MOLECULAR CHARACTERISATION OF ROTAVIRUSES DETECTED IN CHILDREN UNDER THE AGE OF FIVE YEARS WITH DIARRHOEA IN KANO STATE-NIGERIA

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BY

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DECLARATION

I hereby declare that the work presented in this thesis is original. It was carried out by me in the laboratories of Department of Microbiology and Avian Influenza laboratory Asokoro General Hospital, Abuja under the close supervision of Dr. (Mrs) Maryam Aminu.

No part of this thesis has previously been submitted for any degree or qualification. References made to the work of other investigators are duly acknowledged.

Wada-Kura Aminu

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This thesis titled **MOLECULAR CHARACTERISATION OF ROTAVIRUSES DETECTED IN CHILDREN UNDER THE AGE OF FIVE YEARS WITH DIARRHOEA IN KANO STATE-NIGERIA** by Wada-Kura Aminu meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its scientific contribution to knowledge and literary presentation.

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DEDICATION

To the memories of Alhaji Wada-Kura, Mardhiyya and Ja'afar.

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ABSTRACT

Rotaviruses are the most common cause of severe diarrhoeal illness in infants and young children in both developing and developed countries resulting in much death in sub-Saharan Africa and Nigeria. A total of 285 stool specimens were collected from November, 2009 to July, 2010.and analyzed for group A antigen using ELISA and RT-PCR, The prevalence of rotavirus in this study was 36.5% (104/285). Murtala Muhammad Specialist Hospital had 44.4% while Gwarzo General Hospital had the lowest prevalence rate of 21.4%. The virus was detected more in males 37.2% (61/164) than in females 35.4% (43/121). However, there is no statistically significant difference (χ^2=0.11, P>0.05, df =1) observed between the prevalence in males and females. Rotavirus was detected with the highest frequency among children in the age 41-60 months with 50% prevalence. The least prevalence was in age 31-40 months having 15.8%. The prevalence was highest in April with 77.6% and lowest in July 12.2%. Pearson Chi square analysis showed significant difference (χ^2=27.720, P<0.05, df=1) of rotavirus infection in the two different seasons. Three different Rotavirus VP4 P-genotypes (P[8], P[4], and P[6]) were detected in this study. Mixed infection were detected and consist only of dual P-types of P[8]+P[6]. Analysis of the Rotavirus strains indicates that P[6] was the most common Rotavirus infection in the study population with frequency of 50 (48.5%). Six VP7 P-genotypes were detected. The predominant genotype was G2 with a prevalence of 35.0%. The lowest was G9 which had a prevalence of 1.9%. The highest combination was found to be G2P[6] with 19.4% frequency of occurrence. A single GNTP[8+6] (non-typeable G and P[8+6]) mixed combination of infection was also found among the study population. Finally there is need for further studies on the molecular characterization of rotavirus and the need to provide effective vaccine against the viral infection cannot be over emphasized.

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CHAPTER ONE

INTRODUCTION

Diarrhoeal illness is one of the most common illnesses of humans. It is one of the most important causes of morbidity and mortality in children under 5 years of age resulting in approximately 3 million deaths globally with 80% of it occurring in the first 2 years of life (Bern *et al.,* 1992; Murray and Lopez, 1997; Smith *et al.,* 1999; Estes, 2001). Majority of the deaths occur in developing countries - the Indian sub-continents and sub-Saharan Africa (Pager *et al.,* 1999; Bresee *et al.,* 1999; Glass *et al.,* 2001).

Rotaviruses are the most common cause of severe diarrhoeal illness in infants and young children in both developing and developed countries. Rotavirus infection is of special significance in developing countries where they constitute a major cause of mortality among young children. Although diarrhoeal diseases are not a prominent cause of mortality in infants and young children in developed countries, the toll from diarrhoeal disease in developing countries is staggering. Due to the lack of proper disease management and/or the unavailability of health care facilities in the developing world, rotavirus kills over half a million infants a year, about one child each minute (Newall *et al*., 2006). The death toll has been estimated at 610,000 per year, 82% of which occurs in poor countries (Parashar *et al.,* 2006). Rotavirus causes about 10,000 hospital admissions in Australia (Carlin *et al.,* 1998) and 70,000 hospital admissions in the United States annually, or about 5% of all paediatric admissions. It also results in 250,000 visits each year to emergency rooms in the U.S. (Newsrx, 2006). A recent study in Australia has reported thirteen rotavirus related deaths from 1990-2002 due to the severity of infection (Newall *et al.,* 2006).

Rotavirus is ubiquitous and endemic in nature and infections occur in almost all children. It was discovered in Australia in 1973 in a duodenal biopsy from an infected person (Bishop *et al.,* 1973). The rotavirus virion has a round structure with a double-shelled capsid and measures 75nm in diameter. Soon after its discovery, it became apparent that rotavirus was an important etiological agent of diarrhoea in infants and young children causing about 35%-50% of all hospitalizations for this form of gastroenteritis during the first two years of life. The severity of rotavirus infection ranges from no symptoms to dehydrating gastroenteritis that can be fatal. Rotaviruses are also associated with diarrhoea in various animals and birds.

In a thirteen-year survey published in Australia out of 4,637 children from 0 to 14 years of age admitted to a paediatric hospital due to acute diarrhoea, 39.6% of admissions were caused by Group A rotavirus. Fifty five of these children were aged from12 to 23 months. Rotavirus was a frequent cause of acute gastroenteritis in children under 6 months (18.7%) and in those aged 5 to 13 years (16%) (Carlin *et al.,*1998). Rotaviruses were solely responsible for the peak of admissions during winter (Barnes *et al.,* 1998; Carlin *et al.,* 1998). The incidence of hospitalization due to rotavirus infection in New York has been calculated at 8.7% of all diarrhoea-associated hospitalizations (Chang *et al.,* 2003). A total of 136 patients with diarrhoea died during their hospitalization (hospital fatality rate, 1.6 per 1000), and the 12 deaths among patients with rotavirus had a distinct winter pattern. During 1997, 46% of the hospitals reported diarrhoea in children that was due to rotavirus, and 12% of hospitals reported rotavirus in >30% of all diarrhoea-associated hospitalizations. Infants >6 months of age were more likely to get infected than older children. A higher proportion of nosocomial infections with rotavirus were seen in children with congenital malformations (Chang *et al.,* 2003).

Rotaviruses are classified in the family *Reoviridae,* and have distinctive triple-layered icosahedral protein capsids that consist of an outer and an inner layer. Within the inner layer is third layer, the core, which contains the virus genome consisting of 11 segments of double stranded RNA. Rotavirus RNA segments range in size from 0.6 to 3.3 kilo base pairs (Matton *et al.,* 1989). Characteristically, these RNA segments are numbered in order of migration during polyacrylamide gel electrophoresis (PAGE); with the slowest migrating RNA segment designated gene 1 and so on. In the 1980s and early 1990s, electropherotyping of rotavirus became the most common technique for the characterization of different strains of the virus. The resultant RNA migration pattern in polyacrylamide gels is called an electropherotype (e-type). It soon became apparent that human rotaviruses exhibited a wide variety of electropherotypes and that these patterns differed from those observed for animal rotavirus, indicating the existence of huge diversity among rotavirus strains (Holmes, 1996). However, PAGE does not provide accurate or detailed genetic information about the various strains and subsequent studies showed that viruses of the same serotype can exhibit different electropherotypes, and that viruses of the same electropherotype can belong to different serotypes, when molecular typing methods such as reverse transcription-polymerase chain reaction (RT-PCR) are used to classify the virus (Holmes, 1996). The extent of genetic relatedness and variation among rotavirus strains has been studied by using labelled (+) single stranded RNAs transcribed from viral genome as hybridisation probes (Nakagomi and Nakagomi, 1996). These studies have indicated the existence of three major 'genogroups' of human rotaviruses and numerous other genogroups of animal strains.

Molecular epidemiology has become an important tool in studying the various rotavirus strains circulating in different parts of the world at a given time and will aid significantly in determining the appropriate strategies for use in the development of rotavirus vaccines. Such vaccines are urgently needed to prevent the huge number of fatalities caused by this virus. The first licensed vaccine, a tetravalent vaccines based on the rhesus strain, RRV (RRV-TV) incorporated serotype G1, G2, G3 and G4 specificities, the most common serotypes worldwide at the time. The vaccine was subsequently withdrawn in 1999 due to an association with of intussusception in vaccine recipients (Murphy *et al.,* 2001; CDC., 1999a). Extensive research followed to develop a new and effective vaccine and recently, two new rotavirus vaccines, Rotarix and RotaTeq, have been approved in the US and several countries in Latin America (Arvin and Greenberg, 2006). Rotarix, which is a monovalent, attenuated vaccine developed by GlaxoSmithKline has recently been licensed in many European countries (Linhares and Villa, 2006). Both vaccines have been licensed in Australia and have recently been included on childhood routine immunisation schedule. The second generation of vaccines needs to focus on targeting the common types and any of the new and emerging types such as serotype G9, which is now the fifth common global type. Thus, continued surveillance and the genetic and antigenic analysis of the various strains of rotavirus circulating worldwide will aid significantly in assessing the effectiveness of these vaccines and monitor emergence of new strains.

1.1 Statements of Research Problems

There is little information on the epidemiology of human rotavirus which cause sporadic and outbreak associated gastroenteritis in Kano State-Nigeria.

Every year rotavirus causes nearly one million deaths worldwide with most deaths occurring in the Indian sub-continents and sub-Saharan Africa of which Nigeria is part.

Rotavirus infects all children in the world by the age of five indicating that disease transmission will not be limited by improvement in sanitation and hygiene but that vaccines offer the best hope of disease burden reduction.

Rotavirus is current focus of intense research because of the urgent need to develop an effective vaccine to protect against severe rotavirus diseases.

Despite several candidate vaccines currently being evaluated, there is still lack of data in Africa on the antigenic and genomic diversity of rotavirus required for the design of an effective vaccine strategy.

In Africa, Nigeria in particular the epidemiology and diversity of rotavirus VP7 serotypes circulating are poorly understood and have not being established.

The global emergence of uncommon Human rotavirus (HRV) strains, the detection of untypeable HRV strain in many African countries including Nigeria and the observed unexpected diversity among rotavirus strains isolated in Nigeria with their varied and unusual epidemiology have implication for effective vaccine development.

Extended surveillance and continual monitoring of VP7 serotypes of circulating rotavirus strains as well as detecting the emergence of new rotavirus strains is essential for effective vaccine implementation strategy and development of effective rotavirus vaccine to prevent infection and reduce the burden of rotavirus disease.

1.2 Justification

There are few data on the prevalence and epidemiology of rotavirus as etiologic agent of diarrhoea and the contribution of diarrhoea disease to malnutrition in Kano State-Nigeria.

Diarrhoea illness or gastroenteritis in association with malnutrition constitutes major cause of morbidity and mortality in children less than five years of age, in North-Western Nigeria. The major determinants of diarrhoeal deaths are dehydration and malnutrition, so diarrhoeal morbidity has been shown to slow growth in children well beyond the weaning age which suggests that diarrhoea is of great public health significance and therefore, increase attention should be given its study.

1.3 Significance of the 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 Kano state-Northwestern Nigeria and the future management of viral diarrhoea in Nigeria. The study would improve our understanding of the importance of rotavirus considered to contribute to viral diarrhoea episodes in children.

1.4 Aim

The aim of this research is to determine the prevalence and characterise rotavirus infection among children less than five years with diarrhoea in Kano state-Nigeria

1.5 Objectives

1. To determine the prevalence and antigenically characterise the rotavirus strains from infants under 5 years with diarrhoea attending clinics in Kano State, Nigeria using Enzyme Linked Immunosorbent Assay (ELISA).

2. Determination of the VP4 and VP7 genotypes by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

CHAPTER TWO

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*., 2005). Infectious diarrhoea has been with human since the beginning of recorded history. The causative organisms are passed in the stool and subsequently ingested by a new host through consumption of contaminated food or water. Infectious agents can also be picked through contact with infected patients.

The immediate causes of diarrhoeal diseases are often of an infectious nature and involve a variety of pathogenic micro-organisms that include; bacteria, parasites and viruses (Estes, 2001). The most commonly implicated bacterial pathogens of diarrhoea on a global scale are strains of *Escherichia coli* (*E. coli*) (Iruka *et al*., 2003; WHO, 2005). Most strains of *E. coli* are known to be normal intestinal flora of humans and animals. Some strains however are able to cause severe life threatening diarrhoea. 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). 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 diarrhoea are,

Campylobacter, *Shigella, Salmonella, Yersinia and Vibrio* species (Guerrant and Bobak 1991; Iruka *et al*., 2003). The symptoms associated with bacterial diarrhoea include nausea, vomiting, fever, bloody stool and weight loss (Guerrant and Bobak, 1991).

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 diarrhoea in humans are: rotaviruses, astroviruses, adenoviruses and caliciviruses (Estes, 2001). 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 Rotavirus

Rotavirus is the most common cause of severe dehydrating gastroenteritis among infants and young children under five years of age worldwide (Estes *et al.,* 1983; WHO Scientific Working Group, 1980; Hart and Umar, 2002; WHO, 2002) resulting in approximately 660,000 deaths each year (Pager *et al.,* 1999; Miller and McCann, 2000; Molbak *et al.,* 2001). Most of these deaths occur in Africa (Molbak *et al.,* 2000; Jain *et al.,* 2001) where access to rehydration therapy and other medical care are limited (WHO, 2002) and rotavirus may cause up to one million deaths each year accounting for an estimated 20-25% of all deaths due to diarrhoea and 6% of all deaths among children less than 5 years old (Bresee *et al.,* 1999; Glass *et al.,* 2002).

In Africa, West Africa in particular rotaviruses are important aetiological agents of severe dehydrating diarrhoea in young children 1-3 years old and have been reported in Guinea Bissau, Ghana, and Cote D'voire (Asmah et al. 2001, Akran *et al.,* 2002). In Nigeria hospital

and community-based studies have shown that 3.6 –24.2% and 7.7% respectively, of childhood diarrhoea are associated with rotavirus infection (Paul and Paul, 1986; Fagbami *et al.,* 1987; Oyejide and Fagbami, 1988; Adah *et al.,* 1997; Pennap *et al.,* 2000; Gomwalk *et al.,* 1993, 1990) implying that rotavirus infection is endemic in Nigeria.

2.3 Virus structure

When examined by electron microscopy, the morphology of the rotavirus particle resembles that of a wheel, as it consists of three distinct layers. There are about 60 spikes of viral protein VP4 protruding from the outer surface, which has well defined surface rim, giving a wheel-like appearance (Latin 'rota' wheel) hence the name. The virus appears rough when its outer surface is removed showing its intermediate layer which consists of projecting trimers of viral protein VP6. Methods developed by Prasad *et al.* (1988) using cryoelectron microscopy show the reconstruction of three-dimensional structure and have helped the study of the three distinct layers in more detail. The outer layer, made up of molecules of VP7 proteins encoded by one of gene segment 7, 8 or 9 (depending on the strain), forms a triangular shape, surrounding the many aqueous channels present on the outer surface. The thickness of the VP7 layer is a35 Å. Spikes made up of protein VP4, encoded by gene segment 4, surround the outer capsid. The length of the spikes extending from the surface is 120 Å. Each spike has a distinct bilobed structure at the distal arc of diameter a25 Å. Particles containing only the intermediate VP6 layer are 705 Å in diameter. The VP6 and the VP7 molecules interact with one another through their upper domains. The inner layer, or core, is comprised of the protein VP2 and has a radius of 270 Å. The entire genome and the VP1 and the VP3 proteins are contained within the core (Estes and Cohen, 1989; Prasad *et al.,* 1988).

2.4 Viral Proteins

The 11 segments of viral RNA genome code for different structural and non-structural proteins. There are six structural viral proteins (VP1-4, 6 and 7) and 6 non-structural proteins (NSP1-6) with various functions as listed in Table 2.1.

2.4.1 The VP6 protein

The VP6 protein is located on the inner capsid of the virus and is encoded by gene segment 6. This protein constitutes 51% of the total viral protein. Virus classification is based on VP6, which confers group and subgroup specificities and differentiates rotavirus into seven groups (A-G). Within group A, four different subgroups are defined based on the reactivity of subgroup specific monoclonal antibodies: subgroups I, II, I & II and non-I/non-II (Estes *et al.,* 2001). Group A is the major type causing infection in humans. Monoclonal antibodies (MAbs) developed against the human rotavirus strain, Wa (subgroup II), or the rhesus rotavirus, RRV (subgroup I), react with almost all mammalian group A rotavirus (Greenberg *et al.,* 1983). The majority of subgroup II and subgroup I & II rotaviruses exhibit 'long' electropherotypes, based on the mobility of gene segment 11 whereas almost all subgroup I rotaviruses exhibits 'short' electropherotypes (Kapikian *et al.,* 2001).

Table 2.1: Characteristics and functions of rotavirus proteins

Genome Segment	Protein product	Polypeptide (Dalton)	Location In the virus	No. of molecules /virion	Function
1	VP1	125,005	Core	12	RNA Polymerase, SS RNA
2	VP2	94,000	Core	120	RNA binding, required for replicase
3	VP3	88,000	Core	12	guyanylyltransferase,
4	VP4	86,782	Outer Capsid	120	hemagglutinin, neutralisation antigen,
5	NSP1	58,654	NS	-	basic, RNA binding, virus
6	VP6	44,816	Inner capsid	780	hydrophobic, trimer, subgroup antigen,
7	NSP3	34,600	NS	-	acidic dimers, binds 3'end viral mRNA,
8	NSP2	36,700	NS	-	basic, RNA binding,
9	VP7	37,368	Outer capsid	780	integral membrane glycoprotein,
10	NSP4	20,290	NS	-	RER transmembrane glycoprotein, role
11	NSP5/NSP	21,725/12,00	NS	-	basic, phosphoprotein, RNA binding,

Source: www.mediscape.com

A separate domain of VP6 contains the group specific epitopes for group A rotaviruses, corresponding to four different regions on VP6 deduced amino acid sequence: residues 32 to 64, 155 to 167, 208 to 294 and 380 to 397 (Estes, 1996). Rotaviruses that share the group specific antigenic determinants are classified in group A and those that do not share this feature are classified into non group A (Tao, 1988). Presently, non group A rotavirus are classified into groups B, C, D, E, F or G. Group B has been found in children, cattle, sheep, pigs, goats and rats (Gueguen *et al.,* 1996) and also have been reported to cause diarrhoea outbreaks in adults in China (Kuzuya *et al.,* 2005; Su *et al.,* 1986; Tao, 1988; Ward *et al.,* 1992). Group C has been found in pig, cattle and dogs (Otto *et al.,* 1999) and is also been found to cause sporadic infection in humans (Kuzuya *et al.,* 2005; Phan *et al.,* 2004). Group D rotaviruses are found in chickens, pheasants and turkeys (Devitt and Reynolds, 1993). Group E rotavirus is detected in pigs and Group F and G are isolated from chickens and turkeys (Saif and Jiang, 1994).

2.4.2 The VP7 Protein

The VP7 protein, which constitutes 30% of the viral protein, is the major outer capsid protein and is the major neutralising antigen of the virus. Based on antigenic determinants found on this glycoprotein, it serves as the basis for viral classification into G-serotypes. VP7 is encoded by one of gene segments 7, 8 or 9, depending on the strain (Greenberg *et al.,* 1983a; Mattion *et al.,* 1990). So far group A rotaviruses have been classified into 15 G-serotypes based on the reactivity of these antigenic sites in enzyme immunoassay (EIA) (Hoshino *et al.,* 2005). G-serotype identification of rotaviruses has been facilitated by the isolation of VP7 MAbs that are specific for the epidemiologically important human G-serotypes (Hoshino and Kapikian, 1994; 1996).

The amino acid sequences of VP7 of rotavirus strains belonging to each G-serotype have been deduced from VP7 nucleotide sequences. Comparisons among different group A rotaviruses have shown that there are nine regions of the amino acid sequence of VP7 that are highly divergent between strains belonging to different G-serotypes (Nishikawa *et al.,* 1989). Each of these regions is highly conserved among rotavirus strains within the same G-serotypes, allowing prediction of the G-serotype of strains by direct sequence analysis of any two of these variable regions (Green *et al.,* 1988). A common technique used to identify the G-serotype of group A rotaviruses involves multiplex semi-nested PCR using a pool of G-serotype specific primers. This technique is carried out in two steps; the first involves full length amplification of the VP7 gene using gene-specific end primers by RT-PCR which is followed by a second round multiplex semi-nested PCR using one conserved end primer and a pool of G-serotype specific primers (Gouvea *et al.,* 1990; 1991).

2.4.3 The VP4 Protein

The VP4 protein is situated on the outer capsid layer in the form of spikes that protrude from the surface of the virus. There are 60 such spikes and their length is up to 10 –12 nm in a mature virion. The VP4 protein constitutes 1.5% of total viral protein. This protein is susceptible to trypsin proteolytic cleavage yielding its subunits VP5* and VP8*, the asterisk is conventionally used to denote the cleaved subunits. VP4 has a very important function in terms of viral attachment to the host cell and it also induces the production of neutralising antibodies. The protein is coded by the fourth segment of the viral genome (Mattion *et al.,* 1994). VP4 helps the virus to penetrate the host cell by trypsin-associated cleavage and also causes infectivity (Isa *et al.,* 1997). The VP4 protein has neutralising antigenic sites different from those of the VP7

protein. Thus a dual classification system exists based on the VP7 and VP4 neutralising antigens, designating as G-serotypes for the VP7 glycoprotein and P-serotypes for the protease sensitive VP4.

While no correlation between rotavirus virulence and the G-type of the infecting strain is apparent, i.e. each of four major G-serotypes (G1, 2, 3 and 4) are associated with both symptomatic and asymptomatic infections (Hoshino *et al.,* 1985), it was observed that the VP4 gene of rotavirus strains causing asymptomatic infections was highly conserved (95.5% to 97.5% sequence identity) and was distinct from the VP4 gene of strains from symptomatic infections (25.4% to 27.1% sequence divergence) (Gorziglia *et al.,* 1988). Although VP4 antigenic specificities had not been assigned for all rotavirus strains, RNA-RNA hybridisation, reactivities with neutralising monoclonal antibodies and/or sequencing analysis of various rotaviruses have defined at least 26 P-types (Hoshino *et al.,* 2005; Rahman *et al.,* 2005).

The VP8* subunit of VP4 is the location of the major antigenic sites determining P - serotype specificity, whereas the VP5* subunit contains sites responsible for cross-reactivity among various VP4 types (Larrade *et al.,* 1991).

2.4.4 The Core Proteins

The proteins VP1, VP2 and VP3 comprise the rotaviral core proteins and have important roles in viral replication and RNA transcription. Studies have shown that VP1 is the viral RNA-dependant RNA polymerase and functions as both the viral RNA transcriptase and replicase (Estes *et al.,* 1984; Patton *et al.,* 2004). All sequenced rotavirus VP1 proteins have four common motifs conserved in RNA-dependant RNA polymerases and depend on VP2 for replicase activity (Burns *et al.,* 1989; Patton *et al.,* 1993). The VP2 protein binds with RNA non-specifically and

interacts with dsRNA (Boyle and Holmes, 1986; Labbe *et al.,* 1994; Prasad *et al.,* 1996). VP2 plays an important role in replication, as it has the ability to bind to mRNA (Liu *et al.,* 1992; Patton *et al.;* 1996; 1997; 2004). VP3 can bind to the N-terminus of VP2, with labelling experiments using á^{32}P –guanosine triphosphate (GTP), indicating it is a guanyltransferase and also a methyltransferase (Boyle and Holmes, 1986; Chen, 1999 Pizzaro, 1991).

2.4.5 The Non-structural proteins (NSPs)

There are six NSPs coded by the rotavirus genome, namely NSP1-6. With the exception of NSP4, all of these NSPs bind to the RNA. They have various functions in viral replication, assembly of various proteins and in viral packaging during replication (Mattion *et al.,* 1990; Poncet *et al.,* 1993).

2.4.6 The NSP1 protein

NSP1 is encoded by gene segment 5 and its gene size ranges from 1564 bp to 1611 bp in length which encodes a polypeptide of 486-495 amino acids in length (Barro and Patton, 2005). NSP1 appears to be the least important in the replication of virus. However, it has an important role in cell-to-cell spread during infection (Barro and Patton, 2005; Hua *et al.,* 1994) and contains cysteine-rich zinc fingers, involved in binding of RNA. Recently, it has been demonstrated that this protein has an important anti-interferon activity which destroys innate immunity of host cells (Barro and Patton, 2005) and thus has a pathogenic function. This protein is also the least conserved showing more sequence variation among various strains than VP4 and VP7 discussed earlier. NSP1 contains one cysteine-rich region near its amino terminus. The cysteinerich domain of group A and C rotaviruses is the motif C-X2-C-X5-C-X2-C-X3-H-X-CX2-C-X5-C located at amino acids 42-69 (Hua *et al.,* 1993). This motif forms one or two zinc fingers which are highly

conserved among many rotaviruses and are involved in RNA binding suggesting that it plays a critical role in the function of the virus (Hua *et al.,* 1993; Kojima *et al.,* 1996a). The carboxyl half of the protein is not involved in virus replication but has a pathogenic role, such as the anti-interferon activity as mentioned above and viral cell-to-cell spread (Barro and Patton, 2005). Although the rotavirus gene segment 5 is believed to be highly variable in sequence, NSP1 is conserved to some extent among rotaviruses infecting the same host (Kojima *et al.,* 1996a; Mattion *et al.,* 1994). Analysis of 22 NSP1 sequences from various host species showed that this protein exhibits a higher degree of host specificity compared to both VP7 and VP4. The gene 5 nucleotide sequence homology was found to be more specific within a single species than among different species (Dunn *et al.,* 1994) suggesting a role in host range restriction. However, when interspecies relatedness was examined, a high degree of amino acid identity between the human strain, Wa, and the porcine strain, OSU (86%), and between the human strain, AU-1 and the bovine strain UK, (87%), have been observed (Dunn *et al.,* 1994; Nakagomi and Kaga, 1995).

2.4.7 The NSP4 Protein

The NSP4 protein is 175 amino acids in length and is encoded by gene segment 10 (751 bp). It is an endoplasmic reticulum (ER) specific glycoprotein (Ball *et al.,* 2005; Mattion *et al.,* 1994; Tian *et al.,* 1996) and is one of the most important non-structural proteins in relation to virus virulence and pathogenesis and is the first multifunctional viral enterotoxin reported (Ball *et al.,* 1996). This protein acts as an enterotoxin and is responsible for diarrhoea in infants and age-dependant diarrhoea in mice (Ball *et al.,* 1996; Estes *et al.,* 2001). The protein plays a unique role by functioning as an intracellular receptor and converting double-layered viral particles in the cytoplasm into the triple-layered form in the ER (Estes *et al.,* 2001; Horie *et al.,* 1999; Maass

and Atkinson, 1990; Taylor *et al.,* 1996). It is proposed that NSP4 interacts with epithelial cells of the intestine and stimulates a calcium-dependant signal transduction pathway that increases plasma membrane and stimulates chloride secretion resulting in secretive diarrhoea (Ball *et al.,* 1996). The development of antibodies against NSP4 in mice pups immunised with a peptide derived from SA11 NSP4 (residues 114 to 135) resulted in diarrhoea of less severity and shorter duration compared to controls (Horie *et al.,* 1999; Mori *et al.,* 2002a) suggesting that NSP4 induces a protective antibody response in the host.

A computational analysis of the 176 NSP4 genes sequences available in the GenBank database derived from all the different groups of rotavirus, revealed the presence of four different genotypes of NSP4 and many amino acids which were found to be well conserved regardless of genotype (Lin and Tian, 2003). However, considerable variation among the NSP4 from mammalian and avian groups has been observed such that a fifth genotype has been reported. A major difference observed in avian NSP4 was the absence of highly probable coiled–coil region responsible for the quaternary structure seen in mammalian NSP4 (Lin and Tian, 2003; Taraporewala *et al.,* 1999).

2.4.8 Other NSP proteins

Rotavirus non-structural proteins are actively involved in viral replication. NSP2 is an oligomeric nucleotide tri-phosphatase (NTPase), is localised in the viroplasm and possesses helix destabilising activity (Poncet *et al.,* 1993; Taraporewala *et al.,* 1999). NSP3 binds with the 3' end of the viral mRNA, needing only a four nucleotide sequence to recognise the binding site. NSP3 inhibits cellular protein synthesis by binding with the poly A mRNA of the host cell (Piron *et al.,* 1998; 1999, Poncet *et al.,* 1993; Vende *et al.,* 2000). NSP3 also enhances viral translation

(Gonza'lez and Burrone, 1991; Vende *et al.,* 2000). NSP5 is a self-assembled dimer (Arias *et al.,* 1996; Gonza'lez and Burrone, 1991) with an autokinase property that exists abundantly in infected cells in various isomeric forms (Arias *et al.,* 1996; Blackhall *et al.,* 1997; Poncet *et al.,* 1997) and interacts with NSP2 during replication (Afrikanova *et al.,* 1998; Gonza'lez *et al.,* 1998; Mattion 1991; Poncet 1997) NSP6 is encoded by gene segment 11 and is known to interact with NSP5 (also encoded by gene 11) and reside in the viroplasm (Mattion *et al.,* 1991; Torres-Vega *et al.,* 2000). NSP2, NSP5 and NSP6 are involved in RNA encapsidation and movement of viral particles from the viroplasm to the membrane of endoplasmic reticulum (ER) (Estes *et al.,* 2001; Fabbretti *et al.,* 1999).

2.5 Virus Classification

Rotavirus belongs to family *Reoviridae.* It shares the following common general features of the family (Estes, 1996). Rotavirus possess segmented double stranded RNA as a genome, is non-enveloped, the mature virus measures 65-75nm, it has icosahedral particles, the purified viral RNA is not infective and its cultivation is facilitated by protease.

Rotaviruses are classified serologically into several groups based on the VP6 protein (serogroup). Rotavirus groups share the cross-reacting antigens detected by a number of serological tests like Enzyme Linked Immunosorbent Assay (ELISA), immunofluorescence, and immunoelectron microscopy (Estes *et al.,* 2001). Rotaviruses comprise seven distinct groups (A-G) based on the group determinants present in the VP6 protein. Groups A, B and C are found to cause infection in both humans and animals, whereas groups D, E, F and G have been found only in animals.

Viruses within each group are capable of genetic reassortment. Group A rotaviruses have been found as a cause of diarrhoea in significant numbers of children. This group remains the most common type circulating worldwide. Group A is further divided into the following subgroups based the presence or absence of specific antigens: subgroup I; subgroup II; subgroup I & II; and subgroup non I & non II.

Within group A, viruses are classified into serotypes defined by reactivity of virus neutralisation assays using hyperimmune serum prepared in antibody negative animals. Using this assay, 15 VP7 serotypes have been identified (Hoshino *et al.,* 2005). Strains from both human and animal origin may fall within the same serotypes. Neutralisation assays can measure reactivity against the two outer capsid antigens, VP4 (P-type) and VP7 (G-type). However, in many cases, the predominant reactivity is determined against, VP7, simply because VP7 covers a greater percentage of outer capsid and induces highly specific antibodies (Birch *et al.,* 1988; Coulson *et al.,* 1987; Hoshino *et al.,* 1985; Taniguchi and Urasawa, 1985). The minor neutralising antigen, VP4, does not always allow rotavirus strains to react clearly in reciprocal neutralizing assays with hyperimmune antiserum (Hoshino *et al.,* 1985). These two different neutralising antigens thus were made basis for a dual classification system for rotavirus. Rotavirus classification based on neutralising VP7 antigens is termed G-type (since VP7 is a glycoprotein), and classification based on VP4 neutralising antigens is termed P-type (since VP4 is a protease sensitive protein). Genes that encode VP7 and VP4 can segregate separately, resulting in a large amount of antigenic diversity (Hoshino *et al.,* 1985; Su *et al.,* 1986). There are 42 different combination of G and P types that have been recognised so far (Gentsch *et al.,* 2005).

The P-typing system of classification has been hampered by lack of readily available serological typing reagents to the different VP4 serotypes. Instead, the antigenic properties of VP4 have

been studied by gene sequence analysis and have indicated that there are 26 different P-genotypes (Santos, 2005). Genotypes of VP4 and VP7 are determined by sequence analysis or hybridisation, whereas serotypes have been determined by reactivity with polyclonal or monoclonal antisera (Estes, 1996; Imai *et al.,* 1983). For VP7, a correlation between genotypes and serotypes has been established; however such a correlation is not yet available for VP4.

2.6 Viral Replication

Adsorption to the host cell: The first step in viral replication is attachment to host cells with the help of VP4 (Fukudome *et al.,* 1989; Jourdan *et al.,* 1998; Petrie *et al.,* 1981). The type of glycoconjugates that actually function as the *in vivo* receptor for a given rotavirus depends on the strain and host cell type (Franco *et al.,* 1994; Fukudome *et al.,* 1989). Two different methods of cell attachment are known: sialic acid-dependant and sialic acid-independent viral attachment to the host cells. Rotaviruses that are sialic acid-dependent infect only polarised epithelial cells and attack only the apical membrane, whereas sialic acid-independent strains attack non-polarised cells on either the apical or basolateral surfaces (Ciarlet *et al.,* 2000; Ciarlet and Estes, 1999; Franco *et al.,* 1994; Mendez *et al.,* 1999). These results indicate that sialic acid-dependant and independent viruses use different cell receptors and they may share a second receptor (Bastardo and Homes, 1980; Mendez *et al.,* 1999).

Penetration: After binding occurs, the virus is internalised, probably by direct penetration. Virus entry into the cell is a multi-step processes (Mendez *et al.,* 1999). Radiolabelling studies in the monkey kidney cell line (MA104) using the simian virus, SA11, have found that there are 13,000 receptors units per cell; initial binding is sodium dependant, pH sensitive (5.5-8.0) and

dependant on sialic acid residues in the membrane (Bastardo and Homes, 1980; Imai *et al.,* 1983).

Transcription and Replication: Once the virus enters the cells, the synthesis of viral transcripts is initiated by an endogenous viral RNA-dependant RNA polymerase (transcriptase). The enzyme has a number of different enzymatic activities e.g. transcriptase, nucleotide phosphohydrolase, guanyltransferase and methylase (Imai *et al.,* 1983). The transcriptase is a component of VP1 and VP3 localised together in a flower shaped structure attached to the top of VP2 at the axes of VP2 protein unit (Cohen, 1977; Fiore *et al.,* 1991; McCrae and McCorquodale, 1983; Sandino *et al.,* 1986; Spencer and Ariar, 1981). The exact site of transcription within the cytoplasm has not been precisely localised, although it is thought to occur in the viroplasm. Transcription requires ATP for initiation or elongation of RNA molecules and also for polymerisation (Lawton *et al.,* 1997; Spencer and Ariar, 1981). RNA synthesis occurs inside the core of the virion. Newly transcribed RNA exits the core through the type I channels in the core VP2 layer and multiple mRNA transcripts can be released simultaneously from an actively transcribing particle (Lawton *et al.,* 1997; Stacy-Phipps and Patton, 1987). Each genome segment is thought to be transcribed by a specific polymerase complex. The synthesis of positive and negative stranded RNA has been studied in SA11-infected cells (Patton, 1986; Stacy-Phipps and Patton, 1987) in a cell-free system using extracts of infected cells and an electrophoretic system that allows separation of the positive and negative strands in acid urea agarose gels (Patton and Stacy-Phipps, 1986a). RNA synthesis in infected cells results in positive and negative RNA strands that are detectable within three hours of infection (Silvestri *et al.,* 2004; Stacy-Phipps and Patton, 1987). Both newly synthesised and pre-existing positive-stranded RNA can act as templates for negative strand synthesis throughout

infection (Estes *et al.,* 1996; Silvestri *et al.,* 2004). During replication, dsRNA remains within partially uncoated particles, afterwards dsRNA remains associated with sub-viral particles, and thus free dsRNA is not found within the cell at anytime (Estes, 1996; Petrie, 1983).

RNA encapsidation and virion assembly: Sub-viral particles assemble in cytoplasmic viroplasms and bud through the membrane of the ER, acquiring a transient envelope. This is one of the characteristics features of rotavirus that differentiates it from other members of *Reoviridae* family. The temporary envelope is then lost as the particle moves from the membrane to the inner part of the ER and is then coated with outer capsid proteins as the virus matures. Most of the rotavirus proteins, structural or non-structural, are synthesised on free ribosome's that are associated with the membrane of the ER (Richardson *et al.,* 1986). Viroplasms are probably the sites of synthesis of double layer particle that contain RNA. This conclusion is based on the localisation of several viral proteins (VP2, NSP2, NSP5 and NSP6) to the viroplasm and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER (Gonza'lez *et al.,* 2000; Silvestri *et al.,* 2004). Recently, it has been demonstrated by immunofluorescence analysis of bromouridine (BrU) labelled RNA that plus strand RNAs are made in viroplasms (Silvestri *et al.,* 2004).

Virus release: The infectious cycle ends when progeny virus is released by host cell lysis in nonpolarised cells as shown by electron microscopy (Altenburg *et al.,* 1980; Chasey, 1977; Musalem and Espejo, 1985). Extensive cytolysis occurs late during infection and drastic alteration in the permeability of the plasma membrane of infected cell results in the release of cellular and viral proteins (Estes *et al.,* 1996; Musalem and Espejo, 1985).

Rotavirus replication takes place in the cytoplasm of the host cell. The natural host cell of rotavirus is the differentiated enterocyte in the small intestine (Clark *et al.,* 1979; Estes *et al.,*

2001). The various steps of the rotavirus replication cycle have been studied by infecting monkey kidney cells in cell monolayers. In these cells, the replication cycle is fairly rapid with high yields of virus being found after 10 to 12 hours at 37°C when cells are infected with high multiplicities (10 to 20 PFU/cell) (Clark *et al.,* 1979; McCrae and Faulkner-Valle, 1981; Ramig, 1982).

While rotavirus replication takes place entirely inside the cytoplasm, the addition of exogenous protease is required in order to cleave VP4, the outer capsid spike protein. The cell does not have the enzyme to replicate dsRNA, so the virus has to provide the necessary enzymes. Transcripts have two functions: acting as mRNA for the production of proteins, and also acting as a template to form negative strand RNA, which remains associated with the positive strand (Estes, 1996; Silvestri *et al.,* 2004). The nascent sub-viral particles mature by budding through the membrane of the ER, during which they acquire the outer capsid (Estes, 1996; Petrie *et al.,* 1981).

2.7 Molecular Biology of Rotavirus

2.7.1 Genome of Rotavirus

Rotavirus is the only mammalian virus that has a genome of 11 segments of double stranded RNA (dsRNA). The genome is found inside the core. The virus contains its own RNA-dependant RNA polymerase required for replication, thus, deprotenised RNA is not infective. The rotavirus gene sequences are rich in A+U (58%-67%), are base paired end to end and the positive strand contains the 5'- cap sequence, m^7GpppG^mGPy (Estes *et al.,* 1996; Imai *et al.,* 1983; Mccrae and Mccorquodale, 1983; Patton *et al.,* 2004). One of the most important features of rotaviruses is that it co-ordinately replicates and packages the 11 segments with great efficiency. The 11 segments share common cis-acting signals because all the segments of RNA are replicated by the

same polymerase (Estes *et al.,* 1996; Mattion *et al.,* 1991). The nucleotide sequence of all 11 RNA segments is now known for several rotavirus strains. The simian rotavirus strain, SA11, was the first one to be completely sequenced and is the prototype group A rotavirus. Each positive strand starts with a 5'- guanidine followed by a set of conserved sequences that are part of the non-coding sequence. An open reading frame (ORF) then follows, which encodes a structural or non-structural protein. Almost all mRNAs end with the consensus sequence 5'-UGUACC-3', an important signal for RNA replication and gene expression. All the gene segments possess at least one ORF after the first initiation codon. Gene segment 1, 9 and 10, however, has an additional ORF. The entire gene segments are monocistronic, with the exception of gene 11 (Estes and Cohen, 1989; Mattion *et al.,* 1991).

The 11 genome segments can be well segregated by polyacrylamide gel electrophoresis (PAGE). In most cases, the electrophoretic pattern for group A rotaviruses shows a typical pattern of four high molecular weight bands (segments 1-4; 3302, 2690, 2591 and 2362 bp respectively for strain SA11), two middle sized bands (segments 5 and 6; 1611 and 1356 bp respectively for strain SA11), followed by a triplet (gene segments 7-9 ;1 105, 1059, 1062 bp respectively for strain SA11) and two low molecular weight segments (10 and11; 751 and 667 bp respectively for strain SA11). However, the electrophoretic patterns may not always be the same for particular groups, due to various reasons such as gene rearrangements, insertions or deletions of nucleotides during replication. Electrophoretic analysis is however relatively easy, rapid and thus a popular method for virus detection and molecular epidemiology (Bishop, 1996).

Northern blot assays are commonly used to classify rotaviruses into genogroups, based on genome similarities, for identifying the origin of a viral gene in reassorted strains and to identify

viruses involved in cross-species transmission (Ballard *et al.,* 1992; Estes *et al.,* 2001; Nakagomi and Nakagomi, 1991; 1993).

The rotavirus genome undergoes genetic changes by different means. Gene rearrangement is responsible for different electrophoretic patterns within a given group. Around 1,800 nucleotides can be inserted into a particular gene segment without causing any significant changes in the gene expression or particle diameter, but the density of the virion may increase proportionately. Deletions of nucleotides have also been reported. In most cases the rearranged gene does not cause any noticeable changes, which indicates that the rearrangement has left the ORF and its expression unaltered. Gene sequence analysis of rearranged genes has confirmed this (Ballard *et al.,* 1992; Desselberger, 1996; Palombo, 2002).

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2.7.2 Coding Assignment of the Genes of Rotavirus:

Based on *in vitro* translation using mRNA and denatured dsRNA, and by analyses of reassortant viruses, the protein assignments of rotavirus genes and the properties of the encoded proteins have been determined. Much of this information has been obtained from investigations of the prototype rotavirus, SA11. The ability to obtain sequence information directly from dsRNA or ssRNA and the accumulated information on nucleic acid databases has made it possible to

identify similarities among strains based on sequence homology. The structural proteins are known as VP followed by a number and non-structural proteins are name as NSP followed by a number. VP1 has the highest molecular weight.

2.8 Genetics of Rotavirus

The genetics of rotavirus has been studied to better understand the various and unusual combinations of VP4 and VP7, to investigate the natural phenomenon of reassortment occurring between strains *in vivo* and to assign functions to particular genes. Several temperature sensitive (ts) mutants of SA11, UK and RRV have been collected in order to study the genetics of these strains (Estes *et al.,* 2001). The phenotypes of these mutants were consistent with functions assigned for that particular gene segment, based on biochemical or sequence information (Estes *et al.,* 2001). Reassortment, which occurs maximally at the initial phase of infection, can take place at non-suitable temperatures and mutants from all reassortment groups can interfere with the growth of wild-type virus (Estes *et al.,* 2001). *In vitro* reassortment of ts viruses has been carried out successfully to derive reassortant progeny with desired subsets of genome segment derived from each ts parent. These reassortant strains have been used to assign the ts mutation to a specific gene segment and correlate the biological properties of the specific gene segment (Desselberger, 2000; Gonza'lez and Burrone, 1991).

2.9 Genetic Variability of Rotavirus

The rotavirus genome has the potential for rapid genetic change. Different mechanisms create variation among rotavirus populations and result in the evolution of the virus and the development of atypical strains: Point mutation, Genetic rearrangement and Gene reassortment.

The replication of the rotavirus genome occurs via a viral encoded RNA-dependant RNA polymerase (Desselberger, 2000; Ramig, 1997) and the main source of the genetic variation is considered to be mutations due to the error-prone nature of this enzyme. A mutation rate of < $5X10^{-5}$ per nucleotide per replication round has been calculated for rotaviruses (Beards *et al.,* 1989; Ramig, 1997). The contribution of mutations in generating genetic variants of rotavirus isolates collected from various geographical locations worldwide has been well documented (Beards *et al.,* 1989; Desselberger, 2000; Estes, 1996; Palombo, 2002). Genetic rearrangement also contributes to the formation of diverse strains of rotavirus. Although there is no direct evidence for homologous recombination during rotavirus replication, polymerase jumping during plus and minus strand synthesis can result in intermolecular recombination. This leads to the rearrangement of segments, usually their partial duplication (Desselberger, 1996; Ramig, 1997). The rearranged genome may leave the ORF conserved and thus the function of gene remains unaltered (Ballard *et al.,* 1992), the rearrangement could lead to an extended ORF (Tian *et al.,* 1993) or in some cases, the duplication of the gene along with a point mutation in the ORF leads to the premature truncation of the protein (Hua and Patton, 1994a).

The unique segmented nature of the rotavirus genome provides a novel way of genetic variation via the process of genetic reassortment. This occurs during mixed infections where packaging of viral segments into sub-particles can lead to the mixing of genes from two different parental viruses (Palombo, 2002; Ramig, 1997). Reassortment has the capacity to rapidly generate new genetic and antigenic variants and is a distinguishing feature of viruses with segmented genomes (Palombo, 2002; Prasad *et al.,* 1990). The generation of possible reassortant progeny genomes can be detected using electrophoresis.

2.10 Antigenic properties of Rotavirus

Rotavirus consists of two major proteins, VP6 and VP7, which comprise the inner and outer capsid of the virus, respectively. The VP6 protein has contains subgroup specific antigens whereas VP7 has serotype specific antigens. Furthermore, there is third protein known to possess antigenic properties, VP4, which is a spike protein, also found on the outer capsid. VP4 is composed of dimers and has hemagglutinin activity (Greenberg *et al.,* 1983a; Prasad *et al.,* 1990). VP7 and VP4 are known to stimulate neutralising antibodies found in the sera and stool of infected patients, are believed to confer the protection against rotavirus infection (Dyall - Smith *et al.,* 1986; Greenberg *et al.,* 1983; Hoshino *et al.,* 1988) and are therefore the major targets for candidate vaccines. Six major antigenic sites have been located on VP7 and these are designated as regions A, B, C, D, E and F (Dyall-Smith and Holmes, 1984; Estes and Cohen, 1989; Huang, 2000; Lazdins *et al.,* 1995; Kirkwood *et al.,* 1993). These regions contain epitopes that are found to induce protective antibodies (Huang, 2000; Kapikian and Chanock, 1996).

2.11 Molecular analysis of antigenic sites on Rotavirus VP4

VP7 and VP4 are both located on the outer capsid proteins and play important roles in viral attachment and penetration during infection, and both proteins independently induce neutralising antibodies (Jolly *et al.,* 2001; Kapikian and Chanock, 1996). Detailed knowledge of the antigenic properties of these proteins is critical to develop an effective rotavirus vaccine. The VP4 protein modulates important functions such as viral attachment to cellular receptors and helps in consequent host cell penetration (Fiore *et al.,* 1991; Jolly *et al.,* 2001). Prior to cell penetration, VP4 must be cleaved to its subunits, VP5* and VP8*, by treatment with trypsin (Fiore *et al.,* 1991; Fuentes-Panana *et al.,* 1995). Various studies have demonstrated that VP4 is the viral

attachment protein and have provided evidence that amino acid residues 150,180,183, 187 and 194 may be involved in binding to the sialylated cell receptors (reviewed by Jolly *et al.,* 2001). Neutralisation epitopes on VP4 have been identified using serotype specific and serotype cross reactive N-MAbs.

2.11.1 Molecular analysis of antigenic sites on Rotavirus VP7

The VP7 glycoprotein is the second most abundant protein of rotavirus and, as stated earlier, six antigenic regions have been mapped. The locations of these are as follows: region A (aa 87-101), B (aa 143-152), region C (208-221), region D (aa 65-76), region E (aa 189-190) and region F (aa 235-242) (Dyall - Smith *et al.,* 1986; Kirkwood *et al.,* 1993). A reactivity pattern of neutralising antibody escape variants has indicated that region C shows cross reactivity between serotypes G4 and G9 (Kirkwood *et al.,* 1993). Moreover, while all the antigenic regions are widely separated on the linear sequence, some of them are located in close proximity to one another when considering the three dimensional structure of VP7. Regions A and C are juxtaposed in serotypes G1, G2, and G3 and may influence each other (Dunn *et al.,* 1993; Dyall-Smith *et al.,* 1986; Lazdins *et al.,* 1995; Taniguchi and Urasawa, 1995). Also, it has been observed that regions A, C and E are in close proximity with each other in serotype G2 (Das *et al.,* 1993; Kirkwood,1996).
The antigenic region F is reactive to serotype G3, G4 and G9 specific monoclonal antibodies and contains serotype-specific and serotype cross-reactive epitopes detectable in the absence of glycosylation at position 238 (Dunn *et al.,* 1993; Kirkwood *et al.,* 1996a; Kirkwood *et al.,* 1993). The antigenic region E may only be identified in some rotaviruses (Dunn *et al.,* 1993; Lazdins *et al.,* 1995; Caust *et al.,* 1987).

Glycosylation is an important property of VP7 that influences its antigenicity. In one study, SA11 escape mutants selected with specific N-MAbs were found to have a new glycosylation site in region C that led to marked antigenic alterations (Caust *et al.,* 1987; Lazdins *et al.,* 1995). The presence or absence of glycosylation can determine whether a MAb will select substitutions in the antigenic regions A or C, with glycosylation at aa190-192 blocking access of N-MAb to region A (Lazdins *et al.,* 1995; Kirkwood *et al.,* 1993). Antigenicity was also altered by glycosylation in region F (Kirkwood *et al.,* 1993; Mendez *et al.,* 1996).

2.11.2 Antigenic regions on VP7 protein

Furthermore, the two outer capsid proteins of rotavirus are known to interact with each other and thus mutation in one protein have been proposed to affect the antibody binding activity of the other (Mendez *et al.,* 1999; Zhou *et al.,* 1994). An epitope on the VP8* subunit of VP4, at residues 180-183, has been suggested as a site which interacts with VP7 (Zhou *et al.,* 1994).

2.12 Pathogenesis of Rotavirus

Rotavirus infection causes disease by three different pathogenic mechanisms. First, rotavirus infects mature villus tip enterocytes, thus affecting absorption of nutrients and liquid in the gut. Second, rotavirus is able to secrete the virus-encoded non-structural protein, NSP4, which acts as an enterotoxin and mobilises fluid secretion. Finally, effects on the enteric nervous and vascular systems stimulate indirect secretion (Arvin and Greenberg, 2006; Estes *et al.,* 2001; Glass *et al.,* 1996).

2.13 Manifestation of Rotavirus infection

Rotavirus is a major cause of viral gastroenteritis in infants, children and the young of various animals. Rotavirus represents 80% of recognised viral etiologies of gastroenteritis and 140 million cases of diarrhoea per year worldwide. In developing countries, rotavirus disease accounts for one-third of all hospitalisations for diarrhoea (Glass *et al.,* 1996; Jiang *et al.,* 2002; Parashar *et al.,* 2006). In developed countries, where general sanitation and hygienic conditions are better, rotavirus still poses a serious health problem and results in substantial medical costs (Jiang *et al.,* 2002; Parashar *et al.,* 2003). The virus strikes with similar frequency throughout the world but mortality rates are higher in developing countries. Rotavirus causes up to 610,000 deaths annually and leads to 2.3 million hospitalisations each year worldwide with 82% of total rotavirus mortality occurs in developing countries (Ball *et al.,* 2005; Hsu *et al.,* 2005; Parashar *et al.,* 2006). In Australia there are 10, 000 hospital admissions annually as a result of rotavirus infection (Barnes *et al*., 2002). Rotavirus causes 20-40 deaths and 55-80,000 hospitalisations each year in the US (Newsrx, 2006). In Australia, thirteen rotavirus related deaths have been reported (Newall *et al.,* 2006).

2.14 Pathology of Rotavirus-infected cells

One of the rotavirus non-structural proteins, NSP4, acts as a viral enterotoxin, contributing to its pathogenesis (Ball *et al.,* 2005; Estes and Morris, 1999). The enterotoxin can trigger a signal transduction pathway that leads to mobilization of intracellular calcium and chloride ion secretion (Ball *et al.,* 1996; Estes *et al.,* 2001; Estes and Morris, 1999). Rotavirus infects the villi of the small intestine, causing the shortening of the villus and thus mal absorption of fluid. The infection causes disruption of epithelial cell structure and functions within 18 –24 hours

after infection and may lead to cell death. The acute infection usually resolves in 7 days in immunocompetent hosts, however, in severe cases, death can occur due to severe loss of fluid, poor absorption of fluid and nutrients, and shock (Estes *et al.,* 2001; Holmes *et al.,* 1975; Parashar *et al.,* 2003a). The pathological changes observed in the jejunal mucosa in young children hospitalised with rotavirus disease including shortening and atrophy of the villi, cell infiltration in the lamina propria and irregular microvilli (Holmes, 1975; Mebus *et al.,* 1977; Moon, 1994). Detailed information on pathogenic changes has been obtained by directly infecting animals. Within half an hour of infection, morphologic changes such as swelling of the villi and flattening of epithelial cells were observed in the upper intestine, but the lower intestine was intact. Seven hours after the infection, upon onset of diarrhoea, the lower intestine showed morphological changes similar to that of large intestine and forty hours later, the intestine appeared normal (Davidson *et al.,* 1977 Mebus *et al.,* 1977). In another experiment, involving administration of a human rotavirus to a baby pig, physiological alterations in small intestine were observed; glucose transport was affected, sucrose activity was decreased and thymidine kinase activity was increased (Davidson *et al.,* 1977). In permissive cells *in vitro,* cytopathic effect was observed and cell death occurred due to shutting off of host cell DNA, RNA and protein synthesis (Carpio *et al.,* 1981; Ericson *et al.,* 1983; Kim *et al.,* 1977; McCrae and Faulkner-Valle, 1981).

2.15 Age distribution of Rotavirus infection

Rotavirus is a major cause of severe infection in infants and young children under five years of age. Though it is found to cause infection in adults, the number remains comparatively low. Children under six months of age are believed to be protected by maternal antibodies, however,

neonatal strains are commonly identified in hospital nurseries in many countries, including Australia, and cause asymptomatic to mild symptomatic infections in neonates (Bishop, 1996).

The major age group burdened by rotavirus related illness falls between children aged 6-23 months (Bishop, 1996). For example, in a longitudinal epidemiological study of 2,897 patients in the US conducted from 2000-2004 approximately 50% of total population infected with rotavirus ranged between 9-12 months of age and the incidence decreased as the age of patients increased (Fig 2.1).

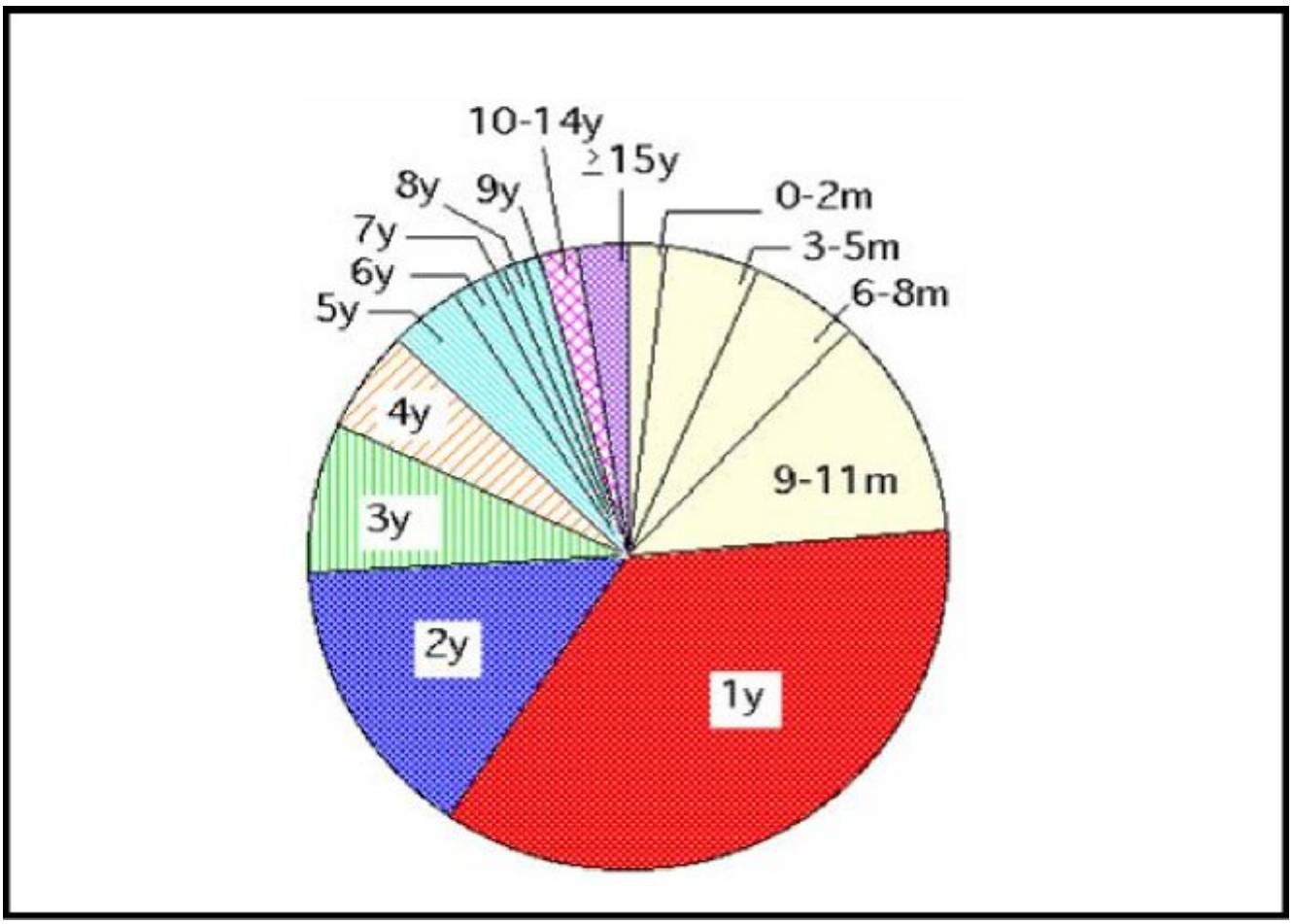


Figure 2.1: Age distribution of rotavirus detected cases from 2000-2004, total cases 2,897. Source: IASR (Infectious Agents' Surveillance Report, 2004, CDC).

2.16 Clinical Features

Rotavirus causes infection in all age groups, but children are more susceptible to the disease. The infection produces subclinical to mild to severe diarrhoea and occasionally fatal dehydrating illness. Rotavirus infection is most commonly characterised by the onset of acute diarrhoea with a temperature of 37.9°C or above and vomiting in most cases, causing severe dehydration. Other clinical symptoms such as irritability, lethargy, pharyngeal erythema, rhinitis and palpable cervical lymph nodes are also associated with the diarrhoea (Estes *et al.,* 2001).

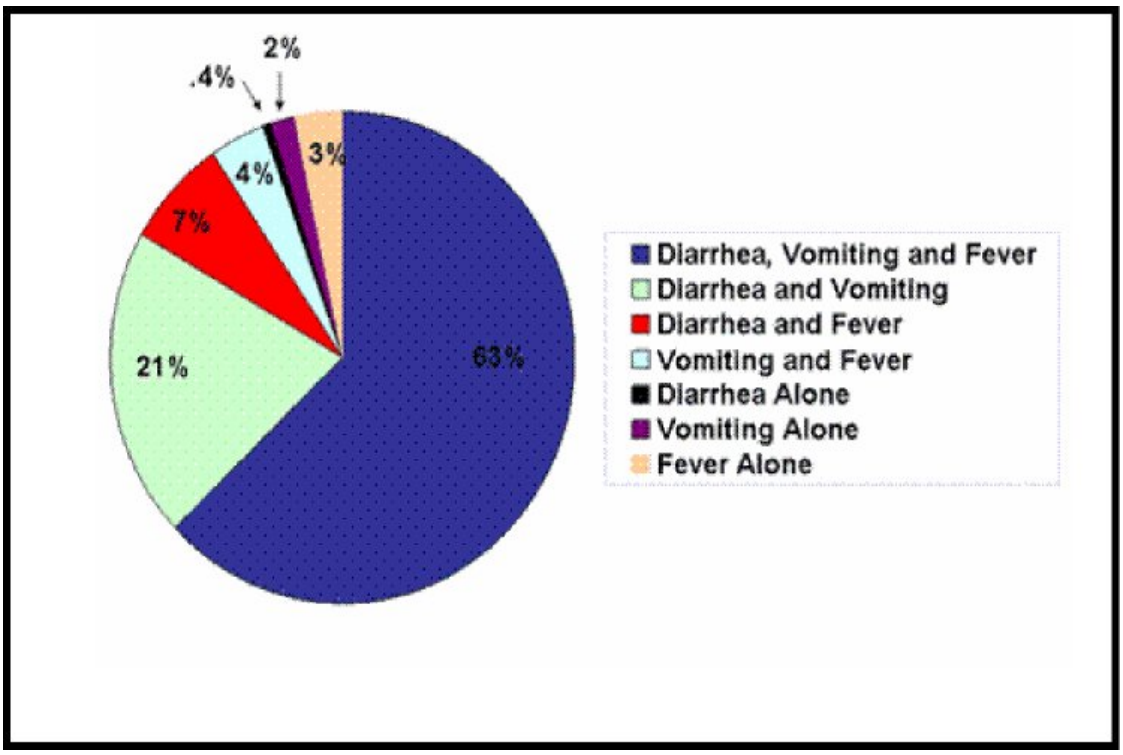


Figure 2.2: Symptoms caused by rotavirus infections. Source: www.medscape.com

Rotavirus can produce chronic illness in some individuals, especially the immunosuppressed, bone marrow transplant recipients, and person suffering from hepatitis (Thea *et al.,* 1993; Yolken and Wilde, 1994). Rotaviruses, however, have not been found to cause any severe illness in AIDS patients (Konno *et al.,* 1977; Konno *et al.,* 1978; Thea *et al.,* 1993). Rotavirus was detected in stools of 11 out of 30 children in Japan with intussusception; 5 of the 7 rotavirus positive children tested developed a serologic response, suggesting that wild type rotavirus might have been the cause of intussusception. Another investigation showed that 33% of intussusception cases (61 patients studied) had rotavirus infection (Konno *et al.,* 1977; Konno *et al.,* 1978; Mulcahy *et al.,* 1982). Three other studies conducted in Australia, France and Germany failed to find a significant association between rotavirus and intussusception (Mulcahy *et al.,* 1982; Staatz *et al.,* 1998; Ward, 1996). The first rotavirus vaccine, RotaShield, licensed in the USA in 1998, was linked to the cases of intussusception in vaccinated children. Simonsen *et al.* (2005) found strong correlation between the Rotashield and cases of intussusception in 80% vaccinated children of age >three months. Similarly, rotavirus was identified in 7 of 8 tissue samples of children who received Rotashield vaccine and developed intussusception (Lynch *et al.,* 2006), due to this link, RotaShield was withdrawn from the market a year later (CDC, 1999).

2.17 Immunity

Rotavirus infection induces both local intestinal and systemic immune responses (Kapikian *et al.,* 2001; Ward, 1996). A rapid humoral antibody response is induced by rotavirus infection that is correlated with intestinal immunoglobulin A (IgA) (Ebina *et al.,* 1983; Kapikian *et al.,* 2001). It is understood that locally produced antibodies in the intestinal mucosa play a key role in protective immunity against rotavirus-induced diarrhoea (Coulson *et al.,* 1990). Rotavirus

specific IgA has been detected in duodenal fluid, serum, faecal material and breast milk (Bernstein *et al.,* 1986; Coulson *et al.,* 1990; Schwartz-Cornil *et al.,* 2002). An interesting observation is that systemic rotavirus antibodies are present in the lumen of the gastrointestinal tract of neonatal calves, if the level of circulating antibodies is sufficiently high. Such serum derived mucosal antibodies can provide protection against experimentally induced infection and diarrhoea (Armstrong and Dimmock, 1992; Besser *et al.,* 1988).

IgA is found to prevent epithelial cell attachment by virus by cross-linking and aggregation of virus particles (Armstrong and Dimmock, 1992; Gilbert *et al.,* 2001). Studies in mouse models have also shown that IgA inhibits the assembly of rotavirus by preventing association with the outer capsid VP7 protein (Blutt *et al.,* 2004; Estes *et al.,* 2001; Gilbert *et al.,* 2001). VP7 induces polyclonal B-cell activation independent of T-cell lymphocytes (Blutt *et al.,* 2004; Franco and Greenberg, 1995). The specific cellular mechanism of the B-cell response is yet to be clearly determined. Although the B-cell arm of the immune response plays a major role in controlling rotavirus infection, experiments involving the infection of B-cell-deficient mice indicate that the actual mechanism itself is not clear (Burns *et al.,* 1996; Franco and Greenberg, 1995; Kapikian *et al.,* 2001; McNeal *et al.,* 1995). It has found that both structural and non-structural proteins of rotavirus induce the production of antibodies, although antibodies to VP6 are non-neutralising but stimulate protective immunity in some animals (Burns *et al.,* 1996; Hjelt *et al.,* 1987). Rotavirus IgA antibody levels were also shown to correlate with resistance to severe diarrhoea (Hjelt et al., 1987; Matson et al., 1993). The association between faecal (IgA) and serum (IgG) anti-rotavirus antibody titres and protection against disease was investigated in 100 children in the US (O'ryan et al., 1994; Matson et al., 1993). During two seasons, seven outbreaks of G1 and G3 rotavirus were observed. These studies concluded that:

a) anti-rotavirus IgA titres of 1:80 and 1:20 correlated with protection against infection or illness, respectively, b) pre- existing serum anti-rotavirus IgA titre of greater than 1:200 or IgG titre of greater than 1:800 was associated with protection against infection and c) a high level of pre-existing G type-specific blocking antibody was associated with protection against infection. Similarly, in a study, a direct correlation between a high level of anti-rotavirus intestinal IgA antibody and a significant degree of protection of children against natural rotavirus infection and illness was observed (González, et al., 2005).

VP4 and VP7 proteins independently induce neutralising antibodies and each antibody plays a role in resistance to disease (Clemens *et al.,* 1993; Hoshino *et al.,* 1988; Ward *et al.,* 1998). Although serotype-specific antibodies have been demonstrated in breast milk, the effect of these on protection is yet to be established (Clemens *et al.,* 1993; Golding *et al.,* 1997; Newburg *et al.,* 1998; Ward, 1996).

Mouse models have been used to study the immune mechanisms involved in rotavirusinduced diarrhoea (Feng *et al.,* 1997; Franco and Greenberg, 1999). The following results have been concluded from those studies: i) rotavirus specific antibodies are of primary importance in protection against rotavirus reinfection, ii) in general, homologous rotavirus strains are far more potent than heterologous rotavirus strains in inducing protective local humoral immune responses, iii) MHC class I-restricted CD8+ áâT cells play a major role in resolution of primary rotavirus infection, iv) after primary infection, CD8+ T cells mediate almost complete protection (up to 2 weeks) or partial protection (3 months or less) from reinfection, however this protection diminishes after 8 months after primary infection, v) perforin and interferon-ã are not essential in antirotavirus activity mediated by CD8+T cells and vi) CD8+T cells expressing the á4â7 marker (an integrin receptor) are more efficient in mediating rotavirus

clearance than á4â7 negative cells (Feng *et al.,* 1994; Franco and Greenberg, 1995; McNeal *et al.,* 1995; Moser *et al.,* 1998; Rose *et al.,* 1998; Sack *et al.,* 1994). These observations indicate that the success of any rotavirus vaccine will depend greatly on its ability to produce mucosal immune responses, since intestinal immunity is found to be protective against rotavirus diarrhoea.

2.18 Treatment

Replacement of lost fluid and electrolytes is the main treatment for rotavirus diarrhoea. Intravenous fluid administration has been used normally with best results. However, in many parts of world, intravenous fluid replacement is not always possible, thus, oral rehydration method using various formulations of oral rehydration salts (ORS) has been used. ORS mainly contains sodium, chloride, potassium, citrate and glucose. The various concentrations of these ingredients have been described by the World Health Organisation (WHO). The standard formula of oral glucose electrolytes recommended by WHO is composed of the following: sodium, 90mmol/L; chloride 80mmol/L; potassium, 20mmol/L; citrate, 10mmol/L; and glucose, 11 1mmol/L resulting in an overall osmolarity of 310 mmol/L (Alam *et al.,* 1999; Prasad *et al.,* 1988; Sack *et al.,* 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.0 STUDY AREA

The study was a hospital based. Approval was obtained from Kano State Hospital Management Board ethics committee and each of the hospital visited was presented with the approval letter before the study was conducted. This consist of infants and young children aged 0-5 years with diarrhoea presented at/or hospitalized in the under listed hospitals in Kano: Murtala Muhammad Specialist hospital, Hasiya Bayero paediatric hospital, Gaya General hospital, Gwarzo General hospital, Wudil General hospital and Bichi General hospital,

3.1 Specimen Collection and Storage

Between November, 2009 and July, 2010 a total of 285 faecal specimens were collected from the various hospitals mentioned above from infants and young children presenting with diarrhoea. The number of specimens collected per hospital largely depended on its population of patients visiting the hospital with diarrhoea cases. The specimens were stored in a refrigerator at 4^{o}C before analysis.

3.2 Detection of Rotavirus Antigen in Stool Specimens

The 285 faecal specimens collected were analysed for rotaviruses using the ELISA kits (ProSpecT^{TM} Rotvirus, REF R240396 Oxoid UK). Group A rotavirus antigen was detected with the test Kit. The test was carried out according to the manufacturer□s instructions.

3.3 Test Procedure

A 10% dilution of each specimen was made by adding approximately 1ml of liquid diarrhoeic stool to 9ml distilled water in sterile screw-cap bottles. The suspension was mixed thoroughly and allowed to settle for 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 manufacturers 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 (calf rotavirus strain 3209176) in buffer containing antimicrobial agent and 100µl negative control, tris buffered saline containing antimicrobial agent and red dye into each separate wells of each plate carefully to avoid cross contamination. Then 100µl of rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxides was added to each of the plate and the plates were incubated for 1 hour at room temperature.

After the period of incubation, the plates were washed 4 times by hand 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 50µl 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 50µl 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 a Universal Microplate Reader. The 10% stool suspensions were kept at 4^oC for any repeat assays. Rotavirus infection was diagnosed if rotavirus antigen was present in a stool specimen. Any specimen well that showed a blue color 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.

3.4 Extraction of RNA from stool specimen

QIAamp Viral RNA Mini Spin Protocol was used for this procedure. The specimens were brought to room temperature (15–25°C), and Buffer AVE was also brought to room temperature for elution. Buffer AW1, Buffer AW2, and Carrier RNA were prepared according to the manufacturer's instructions. All centrifugation steps were carried out at room temperature.

A 560 µl of prepared buffer AVL containing carrier RNA was pipetted into a 1.5ml micro centrifuge tube, from each stool specimen 140 µl was added to the buffer AVL/carrier RNA in the micro centrifuge tube and mixed by pulse-vortexing for 15 sec.

To ensure efficient lysis, the specimen was mixed thoroughly with Buffer AVL to yield a homogeneous solution. The set up was incubated at room temperature (15–25°C) for 10 min. Viral particle lysis was complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in Buffer AVL. The 1.5ml micro centrifuge tubes were briefly

centrifuged to remove drops from the inside of the lid, 560µl of ethanol (96–100%) were added to each of the specimens, and mixed by pulse-vortexing for 15 sec. After mixing, the 1.5ml micro centrifuge tubes were briefly centrifuged to remove drops from inside the lid again. Only ethanol was used since other alcohols may result in reduced RNA yield and purity. The specimens were mixed thoroughly with the ethanol to yield a homogeneous solution.

Carefully 630µl of the solution from the previous step was applied to the QIAamp spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and centrifuge at 6000 x g (8000 rpm) for 1min. The QIAamp spin column was placed into a clean 2ml collection tube, and the tube containing the filtrate was discarded. Each spin column was closed in order to avoid cross-contamination during centrifugation. Centrifugation was performed at 6000 x g (8000 rpm) in order to limit micro centrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. The spin columns containing solutions that had not completely passed through the membrane were centrifuged again at a higher speed until all of the solution has passed through.

The QIAamp spin column, were carefully opened and the above protocol was repeated. The QIAamp spin column, were carefully opened again and 500µl of Buffer AW1 added. The caps were closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp spin column was placed in a clean 2ml collection tube (provided), and the tube containing the filtrate was discarded.

The QIAamp spin columns were carefully opened, and add 500µl of Buffer AW2. The caps were closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3min.

To eliminate any chance of possible Buffer AW2 carryover, the QIAamp spin columns were placed in a new 2ml collection tube (not provided), and the old collection tubes were discarded with the filtrate. These were centrifuged at full speed for 1min.

The QIAamp spin columns were placed in a clean 1.5ml micro centrifuge tube (not provided). The old collection tubes containing the filtrate were discarded.

The QIAamp spin columns were carefully opened and 60µl of Buffer AVE equilibrated to room temperature were added. The caps were closed, and incubated at room temperature for 1min and centrifuged at 6000 x g (8000 rpm) for 1min.

A single elution with 60µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp spin column. Performing a double elution using 2 x 40µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate. Viral RNA is stable for up to one year when stored at –20°C or –70°C.

3.5 Genotyping of Rotavirus Strains

Rotavirus RNA was extracted from 10% (wt/vol) stool suspensions in phosphate buffered saline using a NucliSens automated extractor (Biome´rieux), according to the protocol specified by the manufacturer.

3.6 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription-Polymerase Chain Reaction was used to analyze G and P genotypes on the basis of previously determined conditions. For G genotyping, the primers designed by Das *et al.,* (1994) were used, with the exception of the consensus 9Con1-L which was modified as

reported by Esona *et al.,* (2010) were a new set of primers were used for nested multiplex PCR; they included plus-sense primer 9con1-L (modified from9con1) and type-specific primers (9T1–1, 9T1–2, 9T-3P, 9T-4,and 9T-9B) specific for G1–G4 and G9. In addition, minus sense primer 9T1-Dg specific for G1 was also added to the G primer pool.

For P genotyping, the primers designed by Gentsch, *et al.,* (1992) and some additional primers designed by Esona *et al.,* (2010) were used. Generic primers con3/con2 were used to produce 876-bp fragments of VP8* of the VP4 gene. The 1T-1 to 5T-1 type-specific primers were used against the con3 primer in all nested multiplex PCR analyses. Two additional minus-sense P[8]-specific primers (Jrg237, nucleotides 339–356, 5' CGT GCA ATT GGG TCA TCT 3';1T1-V, nucleotides 339–356, 5' CGT GCA GCT AGG TCA TCT 3'), based on the sequence of genetically distinct P[8] strains, were included in the pool of P genotyping primers.

3.7 Sequencing of Untypeable Strains

Strains that could not be genotyped by these modified methods were identified by nucleotide sequencing. For sequence analysis, a 896-bp length of the VP7 gene and a 876-bp fragment of the VP8* region of VP4 gene of non typeable strains, were generated with the same consensus primers used for seminested genotyping RT-PCR, using a one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions. After denaturation of the double-stranded RNA, RT was carried out for 30 min at 50^{o}C, followed by 15min at 95^{o}C to inactivate the reverse transcriptase and activate the DNA polymerase.

3.8 Polymerase Chain Reaction (PCR)

The complementary DNA generated by reverse transcription was then subjected to 30 cycles of PCR at the following conditions: 30 s at 94^{o}C, 30 s at 50^{o}C, and 45 s at 72^{o}C, followed by a 7-min extension at 72^{o}C and a 4^{o}C cooling step.

Amplicons were run in a 1% agarose gel, and the desired band was cut out and purified with the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's manufacturer's protocol. Cycle sequencing of each amplicon was then conducted with same consensus primers described above, using the BigDye Terminator cycle sequencing kit (Applied Biosystems). Cycle sequencing products were purified using Centri-Sep spin columns (Princeton Separations), dried in a DNA SpeedVac (Savant Instruments), and reconstituted in 15 mL of Hi-Di formamide.

Automated separation and base calling of cycle sequencing products was performed using an ABI 3130 sequencer (Applied Biosystems). Sequences were aligned with the Sequencher program (version 4.8; Gene Codes) and subsequently compared with other sequences by using the University of Wisconsin Genetics Computer Group suite of sequencing software (version 11.1). All mixed infections identified during genotyping PCR were confirmed by employing VP4- and VP7-specific probes and methods. An additional G1-specific probe (G1-P3, nucleotides 152–175; 5'- ATA GAT CTT TGT TGA TTT ATG TAG-3') based on the sequence of a variant G1 strain A91A from GenBank (accession no. M93006) was used for confirmation.

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

CHAPTER FOUR

RESULTS

4.1 Enzyme-linked Immunosorbent Assay (ELISA)

Of the 285 stool specimen tested, group A rotavirus antigens was detected in 36.5% (104/285). The geographical distribution of the prevalence varied between the two hospitals in Kano metropolis. Murtala Muhammad Specialist Hospital had the highest prevalence of 44.4% while Hasiya Bayero Pediatric Hospital had 43.8%, Gwarzo General Hospital had the lowest prevalence rate of 21.4% while Gaya and Bichi General Hospital had 28.9% and 33.3% respectively. The prevalence rate in Wudil was similar to the general prevalence which is 36.4% (Table 4.1).

Pearson Chi Square analysis revealed that no statistically significant difference (χ^2=0.11, P>0.05, df =1) was observed between the prevalence of rotavirus infection in males and females during the study period indicating also that the infection cut across sex, although viral shedding was higher in males 37.2% than females. The distribution of rotavirus infection among different sex during the study period in Kano shows that out of 164 specimens collected from male children 37.2% were found to be infected with rotavirus while 121 specimens collected and analyzed from female children attending these hospitals 35.4% were infected with rotavirus during the study period (Table 4.2).

Table 4.1: Prevalence of rotavirus infection in diarrhoeic children 0 – 5 years old in Kano State, Nigeria

Hospital	Specimen examined	Positive (%)
Murtala Muhammad Specialist Hospital	72	32 (44.4)
Hasiya Bayero paediatric Hospital	64	28(43.8)
Gaya General Hospital	33	12(36.4)
Gwarzo General Hospital	38	11(28.9)
Wudil General Hospital	42	9(21.4)
Bichi General Hospital	36	12(33.3)
Total	285	104(36.5)

Table 4.2: Distribution of rotavirus infection in diarrhoeic children 0-5 years old by sex in Kano State, Nigeria

Sex	Specimen examined	Positive (%)
Male	164	61(37.2)
Female	121	43(35.5)
Total	285	104(36.5)

Although the number of specimens collected based on age (month) were not evenly distributed because of the availability of specimens at the time of sampling, prevalence rate was highest 50% in children aged between 41-50 months, followed by children 11-20 months with 40.4% prevalence rate, 21-30 months having 33.3% prevalence rate and the least prevalence rates were observed in children aged 31-40 months and children 0-10 months 15.8% and 21.4% respectively. However Chi square (χ^2) analysis of the prevalence of rotavirus infection among children aged 0-50 month revealed that no statistically significant difference (χ^2=8.259, P>0.05, df=4) was observed based on the age distribution of the rotavirus infection among the study population showing that the infection cut across different age groups of the children (Table 4.3).

The monthly pattern of distribution of rotavirus infection in this study showed that the prevalence was higher during the month of February to May. The percentage prevalence rises sharply in the Month of February with 42.2% prevalence rate which was almost twice that of the previous month January 23.5% then rises higher in March 66.7% and finally peaks in April 77.6%. The prevalence then starts to decline in the month of May 28.6%. The prevalence of 35% in the month of November was higher than the months of December and January which had prevalence rates of 22.7% and 23.5% respectively, although all were in the dry hamattan season. The lowest prevalence rate were recorded in the months of June and July with 13.5% and 12.2% respectively. Chi Square analysis revealed that there is significant association (χ^2=66.014, P<0.05, df=8) between rotavirus infection and monthly variation (Table 4.4). However Pearson Chi square analysis showed significant difference (χ^2=27.720, P<0.05, df=1) of rotavirus infection in the two different seasons. Rotavirus was detected throughout the study period with higher prevalence rate in the dry season months between November and April.

Table 4.3: Distribution of rotavirus cases in children 0-5 years old by age in Kano State, Nigeria

Age (months)	Specimen examined	Positive (%)
0-10	14	3(21.4)
11-20	183	74(40.4)
21-30	63	21(33.3)
31-40	19	3(15.8)
41-50	6	3(50.0)
51-60	0	0
Total	285	104(36.5)

Table 4.4: Monthly distribution of rotavirus cases in children 0-5 years by month in Kano State, Nigeria

Month	Specimen examined	Positive (%)
Nov	20	7(35)
Dec	22	5(22.7)
Jan	34	8(23.5)
Feb	33	14(42.2)
Mar	21	14(66.7)
Apr	49	38(77.6)
May	28	8(28.6)
Jun	37	5(13.5)
Jul	41	5(12.2)
Total	285	104(36.5)

4.2 VP4 and VP7 Genotyping (RT-PCR)

Three different rotavirus VP4 P-Genotypes were detected in this study. These include P[8], P[4], and P[6] however, P[10] and P[14] were not found to be in circulation in the study area during the period. Mixed infection were detected and consist only of dual P-types of P[8]+P[6]. Analysis of the rotavirus strains indicates that P[6] was the most common rotavirus infection in the study population with frequency of 50 followed by P[8] with a rate of 38.8%. P[4] had a frequency of only 5 and the mixed infection had a combination of P[8+6] with a frequency of 5. Three 2.9% of the entire specimens analyzed using RT-PCR for the VP4 P-Genotyping were found to be negative (Table 4.5).

Out of the 104 rotavirus positive specimens subjected to VP7 G-genotyping, one (1) specimen was lost during processing and 103 were successful. Six different VP7 P-genotypes were detected. The predominant genotype was G2 with a prevalence of 35.0%, followed by G1 with a prevalence of 29.1%. Other VP7 genotypes detected in circulation were G4 with a frequency of 6, G6 with frequency genotypes detected in circulation among the study population are G4 with a frequency of 6, then G6 with frequency of 7, G12 had a prevalence rate of 9 (8.7%). The lowest was G9 which had a prevalence of 1.9%. Similarly mixed infections were detected among the study populations the mixed infections consisted of dual and multiple G-types. The most predominant mixed infection was G2G4 which had a prevalence rate of 4.9% followed by G2G3 that had frequency of 2 and the lowest was G1G2 which had only 1 (1%) case. Out of the 103 ELISA positive specimens analyzed 2.9% where found to be Negative using RT-PCR. Non-typeable (NT) strains were also detected among the specimen analysed that accounted 1.9% of the entire specimen (Table 4.6).

Table 4.5: Distribution of VP4 P-genotypes of rotavirus in children 0-5 years old in Kano State, Nigeria

P – genotype	**Frequency**	**Percentage (%)**
P[8]	40	38.8
P[4]	5	4.9
P[6]	50	48.5
P[8+6]	5	4.9
Untypeable	3	2.9
Total	**103**	**100**

Table 4.6: Distribution of VP7 G-genotypes of rotavirus in children 0-5 years old in Kano State, Nigeria

G-genotype	**Frequency**	**Percentage (%)**
G1	30	29.1
G2	36	35.0
G4	6	5.8
G6	7	6.8
G9	2	1.9
G12	9	8.7
G2G3	2	1.9
G2G4	5	4.9
G1G2	1	1.0
NEG	3	2.9
NT	2	1.9
Total	**103**	**100**

Key: NT= Non-Typeable; NEG = Negative

One hundred and three ELISA positive specimens were characterized by their G and P-genotypes. The highest combination was found to be G2P[6] with 20 (19.4%) frequency of occurrence followed by G1P[8] having 17(16.5%). A single GNTP[8+6] (i.e. non-typeable G and P[8+6]) mixed combination of infection was also found among the study population. Completely non-typeable G and non-typeable P strains were also detected in 2.9% mixed infections i.e. GNTP(NT), indicating that the three (3) were negative for both VP4 and VP7 genotypes which may be an indication of new emerging strains or subtypes. Multiple combinations found include G2G4P[4], G2G4P[8+6], G1G2P[6], G2G3P[6] and G2G3P[8] which all had frequencies 1(1%) each, while G2G4P[6] had 2.9% as shown in (Table 4.7).

The distribution of the P-genotypes of rotavirus in children 0-60 months revealed that the predominant types P[8] and P[6] were distributed among all the age groups although age group 11-20 months had the highest number of P[8] and P[6]. P[4], P[8+6] and the negative specimens were found among children 11-20 months only (Table 4.8). Similarly the distribution of the G-genotypes of rotavirus in children 0-60 months in Kano followed similar pattern with the P-genotypes with age group 11-20 having the highest occurrence of the major G-genotypes of G2 and G1 respectively. The other G-genotypes three (3) non-typeable specimens were also found among the same age group of 11-20 months. The combinations of mixed infections in G2G4 and G1G2 had one case each in 21-30 months age group (Table 4.9).

Table 4.7: Distribution of P and G-genotypes of rotavirus in children 0-5 years old in Kano State, Nigeria

G and P combination	Frequency	Percentage (%)
G1P[8]	17	16.5
G2P[8]	11	10.7
G4P[8]	2	1.9
G4P[6]	3	2.9
G12P[8]	8	7.8
G12P[6]	1	1.0
G9P[6]	2	1.9
G6P[6]	6	5.8
G6P[8]	1	1.0
G1P[6]	12	11.7
G2P[6]	20	19.4
G2P[4]	4	3.9
G2G4P[4]	1	1.0
G2P[8+6]	1	1.0
G2G4P[8+6]	1	1.0
G4P[8+6]	1	1.0
GNTP[8+6]	1	1.0
G2G4P[6]	3	2.9
G1G2P[6]	1	1.0
G2G3P[6]	1	1.0
G1P[8+6]	1	1.0
GNTP[NT]	3	2.9
G2G3P[8]	1	1.0
GNTP[6]	1	1.0
Total	**103**	**100**

Table 4.8: Distribution of P-genotypes of rotavirus in children 0-5 years old by age group in Kano State, Nigeria

					Age group in months		
P – genotype	**0 -10**	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**Total**
P[8]	1	30	8	1	0	0	40
P[4]	0	5	0	0	0	0	5
P[6]	2	29	14	2	3	0	50
P[8+6]	0	5	0	0	0	0	5
Negative	0	3	0	0	0	0	3
Total	**3**	**72**	**22**	**3**	**3**	**0**	**103**

Table 4.9: Distribution of G-genotypes of Rotavirus in Children 0-5 Years Old by Age Group in Kano State, Nigeria

	Age group in months						
G-genotype	**0 -10**	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**Total**
G1	2	22	5	0	1	0	30
G2	0	21	11	2	2	0	36
G4	1	4	0	1	0	0	6
G6	0	6	1	0	0	0	7
G9	0	2	0	0	0	0	2
G12	0	7	2	0	0	0	9
G2G3	0	2	0	0	0	0	2
G2G4	0	4	1	0	0	0	5
G1G2	0	0	1	0	0	0	1
NEG	0	3	0	0	0	0	3
NT	0	1	1	0	0	0	2
Total	**3**	**72**	**22**	**3**	**3**	**0**	**103**

The distribution of G and P-genotypes combination among the age groups is presented in (Table 4.10). Age group 0-10 months had only three (3) cases which include G1P[8], G4P[6] and G1P[6], while age group 31-40 and 41-50 months had two (2) cases each. Age group 31-40 months had G2P[8] and G2P[6] while 41-50 months had G1P[6] and G2P[6]. However, age group 11-20 months and 21-30 months had more distribution of most of the combinations although 11-20 months had the highest distribution of the combinations.

Table 4.10: Distribution of G and P-genotypes of rotavirus in children 0-5 years old by age group in Kano State, Nigeria

	Age group in months						
G and P combination	**0 – 10**	**11 – 20**	**21 – 30**	**31 – 40**	**41 – 50**	**51-60**	**Total**
G1P[8]	1	13	3	0	0	0	17
G2P[8]	0	7	3	1	0	0	11
G4P[8]	0	2	0	0	0	0	2
G4P[6]	1	1	0	1	0	0	3
G12P[8]	0	6	2	0	0	0	8
G12P[6]	0	1	0	0	0	0	1
G9P[6]	0	2	0	0	0	0	2
G6P[6]	0	5	1	0	0	0	6
G6P[8]	0	1	0	0	0	0	1
G1P[6]	1	8	2	0	1	0	12
G2P[6]	0	9	8	1	2	0	20
G2P[4]	0	4	0	0	0	0	4
G2G4P[4]	0	1	0	0	0	0	1
G2P[8+6]	0	1	0	0	0	0	1
G2G4P[8+6]	0	1	0	0	0	0	1
G4P[8+6]	0	1	0	0	0	0	1
GNTP[8+6]	0	1	0	0	0	0	1
G2G4P[6]	0	2	1	0	0	0	3
G1G2P[6]	0	0	1	0	0	0	1
G2G3P[6]	0	1	0	0	0	0	1
G1P[8+6]	0	1	0	0	0	0	1
GNTP[NT]	0	3	0	0	0	0	3
G2G3P[8]	0	1	0	0	0	0	1
GNTP[6]	0	0	1	0	0	0	1
Total	3	72	22	3	3	0	103

CHAPTER FIVE

DICUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

This study shows that rotavirus is an important cause of significant infantile diarrhoea in children less than five years of age. The global death toll due to severe infection caused by rotavirus accounts for 610,000 childhood death a year (Parashar *et al*., 2006; Armah *et al*., 2010). Rotavirus causes more diarrhoea related deaths than any other viral or bacterial pathogens this is worse in developing countries where 80% of total rotavirus related mortality occurs due to inadequate treatment and management of the disease (Howard *et al*., 2000; Esona *et al*., 2003).

In this study 36.5% prevalence rate of rotavirus infection was recorded in Kano, Nigeria. This is generally in agreement with reports of (Ogunsanya *et al*., 1994; Alala *et al.,* 1998 and Audu *et al*; 2002), they reported 22.3%, 37.5% and 45% respectively in Southern Nigeria. However the overall prevalence in this study contradicts the reports of Aminu *et al*., (2008) who reported lower prevalence rate of 18% in Northwestern states of Nigeria. Similarly the rate is much higher than those reported in Ilorin 16.2%, Zaria 16.3%, Ife 13.8%, Maiduguri 11.9% and Jos 11% (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 prevalence of rotavirus was highest in the two hospitals located in Kano metropolis suggesting higher prevalence in the state capital although; slightly higher number of samples was collected in this area. This higher prevalence rate in Murtala Muhammad Specialist Hospital Kano and Hasiya Bayero Pediatric Hospital could be attributed to the more dense population of

the Kano metropolis than the other local government general hospitals, it can also be attributed to the fact that cases are normally referred from the local government general hospital to these hospitals because of reasons such as, qualified medical personel, availability of health care facilities.

The prevalence rate in the four hospitals Gwarzo, Wudil, Gaya and Bichi appeared to be averagely the same. This is due to similar factors obtained in these places such as sanitations, poverty, water supply and socio economic factors.

Pearson Chi Square analysis revealed that no statistically significant difference (χ^2=0.11, P>0.05, df =1) was observed between the prevalence of rotavirus infection in males and females during the study period indicating also that the infection cut across sex, prevalence was higher in males 37.2% than females. The distribution of rotavirus infection among different sex during the study period in Kano showed that out of 164 specimens collected from male children, 37.2% were found to be infected with rotavirus while 121 specimens collected and analyzed from female children attending these hospitals 35.4% were infected with rotavirus during the study period. The prevalence in this study was 36.5% this is in agreement with previous reports (Esona *et al.,* 2003; Aminu *et al.,* 2005). This implies that the infection affects both sexes equally.

In this study rotavirus was detected throughout the study period but it was observed that the prevalence rate was higher during the dry season than the wet season. This agrees with the report that the circulation of rotavirus might be lower during the wet season with slightly increased seasonal peaks during the cooler, dryer months from October to March (Paul and Erinle 1982; Gomwalk *et al*., 1993; Aminu *et al.,* 2010). In sub-saharan Africa, rotaviruses detection were six times more likely to peak during dry season, but the seasonability of virus detection is not clearly

defined in the dry season (Cunliffe *et al*., 1998). However this may be attributed to geographical and environmental factors, low relative humidity, water supply shortages which can affect sanitation.

In this study, rotavirus was detected in all age groups 0-60 months, although higher in some age groups, there was no statistically significant difference $p>0.05$ observed among age groups. This contradicts the reports of (Fagbami *et al.,* 1985; Gomwalk 1990; Audu *et al*., 2002 and Aminu *et al*., 2005) who reported that the detection of rotavirus infection is more often in children under the age of 2 years. However it agrees with some studies that reported rotavirus been detected in older children (Marie-Cardine *et al*., 2002; Esona *et al*., 2003 and Van Man *et al*., 2005).

VP4 P-genotypes

This study has shown that the three out of five most important epidemiologically rotavirus strains P[8], P[6] and P[4] are circulating. However, P[10] and P[14] were not detected in the study population. This shows that P[8], P[6] and P[4] are the most common P-types circulating in the study population. P[8+6] mixed infection was also detected among the study population. P[6] was the most abundant P-type with almost 48.5% in circulation among the population studied. This also agrees with previously studied cases in Nigeria (Adah *et al.,* 1997a, 2001; Audu *et al*., 2002a,b,). Similarly (Armah *et al.,* 2001) also reported that P[6] was the highest type in circulation in Ghana. Genotype P[6] was previously thought to be neonatal strains hence associated only with asymptomatic infection (Gorziglia *et al*., 1988b; Santos *et al*., 1994).

Genotype P[8] was the second most frequently detected P-type in the study area followed by P[4]. As earlier stated P[8+6] mixed infections were detected in the study population with 4.9% prevalence showing cross contamination of strains due to different factors such as overcrowding

and sanitation. Similarly some South African studies showed that genotypes P[6] and P[8] were the most abundant strain found in circulation.

P[4] genotype was detected in 11% each 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). However, 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). It has been reported in Nigeria (Audu *et al.,*2002b) and Botswana (Kasule *et al.,* 2003; Aminu *et al*., 2005) that lower P[4] types were found 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 high prevalence of 71% (Argurlles *et al*., 2000). It is important to note that P[10] and P[14] were not detected completely in the study area. Although previous study in Nigeria and elsewhere reported the circulation of these P-types (Aminu *et al*., 2010; Esona *et al*., 2010).

The present study has shown that rotavirus G1, G2 and G12 are the most frequently occurring G-types in circulation in Kano. This was not unexpected because G1 is the most prevalent VP7 type worldwide (Nakagomi *et al*., 1990; Steele *et al*., 1992; Gentsch *et al.,* 1996). However in the present study G1 was the second most abundant VP7 detected. G2 appeared to be the highest detected and G12 strains were the third.

Aminu *et al.,* (2010) reported that 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

North central region of the country, occurring with a prevalence of 0.86% (Steele *et al.,*2002) but was absent in the Southern part of the country (Audu *et al*., 2002b). In a previous study conducted in Zaria, Kaduna State, Nigeria, G2 was detected (Pennap *et al.,* 2000). This report highlights the varying diversities in the predominance of genotyping distribution in geographical locations particularly in African countries.

In the present study, genotype G9 was the least common genotype circulating at low level and was also reported by (Adah *et al*., 2001) in Nigeria, where G9 was first detected as mixed infection in a single case of diarrhoea. In this study, the G9 strains were detected in children within 11 – 20 months old and this differs from the results of a previous study in Nigeria where G9 was detected for the first time and were reported as the most prevalent VP7 genotype detected in slightly older children (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 age group with higher prevalence in younger children (Armah *et al*., 2003).

A worldwide review of rotavirus studies has confirmed genotype G9 as a globally important rotavirus strain (Gentsch *et al.*, 2005). Other VP7 genotype detected in this study include: G6 6.8%, G12 8.7%, G2G3 1.9%, G2G4 4.9% and G1G2 1%. A high rate of mixed reactivity of rotavirus strains is a very common finding in Northern Nigeria. The strains with mixed reactivity detected in this study were mostly of VP7 genotype: G2P[6], G1P[8] ,G1P[6], G2P[8], G12P[8], G6P[6], G2P[4], G4P[6], G2G4P[6], GNTP[NT], G4P[8], G9P[6], G12P[6], G6P[8], G2G4P[4], G2P[8+6], G2G4P[8+6], G4P[8+6], GNTP[8+6], G1G2P[6], G2G3P[6], G1P[8+6], G2G3P[8], and GNTP[6], in order of decreasing prevalence. In this study, the "Mosaic" rotavirus with G2G4 genotypes predominated 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 study by Aminu *et al*., (2010) also reported the predominance of G1G8 genotypes, Adah *et al*., (2001) also in Nigeria however reported the predominance of the "Mosaic" rotavirus with G1G2. It is important to note that this study reported mixed infection with three genotypes of rotavirus strains. This result however reflects the predominance of G1, G2, G4 and G6 during the study period.

The occurrence of three non-typeable strains in this study is a cause for alarm in view of efforts towards an effective vaccine. This is an indication of emerging strain and can pose serious health threats and affects vaccine production. These strains would require proper sequencing analysis and further studies to ascertain their genotypic status.

5.2 Conclusion

The findings in this study showed rotavirus as an important cause of infantile diarrhoea in the study population as observed in most parts of Nigeria and other developing countries as well as in developed countries. The specimens were analyzed for group A antigen using ELISA and RT-PCR. The prevalence of rotavirus in children in this study was 36.5% (104/285). Murtala Muhammad Specialist Hospital had 44.4% while Gwarzo General Hospital had the lowest prevalence rate of 21.4%. The virus was detected more in males 37.2% (61/164) than in females 35.4% (43/121). However, there is no statistically significant difference (χ^2=0.11, P>0.05, df =1) observed between the prevalence in males and females. Rotavirus was detected with the highest frequency among children in the age 41-50 months with 50% prevalence. The least prevalence was in age 31-40 months having 15.8%. The prevalence was highest in April with 77.6% and lowest in July 12.2%. Pearson Chi square analysis showed significant difference (χ^2=27.720, P<0.05, df=1) of rotavirus infection in the two different seasons. The distribution of P[6] 48.5% and P[8] 38.8% is more among VP4 genotypes occurring most in age group 11-20 months follows P[4] 4.9% and mixed infection of P[8+6] 4.9%. Similarly G1 29.1% and G2 35% are more prevalent in the VP7 genotypes.

The percentage of P and G-genotypes showed that G2P[6] 19.4% as the most prevalent in children 0-5 years old. G-genotypes distribution in children 0-5 years old showed that G1 has the highest prevalence 22% in age group 11-20 months. In the distribution of G and P-genotyping according to age groups the G and P-genotypes co infections occurs in G1P[8] with 13 in 11-20 months old children.

5.3 Recommendation

As a result of the findings in this study conducted in Kano State, Northwestern Nigeria, The following recommendations are made:

1. Adequate supply of portable water and sanitation facilities should be provided.
2. There is need for public enlightenment campaign on adequate sanitation, hygiene and other preventive measures.
3. Exclusive breast feeding should be encouraged in 0-6 month's old children to avoid early exposure to rotaviruses contaminated water and other foods.
4. The study area should be a target for trial of the newly introduced rotavirus vaccine by the pharmaceutical industries.
5. There is need for continuous surveillance as the occurrence of unusual strains may determine the necessity for an alternate vaccine.

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APPENDICES

DATA COLLECTION QUESTIONNAIRE AND RESULTS OF STATISTICAL ANALYSIS

DATA COLLECTION QUESTIONNAIRE

BACKGROUND INFORMATION **Questionnaire No: _____**

Hospital [ ] Date_____

Male [ ] Female [ ]

Age (months) 0-10[ ] 11-20[ ] 21-30[ ] 31-40[ ] 41-50[ ] 51-60[ ]

Is this child breast feeding? Yes [ ] No [ ]

If not, at what age did he/she stop breast feeding?

Age (months) 0-10[ ] 11-20[ ] 21 -30[ ] 31-40[ ] 41-50[ ] 51-60[ ]

Has the child started any other food outside breast milk? Yes [ ] No [ ]

If yes, at what age?

Age (months) 0-10[ ] 11-20[ ] 21-30[ ] 31-40[ ] 41-50[ ] 51-60[ ]

Presence of pet in the household: Cat [ ] Dog [ ] others [ ]

Source of drinking water: well [ ] tap [ ] pond [ ] stream [ ] bore-hole [ ] others [ ]

Main type of toilet: water system [ ] Pit latrine [ ] in the field [ ]

On the average, how many liquid stools per day has the child had? <3 [ ] 3 [ ] 4 [ ] 5 [ ] >5[ ]

Duration of diarrhoea (days)? 1 [ ] 2 [ ] 3 [ ] 4 [ ] >4 [ ]

Any blood in stool? Yes [ ] No [ ]

Was there a change in colour of the child's stool since he/she started diarrhoea? Yes [ ] No [ ]

Has there been vomiting? Yes [ ] No [ ]

How frequent does the child vomited per day? 1 [ ] 2 [ ] 3 [ ] 4 [ ] >4 [ ]

Since when did vomiting start? (days) 1[ ] 2 [ ] 3 [ ] 4 [ ]

Has the child received any treatment? Yes [ ] No [ ]

If yes which type? Traditional [ ] Conventional [ ]

STOOL SAMPLE RESULTS

Rotavirus Positive [ ]
Negative [ ]

RESULTS OF STATISTICAL ANALYSIS

Frequency Table

OPTICAL_DENSITY

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	NEGATIVE	181	63.5	63.5	63.5
	POSITIVE	104	36.5	36.5	100.0
	Total	285	100.0	100.0	

AGE_IN_MONTHS_CODED

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1-10MONTHS	13	4.6	4.6	4.6
	11-20MONTHS	174	61.1	61.1	65.6
	21-30MONTHS	68	23.9	23.9	89.5
	31-40MONTHS	21	7.4	7.4	96.8
	41-50MONTHS	9	3.2	3.2	100.0
	Total	285	100.0	100.0	

Sex

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	MALE	166	58.2	58.2	58.2
	FEMALE	119	41.8	41.8	100.0
	Total	285	100.0	100.0	

MONTH

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	JAN	33	11.6	11.6	11.6
	FEB	33	11.6	11.6	23.2
	MAR	21	7.4	7.4	30.5
	APR	50	17.5	17.5	48.1
	MAY	28	9.8	9.8	57.9
	JUN	37	13.0	13.0	70.9
	JUL	41	14.4	14.4	85.3
	NOV	20	7.0	7.0	92.3
	DEC	22	7.7	7.7	100.0
	Total	285	100.0	100.0	

SEASON

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	DRY	179	62.8	62.8	62.8
	RAINY	106	37.2	37.2	100.0
	Total	285	100.0	100.0	

AGE_IN_MONTHS_CODED * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
AGE_IN_MONTHS_CODED	1-10MONTHS	Count	10	3	13
		Expected Count	8.3	4.7	13.0
	11-20MONTHS	Count	101	73	174
		Expected Count	110.5	63.5	174.0
	21-30MONTHS	Count	46	22	68
		Expected Count	43.2	24.8	68.0
	31-40MONTHS	Count	18	3	21
		Expected Count	13.3	7.7	21.0
	41-50MONTHS	Count	6	3	9
		Expected Count	5.7	3.3	9.0
Total		Count	181	104	285
		Expected Count	181.0	104.0	285.0

85

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	8.259[a]	4	.083	.080		
Likelihood Ratio	9.000	4	.061	.075		
Fisher's Exact Test	8.236			.078		
Linear-by-Linear Association	2.833[b]	1	.092	.100	.053	.015
N of Valid Cases	285					

a. 2 cells (20.0%) have expected count less than 5. The minimum expected count is 3.28.

b. The standardized statistic is -1.683.

Sex * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			85 NEGATIVE	POSITIVE	Total
Sex	MALE	Count	105	61	166
		Expected Count	105.4	60.6	166.0
	FEMALE	Count	76	43	119
		Expected Count	75.6	43.4	119.0
Total		Count	181	104	285
		Expected Count	181.0	104.0	285.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	.011[b]	1	.916	1.000	.508	
Continuity Correction[a]	.000	1	1.000			
Likelihood Ratio	.011	1	.916	1.000	.508	
Fisher's Exact Test				1.000	.508	
Linear-by-Linear Association	.011[c]	1	.916	1.000	.508	.099
N of Valid Cases	285					

a. Computed only for a 2x2 table

b. 0 cells (.0%) have expected count less than 5. The minimum expected count is 43.42.

c. The standardized statistic is -.106.

MONTH * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
MONTH	JAN	Count	25	8	33
		Expected Count	21.0	12.0	33.0
	FEB	Count	19	14	33
		Expected Count	21.0	12.0	33.0
	MAR	Count	7	14	21
		Expected Count	13.3	7.7	21.0
	APR	Count	12	38	50
		Expected Count	31.8	18.2	50.0
	MAY	Count	20	8	28
		Expected Count	17.8	10.2	28.0
	JUN	Count	32	5	37
		Expected Count	23.5	13.5	37.0
	JUL	Count	36	5	41
		Expected Count	26.0	15.0	41.0
	NOV	Count	13	7	20
		Expected Count	12.7	7.3	20.0
	DEC	Count	17	5	22
		Expected Count	14.0	8.0	22.0
Total		Count	181	104	285
		Expected Count	181.0	104.0	285.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	66.014[a]	8	.000	.[b]		
Likelihood Ratio	67.951	8	.000	.[b]		
Fisher's Exact Test	.[b]			.[b]		
Linear-by-Linear Association	7.908[c]	1	.005	.005	.002	.000
N of Valid Cases	285					

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.30.

b. Cannot be computed because there is insufficient memory.

c. The standardized statistic is -2.812.

SEASON * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
SEASON	DRY	Count	93	86	179
		Expected Count	113.7	65.3	179.0
	RAINY	Count	88	18	106
		Expected Count	67.3	38.7	106.0
Total		Count	181	104	285
		Expected Count	181.0	104.0	285.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	27.720[b]	1	.000	.000	.000	
Continuity Correction[a]	26.396	1	.000			
Likelihood Ratio	29.572	1	.000	.000	.000	
Fisher's Exact Test				.000	.000	
Linear-by-Linear Association	27.623[c]	1	.000	.000	.000	.000
N of Valid Cases	285					

a. Computed only for a 2x2 table

b. 0 cells (.0%) have expected count less than 5. The minimum expected count is 38.68.

c. The standardized statistic is -5.256.

AGE_IN_MONTHS_CODED * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
AGE_IN_MONTHS_CODED	1-10MONTHS	Count	10	3	13
		% of Total	3.5%	1.1%	4.6%
	11-20MONTHS	Count	101	73	174
		% of Total	35.4%	25.6%	61.1%
	21-30MONTHS	Count	46	22	68
		% of Total	16.1%	7.7%	23.9%
	31-40MONTHS	Count	18	3	21
		% of Total	6.3%	1.1%	7.4%
	41-50MONTHS	Count	6	3	9
		% of Total	2.1%	1.1%	3.2%
Total		Count	181	104	285
		% of Total	63.5%	36.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.259[a]	4	.083
Likelihood Ratio	9.000	4	.061
Linear-by-Linear Association	2.833	1	.092
N of Valid Cases	285		

a. 2 cells (20.0%) have expected count less than 5. The minimum expected count is 3.28.

Sex * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
Sex	MALE	Count	105	61	166
		% of Total	36.8%	21.4%	58.2%
	FEMALE	Count	76	43	119
		% of Total	26.7%	15.1%	41.8%
Total		Count	181	104	285
		% of Total	63.5%	36.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.011[b]	1	.916		
Continuity Correction[a]	.000	1	1.000		
Likelihood Ratio	.011	1	.916		
Fisher's Exact Test				1.000	.508
Linear-by-Linear Association	.011	1	.916		
N of Valid Cases	285				

a. Computed only for a 2x2 table

b. 0 cells (.0%) have expected count less than 5. The minimum expected count is 43.42.

MONTH * OPTICAL_DENSITY

Crosstab

			OPTICAL_DENSITY		
			NEGATIVE	POSITIVE	Total
MONTH	JAN	Count	25	8	33
		% of Total	8.8%	2.8%	11.6%
	FEB	Count	19	14	33
		% of Total	6.7%	4.9%	11.6%
	MAR	Count	7	14	21
		% of Total	2.5%	4.9%	7.4%
	APR	Count	12	38	50
		% of Total	4.2%	13.3%	17.5%
	MAY	Count	20	8	28
		% of Total	7.0%	2.8%	9.8%
	JUN	Count	32	5	37
		% of Total	11.2%	1.8%	13.0%
	JUL	Count	36	5	41
		% of Total	12.6%	1.8%	14.4%
	NOV	Count	13	7	20
		% of Total	4.6%	2.5%	7.0%
	DEC	Count	17	5	22
		% of Total	6.0%	1.8%	7.7%
Total		Count	181	104	285
		% of Total	63.5%	36.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	66.014[a]	8	.000
Likelihood Ratio	67.951	8	.000
Linear-by-Linear Association	7.908	1	.005
N of Valid Cases	285		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.30.

SEASON * OPTICAL_DENSITY

Crosstab

			OPTICAL DENSITY		
			NEGATIVE	POSITIVE	Total
SEASON	DRY	Count	93	86	179
		% of Total	32.6%	30.2%	62.8%
	RAINY	Count	88	18	106
		% of Total	30.9%	6.3%	37.2%
Total		Count	181	104	285
		% of Total	63.5%	36.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	27.720[b]	1	.000		
Continuity Correction[a]	26.396	1	.000		
Likelihood Ratio	29.572	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	27.623	1	.000		
N of Valid Cases	285				

a. Computed only for a 2x2 table

b. 0 cells (.0%) have expected count less than 5. The minimum expected count is 38.68.