that the rate of viral infection varied significantly with food type. The prevalence of rotavirus infection was highest among children exclusively breast fed and lowest in those fed on solid food or adult diet in both the study and diarrhoeic population. This interesting finding has been reported previously in Nigeria (Gomwalk *et al*., 1990; Omotade *et al*., 1995; Pennap *et al*., 2002c). This result maybe a reflection of the presence of many children on this regimen that are within the rotavirus infection peak age group or, may be highly probable that some children

fell into this group as a result of misunderstanding of the question of feeding. Studies have shown that when asked what a child eats, most mothers do not mention food given as snacks because they equate the term 'eat' with mealtime foods (Hendricks and Badruddin, 1994). An occasional observation was made on the contrary to what the mothers were claiming. For example, some mothers were seen with feeding bottles containing water or even milk but still claimed their children were fed on only breast milk. This claim is attributable to fear of being reprimanded by medical personnel who lecture mothers on exclusive breast-feeding.

It has also been reported that sometimes mothers' statements do not tally with researchers observations (Molbak *et al*., 1994). Hendricks and Badruddin (1994) observed that exclusive breastfeeding is rare because early supplementation with water or other food is common. They also posited that addition of food other than breast milk exposes children to environmental contamination. Breastfeeding has been shown to maintain hydration status during diarrhoea episode, hence help in preventing morbidity and mortality from diarrhoea in infants (Huffman and Combest, 1990). It has also been reported that, if breastfeeding is followed strictly, there will be a reduction in diarrhoea incidence by 8%-20% and in diarrhoea mortality by 24%-27% in the first 6 months of life (Feachem, 1986). In addition, a recent study in Venezuela showed rotavirus to be more frequent in bottle fed children than the breast fed infant (Salinas *et al*., 2004). The enlightenment campaign on exclusive breast-feeding should therefore be intensified. The lower prevalence observed among children fed on solid food maybe as a result of decrease in rotavirus infection with age since children fed on solid food are much older. Primary symptomatic rotavirus infection occurs much early in life (Flores *et al*., 1986; Steele, 2002a) thus, children are protected against subsequent symptomatic re-infection (Bishop, 1996).

There was no association between rotavirus infection and the person that prepared food for the child even though the rate of infection was highest among those for whom food was prepared by anybody. This result is an interesting contrast with an earlier finding by Pennap (2002c) in Zaria where an association was observed. A child whose food can be prepared by anybody excludes all exclusively breast-fed children and those whose mothers particularly have to feed. This again re-emphasizes the importance of exclusive breastfeeding.

It was also observed that 14.5% of children infected with rotavirus had another person in the household with the symptom of diarrhoea. One-fifth (20.0%) and 10.5% of children whose parents or housemaids had diarrhoea at the time of stool collection were shedding rotavirus. Housemaids and parents of a child are usually of an age when rotavirus infection is usually asymptomatic or subclinical; however, immune adults are known to develop rotavirus-induced diarrhoea when immunocompromised or exposed to a large inoculum or new strains (Dodet *et al*., 1997). Some studies have even noted illness in parents of children with rotavirus diarrhoea (Mura *et al*., 1996; Parashar *et al*., 1998). The level of hygiene of housemaids in the study area might not be adequate in protecting the child from the predisposing factors of the viral infection. It has been observed that the highly infectious nature of the virus within households especially where there are predisposing factors like crowding, poor hygiene (Bishop, 1996; Jones *et al*., 2003) and contaminated hands (Bos *et al*., 1995) increase the chances of viral infection. With respect to hand washing as a predisposing factor, it has been recently shown that hand washing with soap prevents the clinical symptoms of diarrhoea (Luby *et al*., 2005).

Similar to the finding of Pennap (2002c) in Zaria, drinking boiled water was not protective in rotavirus infection in the present study. This probably may be due to the fact that the virus is not only transmitted through faecal-oral route. Again, there was no association between rotavirus infection and the type of water supply in area where a child lived. Olusanya and Taiwo (1989) found majority of children infected with rotavirus lived in areas with pipe water in their study in Ile-Ife. Leite *et al*. (1996) had earlier posited that good water supply and sanitation are not likely to reduce infection by rotavirus since it prevails in developed and developing countries alike; this was his reason for the need for a vaccine against rotavirus. Furthermore, the high mortality and morbidity associated with rotavirus diarrhoea, coupled with the fact that improvement in water supply, sanitation or hygiene has not had a significant impact on disease, (Bernstein *et al*, 1995; Joensuu *et al*., 1997; Perez-Schael *et al*., 1997; WHO, 2005a), has been the main reasons why the WHO and Global Alliance for Vaccine Initiative (GAVI) made the development of a vaccine against rotavirus a high priority.

In this study, there was no significant relationship observed between mothers' level of education and viral infection. However, rotavirus infection predominated in both children with mothers with a secondary school education and those with illiterate mothers at similar

level. The prevalence of rotavirus antigen was lowest among children whose mothers had a tertiary school education. The reason for this similar prevalence is not clear. However, it is highly probable that it is because many of these mothers delegate childcare to housemaids as postulated by Pennap (2002c). In the studied area, housemaids are usually illiterate elderly women or young girls. Ojeh *et al*. (1995) has earlier reported illiteracy as one of the factors that enhances the outbreak and spread of rotaviruses.

With respect to a child socio-economic background, the highest prevalence was observed among children from low socio-economic background while the lowest was observed among those from high socio-economic background as earlier reported by Fagbami *et al*. (1987) and Ojeh *et al*. (1995) in Ibadan and Olusanya and Taiwo (1989) in Ile-Ife. On a broader scale, there has been similarity in incidence of rotavirus infection between developing and developed countries, thus the virus has been referred to as 'democratic' (Hoshino and Kapikian, 1994b; Dodet *et al*., 1997).

There was no relationship between a child attending school or not and the rate of viral infection. The rate of infection was however highest among children who were not schooling. The reason for this is not clear. Higher rate of asymptomatic infection has earlier been reported among children in day care centers in Nigeria (Abiodun *et al*., 1985). These authors suggested daycares might serve as reservoirs for the disease.

In this study, the detection of rotavirus RNA by PAGE in only 54% (92/170) of specimens may be due to the presence of insufficient intact RNA in the negative samples, as the sensitivity limit of PAGE stained by the silver nitrate is 3-4ng of viral genomic RNA (Herring *et al*., 1982). It might also be due to destruction of the dsRNA during extraction by staying too long in phenol. The group A ELISA assay also detected all the samples that were positive by PAGE. Furthermore, no ELISA negative specimen was positive by PAGE showing a higher sensitivity of the ELISA method than PAGE.

Both the major rotavirus RNA patterns designated as long and short were observed. As demonstrated in other studies the prevalence of long pattern over the short pattern was observed (Steele and Alexander, 1987; Broor *et al*., 1993; Armah *et al*., 1994; Ojeh *et al*.,

1995; Tietzova *et al*., 1995; Trabelsi *et al*., 2000; De Sierra *et al*., 2002; Kasule *et al*., 2003). In this study the occurrence compared well with those reported in other studies. In addition, the short RNA pattern was seen in a minority (19.6%) of all electropherotypes. This compared well with an incidence of 12% reported from South Africa (Steele and Alexander, 1987), 16% from Ghana (Armah *et al*., 1994), and 16% from India (Broor *et al*., 1993).

The co-circulation of different electropherotypes in the community is a common feature of most epidemiological studies, where one strain predominates in numbers but co-circulates with minor RNA genomic variants (Espejo *et al*., 1981; Beards, 1982; Steele and Alexander, 1987). The presence of a large pool of genomic variants in the community, as indicated by the large number (16) of RNA electropherotypes seen, may be an indication of an active genetic reassortment between strains or may signify the potential for active genetic re-assortment between different viral strains in the locality (Steele, 1989; Watanabe *et al*., 2001). This impressive phenomenon has been reported in Nigeria (Avery *et al*., 1992; Ojeh *et al*., 1995; Adah *et al*., 1997a; Audu *et al*., 2002b; Pennap, 2002c) and other countries (El Assouli *et al*., 1995; Esona *et al*., 2003). The circulation of a single predominant long (L5 = 28.3%) RNA electropherotype with minor co-circulation variants, a typical feature in rotavirus epidemiology (Steele *et al*., 1993a) was also observed in this study.

Extensive genomic diversity of the long RNA profiles was observed with 10 different long RNA migration patterns detected. For gene reassortment to occur a single individual must be infected simultaneously with more than one strain of rotavirus. The variation in the electrophoretic patterns may also be a consequence of either antigenic drift or antigenic shift (Estes, 1996). These two mechanisms may be a common mechanism for the generation of a large pool of viral variants and the high rate of mixed viral strains detected in this region. Furthermore, the continuous viral infection in older (presumably previously exposed) children may facilitate antigenic shift. This large reservoir of susceptible individuals will facilitate and promote reassortment and hence the emergence of new strains as noted by Beards (1982).

In this study, two RNA profiles typical of mixed rotavirus infections were seen. These may represent possible means of emergence of genetic reassortant rotavirus strains. To the best of our knowledge, no such cases have been reported in Nigeria.

The electropherotypes variants that occurred throughout the study period were able to do so probably as a result of the presence of a large pool of susceptible population, which allowed for their persistence. Some predominant electropherotypes observed in July 2002-July 2003 were found to disappear by August 2003-July 2004giving way to new predominant strains, a finding also reported by Chiba *et al*. (1984) and Pennap (2002c). In view of the fact that population is dynamic, it is possible that, the electropherotype that emerged in the second year of study were introduced into the population after the preceding study period. Alternatively as postulated by Pennap (2002c), they could have been circulating at low levels as a result of immunity, but eventually became pathogenic for the population and multiplied to detectable levels. Development of immunity to the prevailing electropherotypes and major antigenic diversity between circulating rotaviruses might be involved in the observed changes.

VP6 subgroup-specificity could be assigned to 87% of the EIA positive strains, similar to recent report from Paraguay where 87% of strains were typed (Coluchi *et al*., 2002). Pennap (2002c) could only assign VP6 subgroup-specificity to 60.7% of specimens analysed. The non-reactivity of 13% (22/168) of the specimens is probably due to low antigen levels in the specimens as reported in previous studies in Africa (Steele *et al*, 1988).

The development of monoclonal antibodies specific to the VP6 protein of rotavirus has made easy the identification of the different rotavirus subgroups in epidemiological studies worldwide. Of the strains that could be sub-grouped, VP6 subgroup II rotavirus strains occurred more commonly (28%) than subgroup I (25%) strains. Unusually and of importance, was finding more than one-third (36%) of the strains reacting to both subgroups I and subgroup II monoclonal antibodies. A significant number (11%) of the isolates exhibited neither subgroup I nor subgroup II reactivity pattern. The presence of 4 forms of antigen in this study confirms the concept of 4 subgroups of human rotaviruses.

This interesting finding of more than one-third of the strains studied reacting to both VP6 subgroup I and subgroup II monoclonal antibody is a striking feature of the results of the present study. Although the occurrence of strains with VP6 subgroup I and subgroup II specificity have been reported in previous studies in Nigeria, they were only detected in a single case (Steele *et al*., 2002) and in 4 cases (Pennap *et al*., 2002c). Similar results have been reported in very low rates such as: 0.6% in South Africa (Steele *et al*., 1989) and 4.3% in Cameroon (Esona *et al*., 2003).

In this study, as expected, most of the strains with subgroup II and those with both subgroup I and II specificity had long electrophoretic patterns while those with subgroup I specificity were associated with the short RNA profile. However, 3 rotavirus strains with VP6 subgroup I specificity had a long RNA electrophoretype. Strains with this rather unusual characteristics, though occur in low frequencies, are not uncommon and have been reported in previous studies in Nigeria (Pennap, 2002c), South Africa (Steele and Alexander, 1988), Australia (Palombo *et al*., 1996), India (Ghosh and Naik, 1989; Broor *et al*., 1993; Krishnan *et al*., 1996; Jagannath *et al*., 2000) and Cameroon (Esona *et al*., 2003). Strains with this type of reactivity are usually of animal origin (Kapikian and Chanock, 1990). It may therefore, be possible that these strains originated from animals. This is because Northwestern Nigeria is predominantly a farming community, and produce livestock in the traditional way both for commercial and subsistence consumption, thus close relationship exist between most people in the study area and their animals.

These strains are also likely to represent naturally occurring reassortants between human and animal rotaviruses with phenotypes that usually results in the emergence of viruses with novel genetic and antigenic properties. They can also spread widely and even cause epidemic in children as suggested by Palombo *et al*. (1996). This result further confirms the speculation that naturally occurring reassortant events may be taking place in Northern Nigeria (Adah *et al*., 1997b). Thus diversity exists among the human rotavirus isolates and there is existence in nature of mixed infections between rotaviruses of the two common human genogroups (Wa and DS-1).

This study has shown that the four most important epidemiologically rotavirus strains (G1, G2, G3 and G4) and the emerging G8 and G9 strains are in circulation in Nigeria. This result is expected especially as G1 genotype is the most prevalent VP7 type worldwide (Nakagomi *et al*., 1990; Steele *et al*., 1992; Gentsch *et al*, 1996), in Nigeria (Avery *et al*., 1992; Pennap *et al*., 2000; Adah *et al*., 2001; Audu *et al*., 2002a), in Africa (Georges-Courbot *et al*., 1988b; Steele *et al*., 1995, 1998; Radwan *et al*., 1997; Cunliffe *et al*., 1998; Trabelsi *et al*., 2000; Kasule *et al*., 2003), in Europe (Iturriza-Gomara *et al*., 1999), in the USA (Griffin *et al*., 2000), in Australia (Bishop *et al*., 2001), in Asia (Fang *et al*., 2002; Bahl *et al*., 2005) and in Canada (Kostouros *et al*., 2003).

In this study VP7 genotype G2 strains circulated as the second most abundant single infection strain and as the third most abundant mixed infection strain. Similarly, Adah *et al*. (2001) in a previous study in North Eastern Nigeria reported G2 as the third most abundant genotype. However, a year after the genotype was reported to be the least circulating in Northcentral region of the country, occurring with a prevalence of 0.86% (Steele *et al*., 2002) and was even absent in the Southern part of the country (Audu *et al*., 2002b). In a previous study conducted in Zaria, Kaduna State, Nigeria, no G2 was detected (Pennap *et*

al., 2000). This report highlights the varying diversities in the predominance of genotype distribution in geographical locations, particularly in African countries.

In the present study, genotype G3 was the least common genotype circulating at low levels as both single and mixed infection strain. This contrasts previous study in Nigeria carried out between 1993 and 1995 where genotype G3 was reported as the most common strain in circulation in Southern Nigeria occurring in 39% of infected children (Adah *et al*., 1997a). The genotype has also been reported as the second most abundant strain in some studies in Nigeria (Audu *et al*., 2002b; Steele *et al*., 2002).

VP7 genotype G4 are generally low in circulation and have been reported only in a few studies in West, East and South Africa. This is the second report of the circulation of G4 genotype in Nigeria, which occurred at low frequency and as mixed infection in this study. Genotype G4 was reported in circulation for the first time in Nigeria in a single strain in Ilorin (Audu *et al*., 2002b). Similarly genotype G4 has been reported to circulate in low numbers in South Africa (Mnisi *et al*., 1992) and Malawi (Cunliffe *et al*., 1999).

In the present study, the detection of G8 as the second most predominant strain in both single and mixed infections contrasts reports of previous studies in Nigeria (Adah *et al*., 1997c, Steele *et al*., 2002a), Kenya (Nakata *et al*., 1999), South Africa (Steele *et al*., 1999), Guinea Bissau (Fischer *et al*., 2000), Ghana (Armah *et al*., 2001) and Cameroon (Esona *et al*., 2003). These G8 genotypes have, however, been reported in an earlier study in Nigeria where it was circulating in levels similar to that of the present study as the second most predominant strain (Adah *et al*., 2001). Genotype G8 were before now rarely found in man. They have a wide distribution in animal species and have been reported in pigs, horses and cattle (Cooney *et al*., 2001). The detection of this genotype as the second most prevalent in circulation may be interpreted as an evidence for accidental animal to human transmission. This is because such transmission cannot be ruled out in the study area as many of the people lived in close proximity with their animals and share a common source of drinking water.

In addition, about 24.7% of the children infected with rotavirus had parents who owned animals while 18.24% of these infected children had contact with these

animals. This has been reported to be common in developing countries (Urasawa *et al*., 1992). Such unhygienic circumstances might have facilitated the emergence of reassortant viruses as a result of simultaneous infection in children by human and animal viruses or multiple human rotaviruses. This might explain why G8 was detected as the second most prevalent mixed infection strain occurring in 30% of cases and G1G8 was the predominant mixed infection strain in this study. Cooney *et al*. (2001) earlier pointed out that the frequent identification of these genotypes among human clinical isolates suggests inter-species transmission has led to their emergence in man. This point is buttressed by the fact that Adah and co-workers (2003) recently found a close relationship between bovine G8 and human rotaviruses in Nigeria. However, human strains with a high degree of genetic homology to animal strains have also been reported (Gerna *et al*., 1994; Parashar *et al*., 1998). This result may also be attributable to the use of improved typing method (specific RT-PCR) for rotaviruses in this study, and it is likely that the detection of rare or emerging genotypes will continue.

The detection at low levels as mixed infections of the more novel unusual rotavirus strain with G9 specificity in this study extends its global distribution and suggests a continued evolution of rotaviruses. This result is similar to the report of Adah *et al*. (2001) in Nigeria where G9 was first detected as mixed infection in a single case of diarrhoea. A recent worldwide review of rotavirus studies has confirmed genotype G9 as a globally important rotavirus strain (Gentsch *et al*., 2005). This increased detection only confirms their emergence as the fifth most abundant strain.

The G9 strains were found to infect the normal target age for rotavirus infection, showing that they were competing successfully with the more usual circulating rotaviruses. This differs however from the results of a previous study in Nigeria where G9 was detected for the first time in slightly older children and was the most prevalent strain (Steele *et al*., 2002). The result also differs from that reported in the United Kingdom (Cubitt *et al*., 2000). However, a study in Ghana detected G9 in all group with higher prevalence in younger children (Armah *et al*., 2003).

The emergence of G9 as an epidemiologically important genotype and the importance of genotype G8 in focal areas is the reason Kapikian *et al*. (2005) propose to add human-bovine (UK) reassortants with G8 and G9 specificity to their previously developed

tetravalent vaccine thereby formulating a 'designed' hexavalent vaccine for universal use. This recent report is a welcome development in view of the fact that in this study, a significant number of genotype G9 were found in circulation while G8 was the second most predominant strain detected.

A high rate of mixed reactivity of rotavirus strains is a very common finding in Northern Nigeria. The VP7 genotype strains with mixed reactivity detected in this study were G1G8, G1G2G8, G2G4 and G2G8, G1G2, G1G2G8G9, G1G3, G1G9 and G4G9 in order of decreasing prevalence. The "mosaic" rotavirus with G1G8 genotypes predominated in this study unlike other Nigerian studies, where the "mosaic" rotavirus with G1G3 was reported to be dominating (Adah *et al*., 1997a, Pennap *et al*., 2000; Audu *et al*., 2002b; Steele *et al*., 2002). A later study by Adah *et al*. (2001) in Nigeria however reported the predominance of the "mosaic" rotavirus with G1G2. Notably, none of the above studies reported mixed infection with three or four genotypes of rotavirus strains as observed in this study. Again none had a dual infection involving the G8 and G9 genotypes. It is important to note that these previous studies have used different techniques including antigenic analyses with monoclonal antibodies (Pennap *et al*., 2000), RT-PCR typing with various primers (Adah *et al*., 1997a; Asmah *et al*., 2001) and probe hybridisation (Steele *et al*., 1995). Thus it seems unlikely that this is a phenomenon of these techniques, but rather a real characteristic of rotavirus strains in Africa. This result, however, reflects the predominance of G1, G2 and G8 during the study period.

Almost one quarter (30%) of the rotavirus positive specimens could not be assigned any VP7 genotype. This may be an indication of the existence of genotypes, which the genotype-specific primers of Gouvea *et al*. (1990b) used in this study could not detect. This primer mis-matching has been reported previously in Nigeria (Adah *et al*., 1997c; Audu *et al*., 2002b). Similar findings have been reported in Egypt and Ghana where 38.7% and 24% of EIA positive specimens respectively could not be genotyped (Radwan *et al*., 1997; Armah *et al*., 2001). Non-typeable G-genotypes have been reported in India (Ramachandran *et al*., 1996; Das *et al*., 2002), Brazil (Cardoso *et al*., 2000), Paraguay (Coluchi *et al*., 2002) and China (Fang *et al*., 2002, 2005).

These non-typeable strains occurred more frequently in the older children than the younger ones with the highest and lowest prevalence observed in the 37-48 months and 0-6 months

old children respectively. The occurrence of non-typeable strains in this study is a cause for alarm in view of efforts towards an effective vaccine. Such strains should have been subjected to sequencing analysis for further studies to ascertain their genotype status.

The detection of rotavirus infection in children older than 37 months in this study raise the interesting question of whether, re-infection in African children is commonly symptomatic. Some of the reasons older children acquire a symptomatic rotavirus re-infection in Africa, may include, malnutrition (Mata *et al*., 1982) and high background levels of HIV or other enteric pathogens (Dodet *et al*., 1997).

The detection of G1 and G8 in all the states studied is attributable to the predominance of these genotypes during the study period while the highest prevalence recorded for Kaduna state maybe as a result of a large and susceptible study population. This thus expands the diversity of VP7 genotype in these states. The absence of G2, G3, G4 and G9 from Kebbi State and G3 and G4 from Zamfara State may be due to fact that 57.1% and 40% of the EIA positive specimens from Kebbi and Zamfara state respectively were non-typeable. Genotypes have been observed to vary between places of study (Adah *et al*., 1997b; Liddle *et al*., 1997). This result may also be transitional and may change in the future because VP7 genotype prevalence depends on the locale as observed in both developing (Urasawa *et al*., 1987) and developed countries (Noel *et al*., 1991).

The present study detected genotype P[6] as the most abundant P-type in Nigeria as previously reported in Nigeria (Adah *et al*., 1997b, 2001; Audu *et al*., 2002a, 2002b; Pennap *et al*., 2002c) and Ghana (Armah *et al*., 2001). Genotype P[6] was previously thought to be neonatal strains and hence thought to be associated with asymptomatic infection (Gorziglia *et al*.,1988b, Steele *et al*.,1992, Santos *et al*.,1994). However, the present and previous studies conducted in Nigeria and other studies conducted in South Africa, India, and Ghana has increasingly shown them to be associated with diarrhoea (Steele *et al*., 1995; Ramachandran *et al*., 1996; Armah *et al*., 2001). This result questions the validity of the putative association of the P[6] rotavirus in asymptomatic infections, which leads to important considerations for this phenotype as a vaccine candidate. This, therefore, suggests that this genotype might be common in symptomatic infections of children in some more countries after all. This result, however, contrasts results from neighbouring Cameroon (Esona *et al*., 2003) where they circulated at low levels (only in a

case) and were the least detected. As suggested by Pennap *et al*. (2002c), detailed study of the virulence genes of P[6] isolates from children of different ages and different regions might explain the discrepancies.

Genotype P[8] was the second most frequently detected P-type in this study. In Africa, only limited studies have reported on the occurrence of the VP4 genotypes. These studies, however, showed genotype P[8] to predominate and circulate at higher levels compared to the present study. In Southern Africa, two South African studies revealed that P[8] genotype were predominant (Steele *et al*.,1993b; Mphahlele and Steele, 1995). However, in a recent Southern Africa study conducted in Botswana by Kasule *et al*. (2003), genotypes P[6] and P[8] were the most abundant strains found circulating at similar level (33% each). In Zambia, P[8] genotype was also prevalent in 65% of cases (Steele *et al*., 1998) and in Malawi during the 1997-99 rotavirus season P[8] was observed as the most common strain in circulation (Cunliffe *et al*., 2001a). A similar picture of P[8] predominance has also been observed in Tunisia and Cameroon where the P[8] genotype was detected in 18.5% and 20% of cases (Trabelsi *et al*., 2000; Esona *et al*., 2003). This study reflects the widespread distribution of VP4 genotype P[8] in Africa.

VP4 genotype P[4] was the third most commonly detected P-type in this study. Similar levels have been detected previously in Nigeria (Audu *et al*., 2002b) and Botswana (Kasule *et al*., 2003). The figure is, however, lower than that reported in many studies in developing countries and in Asia. In a study in Argentina, P[4] was reported to be the most abundant genotype circulating at a high prevalence of 71% (Arguelles *et al*., 2000). In Taiwan, the genotype P[4] was detected as the second most important strain in circulation occurring in 34% of cases (Tsai *et al*., 2000). Similar results have been reported in Bangladesh (Bern *et al*., 1992b), Japan (Ushijima *et al*., 1994), India (Das *et al*., 2002) and China (Fang *et al*., 2002). Similar trend has also been observed in Ireland (O'Mahony *et al*., 1999) and UK (Iturriza-Gomara *et al*., 1999).

In Africa, the VP4 genotype P[4] has been reported in very few studies. Unlike results from the present study, genotype P[4] was detected in Ghana as the most predominant strain in circulation in 1998 by Asmah and colleagues (Asmah *et al*., 2001). Like the present study, P[4] genotype was detected in 11% each of samples tested in Malawi (Cunliffe *et al*., 1999) and Tunisia (Trabelsi *et al*., 2000). In Zambia, Steele and co-workers (1998a) reported 12%.

Studies in South Africa however, reported higher (21%-23%) detection rate of P[4] (Steele *et al*., 1993b; Mphahlele and Steele, 1995).

Significantly, the detection of genotype P[10] as mixed infections in symptomatic and a higher proportion of asymptomatic children in Nigeria for the first time adds to the increasing detection of this unusual VP4 genotype in Africa. Low-level detection of the strain has been reported from studies in Ghana, Kenya and Malawi (Steele *et al*., 2003) and recently in Cameroon (Esona *et al*., 2003). It may however, be interesting to investigate why this genotype was detected mainly in children <18 months of age in this study in contrast to previous study where it was detected in older children.

More significantly, P[14] genotype was detected for the first time in Nigerian as part of mixed infections mainly among asymptomatic children impling a greater diversity in VP4 strains circulating in Nigeria. The first identification of human rotavirus strain with P[14] type was reported in 1992 in Italy (Gerna *et al*., 1994). They were later recovered by nucleic acid sequencing or by hybridization analysis from Thailand, South Africa, Australia and Italy (Urasawa *et al*., 1993; Mphahlele and Steele, 1995; Palombo and Bishop, 1995; Arista *et al*., 1999). So far, reports on the identification of human rotavirus strains with P[14] type have been limited. They have however been detected in similar level with the present study in Italy (Arista *et al*., 1999).
The identification of novel rotavirus strains in this study is not unexpected as many of the people in the studied area live in close proximity with their animals and share a common source of drinking water. In addition, about 24.7% of the children infected with rotavirus had parents who owned animals while 18.24% of these infected children had contact with these animals. Rotaviruses are ubiquitous in the animal kingdom, and therefore, interspecies transmission and more importantly, exchange of genetic material between animals and human strain through reassortment can lead to emergence of novel rotavirus strains of epidemiological significance (Iturriza- Gomara *et al*., 2004).

It is worth noting that 21.8% of specimens had a rotavirus strain, which reacted with a mix of VP4 specificities. In a previous study in Nigeria (Audu *et al*., 2002b), mixed VP4 genotype of only a single P[4][6] combination was reported in 28% of cases compared to the present study where a cocktail of 8 mixed infection strain specificity was observed. Similarly Adah and co-workers (2001) reported a single P[6][8] combination in 5.6% of

cases in Nigeria. However, Pennap (2002c) detected 2 mixed infection strain type with the P[4][6] predominating over the P[6][8]. Several researchers worldwide have reported the occurrence of mixed strain specificity (Steele *et al*., 1995; Santos *et al*., 1994; Armah *et al*., 2001; Coluchi *et al*., 2002; Kasule *et al*., 2003; Fischer *et al*., 2005). Dual infections have been reported in most studies in Africa but not in rates as high as that observed in this study. In South Africa, Botswana and Cameroon, the most common dual infection were found to occur between strains bearing a P[4] and a P[8] VP4 genotype (Steele *et al*.,1995; Kasule *et al*., 2003; Esona *et al*., 2003), while the most common dual infection in the present study occurred between strains bearing a P[4] and a P[6] and also a P[6] and a P[10]. The second most common dual infection was found to occur between strains bearing a P[6] and a P[8] genotype.

The age distribution of the VP4 genotypes among children infected with rotavirus, showed that only VP4 P[6] genotype circulated in all the age groups. This finding is unusual, as this strain is known to be common in neonates. The strain predominantly affected children less than 24 months old. This differs to some extent from the observation made by Esona *et al*. (2003) where P[6] was detected in a 6 month old child. Genotype P[8] was detected in children less than 49 months old. This also differs from the observation made previously in Nigeria by Adah and co-workers (1997b) in which P[8] genotype was commonly detected in children between the ages of 6 and 9 months.

Genotype P[6] was the only genotype that occurred in all the states studied as single infection. Other genotypes occurred in all states studied either as single or mixed infections. The genotypes were significant distribution in States with P[6] and P[8] predominating in Kaduna State followed by a combination of P[4][6][8]. Differences in the distribution of the VP4 genotypes have been previously reported in Nigeria (Adah *et al*., 1997a, Audu *et al*., 2002b). However, majority of the mix infection specificities were detected in Kaduna and Zamfara States. This may be due to the large and susceptible number of specimens collected from these states.

Seven distinct G and P strain combinations of four different G-types and three different P-types were identified in this study. This is similar to what was observed in rural Guinea Bissau and Cameroon, where nine and 11 different G and P-type combinations were respectively described (Fischer *et al*., 2000; Esona *et al*., 2003). The detection of G1 P[8] strain as the most predominant in Nigeria correlates well with reports from previous study

in Nigeria (Adah *et al*., 1997b, 2001) and contrast the report of Audu *et al*. (2002a) where G1 P[6] was the predominant strain combination detected. In Africa, similar pictures of G1 P[8] predominance have been reported in Kenya (Nakata *et al*., 1999, Cunliffe *et al*., 2001b) and Tunisia (Trabelsi *et al*., 2000). It has also been detected as predominant circulating strain in the USA (Santos *et al*., 1994), Japan (Ushijima *et al*., 1994), Mexico (Castillo *et al*., 2000), France (Bon *et al*., 2000), Ireland (O'Halloran *et al*., 2000), India (Das *et al*., 2002; Bahl *et al*., 2005), and Argentina (Castello *et al*., 2004). The detection of G1 P[8] strain as the most prevalent strain in Nigeria further confirms its global predominance in circulation. However, genotypes are known to vary from place to place and from time to time (Liddle *et al*., 1997). Rotavirus vaccine based on rotavirus G1 P[8] strain formulation are presently on trial as a possible intervention tool for rotavirus diarrhoea.

Genotype G8 P[6] was detected in 9 symptomatic and one asymptomatic child in the present study, a finding also documented previously in Nigeria (Pennap, 2002c) and Bangladesh (Unicomb *et al*., 1999). However, this strain combination has only been reported in a very few studies in certain regions in Africa. The detection of G8 P[6] strain combination as the second most predominant strain in this study is attributed to the predominance of G8 and P[6] genotypes circulating in the country at this time. Comparative studies of the genes of the G8 P[6] strain recovered from symptomatic and asymptomatic children will be necessary to give an insight as to what gene causes virulence in the strain and the difference between virulent and avirulent G8 P[6].

Rotavirus strain G2 P[4], which is another globally common strain, was detected as the third most predominant strain in this study. Some studies in other countries have reported the occurrence of strain G2 P[4] in much higher percentage than observed in the present study (O'Mahony *et al*., 1999; Arguelles *et al*., 2000; O'Halloran *et al*., 2000; Araujo *et al*., 2001; Jain *et al*., 2001; Das *et al*., 2002; Castello *et al*., 2004; Bahl *et al*., 2005). The G2 P[4] prevalence observed in the present study is lower than that observed in other studies in Africa such as South Africa (Steele *et al*., 1993b,1995), Kenya (Nakata *et al*., 1999) and Guinea Bissau (Fisher *et al*., 2000).

In this study, 3.6% of unusual and 50.9% of mixed P and G combinations types were observed. Similar results have been reported from Brazil (Mascarenhas *et al*., 1998l), USA

(Ramachandran *et al*., 1998), Ireland (O'Mahony *et al*., 1999), Argentina (Arguelles *et al*., 2000) and Denmark (Fischer *et al*., 2005). In Africa, few studies (Armah *et al*., 2001; Fischer *et al*., 2003) have actually reported the predominance of mixed genotype combination. In Ghana, the unusual strains G3 P[4] and G2 P[6] were reported as the most important strains causing rotavirus infection in the year 1998 (Asmah *et al*., 2001) and 1999 (Armah *et al*., 2001). In a very recent study in India, the globally uncommon strain G4 P[6] was the most prevalent genotype combination isolated primarily from neonates (Kang *et al*., 2005b). Uncommon strains have been observed in studies conducted in Argentina, Tunisia, Ireland, Malawi, Brazil, India and China (Arguelles *et al*., 2000; Trabelsi *et al*., 2000; O'Halloran *et al*., 2000; Cunliffe *et al*., 2001; Araujo *et al*., 2001; Das *et al*., 2002; Fang *et al*., 2002).

The occurrence of mixed infection genotypes in high frequency in this study is not unusual, as mixed infections have been reported to be more common among symptomatic children in Northern Nigeria (Adah *et al*., 1997b). A similar frequency of mixed infections has also been observed in Guinea-Bissau (Fischer *et al*., 2003). This mixed infections, most likely represent naturally occurring reassortants of rotavirus strains, which could have resulted from multiple infections by multiple type isolates. Dodet *et al*. (1997) had posited that mixed infections with several strains are common in developing countries. The highly dusty and windy nature of the study area especially during the dry season could have increased the aerosol transmission of various types of rotavirus strains with a consequent high possibility of mixed infections. It could also have been as a result of the communal life usually found in the study area where there is a lot of sharing (Pennap, 2002c). The practice where people live in close proximity with their animals could have been a predisposing factor for the mixed infections observed in this study. Also the presence of many unusual isolates known to be reassortants may depict a high rate of multiple infections in this region. Furthermore, the very high diversity of unusual strains seen in this study, strengthen the hypothesis that West Africa could be a hot bed for genetic recombination as stated by Armah and co-workers (2002b).

Age distribution of single G/P constellation in rotavirus infected children show strain G1 P[8] which is the overall predominant strain seen in this study to occur more often in children below 18 months and was not detected in children older than 37 months. This contrasts results of a previous study in Nigeria where this strain was detected in children in all age group peaking in the 3-9 months old children (Adah *et al*., 1997b). The absence of G8 P[6] in the age group 0-6 months is very unusual as the P[6] strains are considered to be classical neonatal strains.

Generally rotavirus strains were detected predominantly in children less than 24 months old in this study, a notable finding documented by several researchers worldwide. This result might suggest that by 2 years of age, most of the children in the study area would have had an infection, hence, the development of immunity to the infection. Natural immunity has been suggested as the reason for decreases incidence with age (Parashar *et al*., 1998). The detection of rotavirus infection in children older than 37 months in this study could have been as a result of exposure to new genotypes, malnutrition, and high background levels of HIV or infection with other enteric pathogens. The result however, raises the interesting questions of whether re-infections in African children are commonly symptomatic. This has not been addressed in any study in Africa to my knowledge.

The changes observed in the occurrence of predominant strains over time and place of study have been reported in a few studies in Nigeria and elsewhere (Fischer *et al*., 2005). Strains observed throughout the study period and those that prevailed in all the states studied were those that might have found a large pool of susceptible children that allowed for their persistence. On the other hand, those that could not be detected after a certain time or in certain states could have been as a result of type specific immunity among children included in the study. Strains that were initially undetected but emerged at another time at other places could have just been introduced into the population or were at undetectable levels and which could now be detected. The presence of susceptible populations could have enabled such strains to establish themselves to detectable levels. Annual and seasonal variations in the occurrence of strains may provide useful information on the reservoir and mode of viral transmission, which are useful in the provision of an effective vaccine.

High proportion of untypeable strains constellation is a common feature of rotavirus characterization in most developing countries where more than 30% of all strains remain untypeable. The incompletely typed (GNT and PNT) isolates were evenly distributed in the various age groups while the NT strains increased in prevalence with age. In addition the NT strains were more prevalent in asymptomatic children. Such isolates must be considered important and worth subjecting to further studies as they may have important implications for vaccine applications in Nigeria. They may belong to a group of yet undetermined genotype for which no primer(s) was included during the molecular study. It has also been suggested that some of them could have been of animal strains that have infected young children. This point is buttressed by the detection of an animal rotavirus strain (G5) in a Cameroonian child (Esona *et al*., 2004), which points to the likelihood that a large proportion of untypeable strains in Nigeria and other African studies of rotavirus,

particularly in Central Africa, may be G5 and other "animal" rotavirus strains that have infected young children. Some may also be typeable isolates but with a mutation and therefore require sequencing (Adah *et al*., 1997c).

The availability and affordability of sequencing methods now provide an alternate tool for characterization of these unusual strains to provide more information on circulating types and the natural reservoirs for these strains.

Genotypes G1, G3 and G4, G8 and G8 are always associated with SG II and the 'Long' electropherotic pattern while genotypes G2 is always associated with SG I and the 'Short' electrophoretic pattern. While some studies indicated correlation between electropherotype and VP7 genotypes, others reported their independence (Steele *et al*., 1998). In this study, viral strains with identical electropherotypes did not always have the same genotype specificities, a finding also documented by Georges-Courbot *et al*. (1988b) and Pennap (2002c). Some G2 genotypes detected in both single and mixed infections were associated with a SG II specificity and the 'Long' electropherotic pattern while some G1 and G8 were associated with SG I. The combination strain G1 P[8] and G8 P[6] were frequently associated with subgroup I, while G2 P[6], G2G9 P[6] exhibited a 'Long' electropherotic pattern. The multiple mixed infection strain G2G4G9 P[6][8][10] exhibited a SG II specificity and were associated with the 'Long' electropherotic pattern. This anomaly might likely have resulted from naturally occurring reassortment between human and animal rotaviruses and shows that electropherotype cannot be used to predict genotype of any one strain, at least for long RNA patterns (Georges-Courbot *et al*., 1988b).

5.2 Astroviruses

The prevalence rate (19.1%) of astrovirus detected in this study is higher than previous rates reported in Nigeria where the virus circulated at low rates of 1.5%, 6.4% and 1.2% (Avery *et al*., 1992; Pennap *et al*., 2002a; Audu *et al*., 2002b). Similarly, the virus has been detected in much lower rates in studies conducted worldwide: 7.0% in South Africa, 6.0% in France, 6.7% in Australia, 4.9% in Spain, 5.2% in the United State, 2.7% in Botswana, 4.0% in Germany, 4.6% in Mexico, 1.6% in Hungary and 5.5% in Mongolia (Steele *et al*., 1998; Bon *et al*., 1999; McIver *et al*., 2000; Guix *et al*., 2002; Rodriguez-Baez *et al*., 2002; Basu *et al*., 2003; Oh *et al*., 2003; Mendez-Toss *et al*., 2004; Jakab *et al*., 2004; Hansman *et*

al., 2005). This very high prevalence shows astrovirus to be an important cause of gastroenteritis in Nigerian children.

Another notable finding was the detection of astrovirus antigen in half (50%: 3/6) of the intussusception cases while rotavirus and adenovirus were detected in a single case each. This result is very interesting because adenoviruses are frequently associated with intussusception as reported in a previous study in Nigeria (Bode and Omilabu, 2002) and in studies in other countries (Bhisitkul *et al*., 1992; Montgomery and Popek, 1994; Hsu *et al*., 1998). More so rotaviruses have been widely reported as the cause of intussusception in recipient of rotavirus vaccines (Murphy *et al*., 2001). This result might however, suggest an association between astrovirus and primary intussusception in Nigerian children.

Human astroviruses were detected in all the 4 States studied and occurred more frequently in Kaduna State. This uncommon finding reported elsewhwere (Mendez-Toss *et al*., 2004) might indicate that astroviruses are common in Northwestern Nigeria. The frequent occurance of astrovirus in Kaduna State might be attributable to the presence of more susceptible children to astrovirus in the State.

Astrovirus infection predominated in children less than 18 months in this study with the highest prevalence in the 0-6 months old. This is not unusual as the virus is usually associated with children less than 24 months old as reported by most studies (Marx *et al*., 1998; Steele *et al*., 1998b; Qiao *et al*., 1999; McIver *et al*., 2000; Basu *et al*., 2003). However, in the study of Pennap *et al*. (2002b), astrovirus was reported to predominate in children one to four years old.

There was no major variation in the monthly prevalence of astrovirus observed in this study with no virus isolated in the first quarter of the study. However, viral shedding occurred more frequently in the dry season. This might be attributable to the highly dusty and windy nature during the dry season in the study area, which could have increased the aerosol transmission of the virus. This finding provides an interesting contrast with the findings of Pennap *et al*. (2002b), where the peak of rotavirus detection was the cold season and to that of Steele *et al*. (1998b) and Guix *et al*. (2002) where astroviruses were isolated throughout the study period and peaked in the winter months. The absence of astrovirus in the first quarter of the study may be due to loss of the group - specific antigen during storage of

specimens as a result of repeated freeze thawing. It might also be attributable to fewer numbers of samples collected during this period or to a less susceptible population. Bates *et al*. (1993) and Steele *et al*. (1998b) have reported lower astrovirus positive rates on stored and frozen samples in the early years of study. In addition, unlike rotaviruses that can survive storage in ambient tropical temperature for more than 2 months (Fischer *et al*., 2002), astrovirus particles easily disintegrats losing their antigenicity. Thus, the true prevalence of astrovirus in these children might be even higher than recorded here.

Dual or co-infection with rotaviruses and astroviruses was observed in one third (33.3%) of cases. This finding is not unusual but the prevalence rate is much higher than any reported in previous studies worldwide such as South Africa (Steele *et al*., 1998b), Argentina (Giordano *et al*., 2001) and Mexico, (Mendez-Toss *et al*., 2004). Despite the fact that dual infections by human astroviruses and rotaviruses in children with diarrhoea are common (Gaggero *et al*., 1998; Marx *et al*., 1998; Steele *et al*., 1998b; Bon *et al*., 1999; Qiao *et al*., 1999; Mendez-Toss *et al*., 2004), it is not clear whether infection with one of these viruses favors infection by the second. There was however no significant relationship between co-infection and the prevalence of clinical symptoms observed in these children as Bon *et al*. (1999) earlier observed. This suggests that, a dual infection with astroviruses and rotaviruses has no effect on the severity of diarrhoea illness in this study.

5.3 Adenoviruses

This study to delineate the clinical features of EAds gastroenteritis in Northwestern Nigerian children detected non-enteric adenoviruses in 22.3% of the specimens compared to 23.4% infected with rotaviruses in the same cohort. This figure is relatively higher than those obtained in previous studies in Nigeria (Nimzing *et al*., 2000; Audu *et al*., 2002b). In Africa, lower prevalences have been observed (Mhalu *et al*., 1988; Steele *et al*., 1998b; Moore *et al*., 1998; Basu *et al*., 2003). The prevalence in this study is also higher than reports from other countries such as Finland, Brazil, Australia, Germany, Taiwan and Denmark (Ruuska and Vesikari, 1991; Stewien *et al*., 1993; Grimwood *et al*., 1995; Oh *et al*., 2003; Chen *et al*., 2005; Olesen *et al*., 2005).

In other African countries, enteric adenovirus 40/41 was detected in 8.0% of the positive specimens (Mhalu *et al*., 1988; Moore *et al*., 1998). However, lower rates have been

reported in South Africa, Argentina, United States and Botswana (Marx *et al*., 1998; Giordano *et al*., 2001; Rodriguez-Baez *et al*., 2002; Basu *et al*., 2003).

Non-enteric adenovirus antigen was detected in 23.0% of the diarrhoeic and in 17.6% of the non-diarrhoeic specimens. This result is similar to rate reported previously in Nigeria (Audu *et al*., 2002b). A notable finding was the detection of adenovirus in a single intussusception patient. This finding provides an interesting contrast with the findings of Bode and Omilabu (2002) in a previous study in Nigeria where adenoviruses were detected in 58.8% (10/17) of intussusception patients. Higher prevalence of non-enteric adenoviruses has also been reported in studies conducted in the United States where they were detected in 20% (Bhisitkul *et al*., 1992) and 31.2% (Montgomery and Popek, 1994) of intussusception patients. Similarly, Hsu *et al*. (1998) reported a prevalence of 44.3% of non-enteric adenoviruses in intussusception patients in Taiwan.

Adenovirus infection was more prevalent in the older children with almost a third of patients (32%) infected in the age group 25-36 months. This is an interesting contrast with the findings of most studies conducted worldwide where children < 2 years of age are more vulnerable to adenovirus infection (Qiao *et al*., 1999; Nimzing *et al*., 2000; Audu *et al*., 2002b; Basu *et al*., 2003). This incompatible result may be attributed to breastfeeding, socio economic status, hygiene, culture and climate. Steele *et al*. (1998b), however, in their study found adenovirus to be more predominant in children 3-30 months old with a mean of 16 months.

Adenoviruses were isolated throughout the study period and in all the states studied with a marginal significant association in the prevalence by sex. The male to female ratio was 2.2. This does not correlate well with a previous report in Nigeria (Audu *et al*., 2002b) where the virus was isolated all year round except in the month April and there was no difference in the prevalence between males and females. Isolation of the virus in all the states studied indicates that these viruses are widely distributed throughout the region.

Co-infection with rotaviruses was observed in 14.3% of adenovirus-infected children. This result has been previously reported (Marx *et al*., 1998; Qiao *et al*., 1999; Nimzing *et al*., 2000). However, dual infections occur in low frequency (a case or in 2 cases) in these

previous studies. In a study conducted in South Africa (Steele *et al*., 1998b), no dual infection with adenovirus and astrovirus was observed.

Almost all (89%) of the co-infected children were from the diarrhoeic population and merely required rehydration and treatment. There was no significant relationship between co-infection and the prevalence of clinical symptoms observed in these children as previously observed elsewhere (Marie-Cardine *et al*., 2002). The dual infection observed in this study raise the question of whether a single virus is responsible for illness or whether two viruses act in synergy.

The EAd is a relatively unknown entity in gastroenteritis epidemiology in Africa with the exception of South Africa where the etiological importance in paediatric gastroenteritis has been established (Steele *et al*., 1990; Moore *et al*., 1998, 2000; Rossouw and Steele, 2003). The prevalence of adenovirus in this study, confirms the speculations of Moore *et al*. (2000) that the detection of adenovirus strain in stools of African children is probably under-reported due to low or lack of specificity of commercially available EIA for all circulating adenovirus strains.

5.4 Co-Infection

Co-infection with adenoviruses, astroviruses and rotaviruses was observed in 1.8% (5/282) of cases. This is the first co-infection with multiple gastroenteritis viruses that is being reported in Nigeria or elsewhere in Africa to the best of my knowledge. Although multiple infections have been reported in Spain by Guix *et al*. (2002), they were, however, between enteric pathogens. Multiple infections observed in this study may be attributable to poor hygienic conditions and overcrowding peculiar to the study area. Poor hygienic condition has been suggested to contribute to multiple infections in developing countries (Guix *et al*., 2002). Multiple infections were more prevalent in males than females and occurred only in children less than 12 months old. This result might suggest that most of the children in the study area would have had an infection, hence, the development of immunity to the infection. Natural immunity may be responsible for decreases in disease incidence with age.

CHAPTER SIX

6.0 CONCLUSIONS

6.1 Rotaviruses

One of the public health tools targeted for the reduction of morbidity and mortality associated with rotavirus infection is the development of an effective polyvalent rotavirus vaccine. However, in Africa, there is still a need to define the epidemiology of rotavirus infection and to investigate the diversity of strains both at molecular and antigenic levels in specific areas.

In this study, the first from Kebbi, Sokoto and Zamfara States, Northwestern, Nigeria, rotaviruses were shown to be an important cause of infantile diarrhoea as observed in most countries in the developing and developed world where rotavirus research have been conducted. Similar to other studies in Africa continent, rotavirus infection predominated in infants less than 24 months of age in this study confirming the notion that rotavirus infections occur early in life.

The prevalence of rotavirus infection, found to be more abundant in Kaduna state was higher during the cool drier months of October and March.

Rotavirus strains with 'long' electropherotypes predominated in this study. Extensive genomic diversity in terms of the long RNA electropherotype was also observed. Mixed infections were detected in very small proportion (2.2%) in the study and are probably the most common means by which genomic diversity arises.

The four forms of VP6 subgroup antigen were detected in all children in all the states studied. Of importance, perhaps, is that majority (over 50%) of the strains in the study belonged to subgroup I and subgroup II specificity. On a whole, it confirms the concept of 4 subgroups of human rotaviruses.

Existence of an association between subgroup II rotaviruses and long electropherotype, and subgroup I and the short electropherotype was confirmed in this study. This supports the existence of two rotavirus "families". However, three rotavirus strains were found that did not adhere to the pattern. These strains with VP6 subgroup I specificity were found to possess a 'Long' RNA electrophoretic pattern. These strains are likely to represent naturally occurring viral reassortants. Furthermore, this study highlights the diversity that exists among the human rotavirus isolates and has shown the existence in nature of mixed infections between rotaviruses of the two common human genogroups (WA and DS-1).

Genotype G1 was the most predominant VP7 genotype identified. This result is expected especially as G1 genotype is the most prevalent VP7 type worldwide. Of interest is the circulation of genotype G2 and the unusual genotypes G8 at similar level as the second most abundant strain detected in the study. The high prevalence of the unusual genotype, G8 has implications on vaccines application for the population.

A significant no of the unusual genotype G9 strains were found co-circulating. The increased detection globally, of rotavirus with G9 specificity, confirms the hypothesis that this unusual rotavirus strain is emerging as the fifth most abundant strain worldwide. Hence, there is a need to include this genotype in the development of next generation vaccine.

Genotype P[6] was the predominant VP4 genotype detected in the study. This finding highlights the proposed role of the VP4 P[6] genotype in the natural attenuation of the neonatal strains. This result questions the validity of the putative association of the P[6] rotavirus in asymptomatic infections, which leads to important considerations for this phenotype as a vaccine candidate.

Significantly, the detection of the unusual VP4 genotype P[10] and P[14] for the first time in Nigeria as mixed infections, suggest a greater diversity in rotavirus strains currently circulating in Nigeria. This adds to the global distribution of these strains and strengthens the need to continue strain surveillance in developing countries to further understand the extent of strain distribution and diversity. However, the significance of these unusual strains in relation to severity of the diarrhoea is not clear.

Seven distinct (single) G/P combination strains were identified. In consistence with reports of global studies, strain G1 P[8] was the most predominant in the study area. The significance of this finding underscores the need for a rotavirus vaccine based on the rotavirus G1 P[8] strain, presently on trial, as a possible intervention tool for rotavirus diarrhoea. In addition, a total of 41 exotic mixed G and P genotypes combinations were detected in an amazingly high percentage while, unusual multiple combinations were observed. This was a striking feature of this study which stresses the need to put in place a surveillance system for the detection of new and exotic rotavirus strains. The presence of unusual multiple combination strains are suggestive of an extensive reassortment of rotaviruses with either other human or animal viruses in Northwestern Nigeria.

Intriguingly, untypeable and partially typed isolates of rotaviruses were found in circulation. This may be an indication of the existence in Northwestern Nigeria of some yet undetermined genotype of rotavirus, which the primer(s) utilized in this study, could not detect.

The availability and affordability of sequencing methods now provide an alternate tool for characterization of these unusual strains to provide more information on circulating types and the natural reservoirs for these strains. Therefore, such isolates must be considered important and worth subjecting to further studies as they may have important implications for vaccine applications in Nigeria.

Finally, this study suggests that a diversity of primer sequence is needed to identify untypeable strains, hence, the need to design primers specifically for the numerous untypeable rotaviruses in Nigeria. Also, all untypeable rotavirus positive samples should be subjected to sequencing reaction. In addition, in view of the fact that periodic variation has been observed in the circulating genotypes, it is important to be cautious in drawing conclusion about the prevailing genotypes in Northwestern Nigeria based on this study only. Therefore, there is a need for an extensive study in Northwestern Nigeria in order to further highlight the diversity of rotavirus strains in Nigeria. This would optimize rotavirus vaccine strains and provide prospects for future paediatric needs and health policies.

6.1.2 Astroviruses and Adenoviruses

Astroviruses circulated at a high prevalence in association with diarrhoea and intussusception in children less than 18 months old in this study. This high prevalence, which has not been previously reported in any study, was similar to that of rotaviruses in the same cohort, suggesting, astrovirus might be equally important as rotavirus in the cause of diarrhoea in Nigerian infants.

Adenoviruses were also shown to circulate at a higher incidence rate in association with gastroenteritis in children less than five years old. This study provided the much-needed epidemiologic data on astroviruses and adenovirus infections in Northwestern Nigeria. However, to establish the relative epidemiological importance of these viruses in diarrhoea infections in Nigeria, further studies are required.

The application of simple EIA technology for testing for viral co-proantigen has increased the testing for rotavirus, astrovirus and adenovirus in clinical isolates. As found in this study, the increased isolation of astrovirus provides an insight into the significance of the agent as a pathogen and a causative agent of paediatric gastroenteritis. Understanding the

incidence of different pathogens in gastroenteritis is essential to measure the magnitude of the public health impact and the effectiveness of intervention strategies.

6.2 Recommendations

As a result of the findings from this first study conducted in Kebbi, Sokoto and Zmfara States, Northwestern Nigeria, the following recommendations are made:

1. The use of ORS should be encouraged in all diarrhoeal cases.
2. In view of the prevalence of rotavirus observed in both symptomatic and asymptomatic children, vaccination against the infection is recommended. Hence the study area should be a target for the trial of the newly introduced rotavirus vaccine.
3. Because of the availability and affordability of sequencing methods, which now provide an alternate tool for characterization of unusual and untypeable strains, all the untypeable strains should be subjected to sequencing analysis.
4. Since, this study suggests that, a diversity of primer sequence is needed to identify the numerous untypeable strains in Nigeria, there is the need therefore, to design cocktail of primers and use animal virus primers specifically for these untypeable rotaviruses. A complete characterization of circulating strains is necessary for the formulation of an effective vaccine.
5. Continuous surveillance is necessary as the occurrence of unusual isolates may determine the necessity for an alternate vaccine. It is also required to monitor changes as vaccine put new pressure on evolution.

6.3 Limitations

1. Due to time and cost implications, samples were only collected from four of the seven states in Northwestern Nigeria.
2. It was not possible to collect specimens from all children presented or hospitalized at the various clinics/hospitals because not all mothers were willing to have their children enrolled in the study. Some mothers who even consented never produced the specimen nor returned the specimen containers. As a result half of the specimen bottles were lost in this study.
3. It was not possible to verify the information given by parent or caregiver of a child.

4. Inability to get to the central storage point of specimens because of the distance between the numerous clinics/hospitals and the various states from which specimens were collected, led to loss of so many specimens. These specimens were discarded because of fear of spoilage/disintegration attributable to non-functional cold storage facilities or to the epileptic power supply peculiar to Nigeria. This was the sad and most painful aspect of the study considering the effort put in to convincing mothers to consent and produce specimens.

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APPENDICES

Appendix 1: Questionaire

Department of Microbiology, Faculty of Science,
Ahmadu Bello University, Zaria

A Survey of Viral Gastroenteritis in Infants in Northwestern Nigeria

Form No.: _____

Eligibility: Child ≤ 60 Months who passed ≥ 3 Loose Stool in the last 3 days.

1. BASIC DATA

1.1 **Date of Visit:** _____

1.2 Study Child 1D (Sample No.): _____

1.3 Study Child Name:

_____

1.4 Age (In Months): _____

1.5 Sex: (i) Male: _____ (ii) Female: _____

1.6 Parent Name and Address: _____

_____

2. HISTORY OF CURRENT ILLNESS

2.1 Please describe your child's illness (Do not prompt).

(a) Diarrhoea (i) Yes { } (ii) No { }

(b) Fever (i) Yes { } (ii) No { }

(c) Vomiting (i) Yes { } (ii) No { }

2.2 Duration: Ask if the child had the following symptoms and if present, ask how many days ago it began. If symptom is not present, enter Nil.

(a) Diarrhoea duration: _____

(b) Vomiting duration: _____

2.3 When the diarrhoea was at its worst:

(i) How many times did the child pass stool during the day? _____

(ii) How many times did the child pass stool during the night? _____

(iii) Was it continuous? Yes { } No { }

2.4 Has the child had diarrhoea before? Yes { } No { }

2.5 If the child vomited (when the vomiting was at its worst):

(i) how many times did the child vomited during the day? _____

(ii) how many times did the child vomited during the night?: _____

2.6 Has the child taken Oral Dehydration therapy? Yes { } No { }

2.7 Has the child received any antibiotic or antiparasitic treatment before?
Stool collection? Yes { } No { }.

2.8 If Yes, What is the name of the drug _____

3. SAMPLES

3.1 Stool character: (i) Watery { } (ii) Loose { } (iii) Normal { } NA { }
Blood present: (i) Yes { } (ii) No { } (iii) NA { }
Mucus present: (i) Yes { } (ii) No { } (iii) NA { }

4. EXAMINATION: MEDICAL ASSISTANT OR DOCTOR TO COMPLETE

4.1 General condition: (i) Normal { } (ii) Ill { } (iii) Very Ill { } (iv) Moribund { }

4.2 Mental status: (i) Normal { } (ii) Irritable { } (iii) Drowsy/lethargic { }
(iv) Unconscious { }

4.3 Maculopapular rash: (i) Yes { } (ii) No { }

4.4 Sunken eyes: (i) Severe { } (ii) Some { } (iii) No { }

4.5 Skin turgor decreased: (i) Severe { } (ii) Some { } (iii) No { }

4.6 Mouth dry: (i) Yes { } (ii) No { }

4.7 Tears seen: (i) Yes { } (ii) No { }

4.8 Fontanel: (i) Sunken { } (ii) Normal { } (iii) Bulging { } (iv) NA { }

4.9 Dehydration status: (i) None { } (ii) Mild { } (iii) Moderate { } (iv) Severe {}

4.10 Respiratory symptoms Yes { } No { }

5. ACTION TAKEN

5.1 Oral rehydration salts (ORS) given under observation in clinic: Yes{ } No{ }

5.2 Weight of Child? _____kg.

5.3 ORT packets given to take home: Yes { } No { }

5.4 Other medication prescribed: Yes { } No { }

(a) Antibiotics: Yes { } No { } NA { }

(b) Antipyretics: Yes { } No { } NA { }

(c) Antimalarials: Yes { } No { } NA { }

(d) Others: Yes { } No { }

6 PERSONAL DATA

6.1 Child's Food: (a) Exclusive breast feeding { } (b) Breast Milk plus solid food { }
(c) Solid foods { } (d) Breast Milk and Water { }

6.2 Does any other person in the household have diarrhoea? (a) Yes { } (b) No { }

6.3 If Yes, who? (a) Parents { } (b) Siblings { } (c) House help { }
(d) Others specify: ----------

6.4 Source of water: Well { } Tap { } River { } Pond { }

6.5 Drinking water: Boiled { } Not boiled { }

6.6 Animal ownership: (a) Dogs { } (b) Cats { } (c) Cows { } (d) Goats { }

6.7 Child's contact with the animal (s): Often { } Rarely { } Not at all { }Others { }

6.8 Mother's Education: (a) Illiterate { } (b) Primary education { }
(c) Secondary Education { } (d) Tertiary education { }

6.9 Occupation of the head of the family: _____

6.10 Father's education: (a) Illiterate { } (b) Primary Education { }
(c) Secondary Education { } (d) Tertiary Education { }

6.11 Socio-economic status of parents: (a) Low { } (b) High { }

6.12 Who prepares baby's food? (a) Nanny { } (b) Mother (c) anybody

6.13 Does baby go to school? (a) Yes { } (b) No { }

7. OUTCOME OF THIS VISIT

7.1 Treated at clinic or distant hospital OPD: { }

7.2 Referred to district hospital: { }

7.3 Admitted at district hospital in patient department: { }

7.4 Others

(specify):_____

_____

Appendix 2: Preparation of reagents for negative staining

1. **3% phosphotungstic Acid (PTA):** Dissolve 0.75g PTA (Merck) in 25 ml distilled water. Adjust to Ph 5.5-6.3. Filter using 0.22 pore size filter paper (Cameo 25AS Acetate)
2. **1 M Potassium Hydroxide (KOH)**

   Dissolve 2.8g KOH in 40 ml distilled waater Make up to 50 ml with distilled water

Appendix 3: Reagents for extraction of rotavirus dsRNA from stool specimens

1. **10% sodium dodecyl sulphate (SDS) stock:** 10g of SDS was added to 100ml distilled water and the mixture was dissolved in a 65^{o}C water bath.
2. **1M sodium acetate containing 1% sodium dodecyle sulphate (SDS)**

8.2g of sodium acetate was dissolved in 60ml distilled water. 1ml of 10% SDS stock was added and then mixed. The pH was adjusted to 5.0 with glacial acetic acid and the solution was made up to a final volume of 100ml by the addition of distilled water.

3. **Phenol /Chloroform (1:1)**

Equal volume of saturated phenol and chloroform were mixed in a cellophane covered bottle and stored at 4^{o} C.

4. **3M Sodium acetate (NaAc)**

24.6g of NaAc was dissolved in 60m1 of distilled water and made up to a final volume of 100m1 with distilled water.

5. **PAGE sample dye**

10 mg of bromophenol blue (BPB) was mixed with 5ml of spacer gel buffer and 1m1 glycerol. They were dissolved and final volume made to 10ml with distilled water.

Appendix 4: Reagents required for PAGE

1. **30% Acrylamide stock:** 30g of acrylamide (Fluka Biochemika, Switzerland) and 0.8g N, N' Methylene bis-acrylamide (USB Amersham Life Sciences, Cleveland, Ohio) were dissolved in 50ml of distilled water. Final volume was made to 100ml by the addition of distilled water. Before use, the solution was filtered and stored at $4^{o}C$ in the dark.
2. **1N Hydrochloric acid (HCI):** 86ml of concentrated HCI was mixed with 910ml of distilled water.
3. **Resolving gel buffer:** 36.3g of Tris base (USB^{TM} United States Biochemical, Cleveland OH 44128) was dissolved in 4.8ml IN HCI and the PH was adjusted to 8.9 with HCI or sodium hydroxide (NaOH). Distilled water was added to give a final volume of 100ml.

4. Spacer gel buffer: 5.98g of Tris base was dissolved in distilled water and the Ph was adjusted to 6.7 with 1N HCl. Distilled water was added to give a final volume of 100ml

3. **10% (W/V) Ammonium persulphate (APS):** 0.1g of ammonium persulphate (USB Amersham Life Sciences, Cleveland, Ohio) was dissolved in 1ml of distilled water prior to use.

6. 5x Tris-glycine running buffer: 5.1g of 25mM Tris and 94g of 250mM glycine (USB Amersham Life Sciences, Cleveland, Ohio) were dissolved in distilled water and the solution was made up to 1000ml.

7. 1 X Tris-glycine running buffer: 200ml of the 5x Tris-glycine running buffer was added to 800ml of distilled water.

Appendix 5: Preparation of reagents required for Silver Nitrate ($AgNo_3$) staining of one gel

1. **Fixing solution 1:** About 80ml of 96% ethanol was mixed with 110ml of distilled water and 10ml of acetic acid.
2. **Fixing solution 2:** 20ml of 96% ethanol was mixed with 180ml of distilled water and 10ml of acetic acid was added.
3. **11mM silver nitrate ($AgNo_3$) staining solution:** 0.37g of $AgNO_3$ was dissolved in 200ml of distilled water just before use.
4. **Developing solution:** 7.5g of sodium hydroxide (NaOH) was dissolved in 250ml of distilled water and 2ml of 36% formaldehyde was immediately added
5. **Stopping solution:** 10ml of acetic acid was mixed with 200ml of distilled water

Appendix 6: Reagents Required for Subgroup (VP6) ELISA

1. 0.05M carbonate/bicarbonate buffer: 0.397g of sodium bicarbonate (Na_2CO_3) and a 0.7325g sodium carbonate ($NaHco_3$) were dissolved in distilled water. The pH was adjusted to 9.6 with HCl or NaOH and the solution was made up to 250ml with distilled water. The solution was then stored at 4^o C for up to 14 days.

2. 10X phosphate buffered saline (PBS): 80g of sodium chloride (NaCl), 2g Potassium chloride (KCl) 2.4g potassium phosphate (Na_2PO_4) and 14.4g of sodium phosphate were dissolved in distilled water. The pH was adjusted to 7.2 with HCl or NaOH and the solution was made up to 1000ml. The solution was stored at 4^o C for up to 2 months.

3. 1X phosphate buffered saline (PBS): 100ml of 10 X PBS was mixed with 900ml of distilled water and pH was adjusted to 7.2 with HCl or NaOH. The solution was stored at 4^o C for up to 2 months.

4. Phosphate buffered saline/Tween (PBS/T): 500µ1 of Tween was mixed in 1000ml of 1X PBS at pH 7.2 and the solution was stored at 4^o C for up to 2 months.

5. 3M Sodium hydroxide (NaOH): 12g of NaOH was dissolved in 100ml of distilled water.

6. Phosphate buffered saline /Tween /EDTA (PBS/T/EDTA): 1.86g of EDTA was dissolved in 500ml PBS with 500lµ Tween and the pH was adjusted to 7.2 with 3M NaOH. The solution was made to a final volume of 1000ml with PBS and stored at 4^o C for up to 2 months.

7. Phosphate buffered saline /Tween /Bovine serum albumen (BS/T/BSA): 5g of bovine serum albumen (BSA) was dissolved in 1000 PBS/T and the pH was adjusted to 7.2 with HCl or NaOH. The solution was stored at –20˚C until ready to use.

Appendix 7: VP7 and VP4 RT-PCR Reagents

1. 20 X Tris-acetic acid-EDTA buffer (TAE): 48.44g of 0.4M Tris, 6.81g of 0.05M NaCl, 3.72g of 0.01M of EDTA were dissolved in distilled water. The pH was adjusted to 7.9 with glacial acetic acid and volume made up to 1000ml with distilled water.

2. **1 x Tris-acetic acid-EDTA buffer (1x TAE):** 50ml of the 20x TAE buffer was added to 950ml of distilled water.

3. Ethidium bromide stock: 1 tablet (100mg) of ethidium bromide was dissolved in 10ml of distilled water and the solution stored in a foil-covered bottle.

4. 1.2% Agarose gel: 0.24g of agarose (Gibco BRL ultra PURE, Life Technologies, Scotland) was mixed in 20ml of 1x TAE buffer at pH 7.9. The mixture was heated in the microwave oven until the agarose was completely dissolved and solution made up to 20ml with distilled water. The solution was cooled under cold running water and then 2µ1 of ethidium bromide stock was added. The solution was mixed and poured into cassette tray, comb was inserted and gel allowed to set for at least 30 minutes before use.

5. Bromophenol blue tracking dye: 0.001g of bromophenol blue was dissolved in 1ml of distilled water. 1ml of bromophenol blue stock was then added to 9ml of 40% sucrose solution made in 1x TAE. The final solution was filtered and stored in a foil-covered bottle.

Appendix 8: Sample size for adequate power

$$\text{Sample size (n)} = \frac{(Z_1 - Z_2)^2 \times 2p(1-P)}{(p_2 - P_1)^2} \times \frac{C+1}{2C}$$

P_2 = Proportion with disease in unexpected group

P_1 = Proportion with disease in exposed group = (7% of 1063)

P = Average of P1 and $P_2 = \frac{P_1 + P_2}{2}$

C = Ratio of unexposed to exposed $= \frac{P_2}{P_1}$

Z_1 = 95% confidence = 1.96

Z_2 = 90% power = 1.28

n = Derived sample size