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Molecular characterization of Human Rotavirus Strains circulating among Children less than 5 years attended with Diarrhea to Mohammed Alamin Hamid Pediatric Hospital, Khartoum.

A thesis Submitted in Fulfillment for the Requirements of PhD Degree in Microbiology

By

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الآية

بسم العلي القدير امين

لان رحمتك افضل من الحياة شفتاي تسبحانك

مزمور 3:63

Dedication

I dedicate this work to my parents
Who taught me the value of education and hard work

And to my sisters Queen, Suhair and Lana
Who make my life shining

TO MY WIFE: HIBA SAEED MUSA Who gave me love and respect

TO MY SON FADY who bring happiness to my life

To the soul of my cousin; JEMO MUSA

And to everyone who smile on my face and help me.

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Abstract

Rotavirus is the leading cause of severe acute diarrhea in children throughout both developed and developing countries. Every year, rotavirus is estimated to cause 500,000 deaths worldwide among children aged less than 5 years, with 80% of these deaths occurring in developing nations.

The aim of the study is to characterize human rotavirus strains circulating among children below 5 years of age presented with Diarrhea to Mohammed Alamin Hamid Pediatric Hospital using molecular methods.

Descriptive cross-sectional analytical hospital based study was conducted and a total of 150 fecal specimens from children less than 5 years were collected and analyzed. The samples were screened for rotavirus using antigen based enzyme immune-sorbent assay (ELISA), genotyping was done by RT-PCR to determine rotavirus genotypes using specific primer sets targeting VP7 gene.

The study revealed that the highest percentage of rotavirus infection was among age of 13-24 months. This study emphasizes that the rate of Rotavirus infection in males is nearly equal to females. The study found that the prevalence of rotavirus attributed diarrhea is 28%, and G2 genotype was the predominant type that circulates among vaccinated children, while G1 genotype is the predominant type that circulates among nonvaccinated children.

The study concluded that the reduction of G1 genotype among the immunized children reflecting the effectiveness of immunization and the existence of uncommon rotavirus strains such as G3 and G9 will provides baseline data for future vaccine studies.

المستخلص

فيروس الروتا هو السبب الرئيسي الأول للأسهال الشديد الحاد لدي الأطفال الموجودين في الدول المتقدمة والدول النامية معا. سنويا يتسبب فيروس الروتا في 500000 حالة وفاة في الأطفال الذين أعمارهم أقل من 5 سنوات، وتحدث 80% من هذه الوفيات في الأمم النامية.

الهدف من الدراسة هو توصيف سلالات فيروس الروتا البشري الجائلة في الأطفال الذين أعمارهم أقل من خمسة أعوام بمستشفى محمد الأمين حامد للأطفال المصابون بالاسهال باستخدام الطرق الجينية. أجريت هذه الدراسة المقطعية التحليلة بجمع 150 عينة براز من أطفال أعمارهم أقل من خمسة سنوات في مستشفى محمد الأمين حامد للأطفال وتمت تحليلها. تم فحص العينات باستخدام تقنية ألأليزا (ELISA) وتم التنميط الجيني بواسطة RT-PCR لتحديد مورثات فيروس الروتا باستخدام مجموعات (primers) النمط الجيني المحدد تستهدف الجين VP7.

أوضحت الدراسة أن النسبة الأكبر من أصابات فيروس الروتا تكون في المدى العمري 13-24 شهرا. كما بينت الدراسة معدل الأصابة بالروتا في الأطفال الذكور تقريبا تساوي معدل الأصابة في الأطفال الأناث. كما أظهرت الدراسة أن نسبة الأسهالات التي يسببها فيروس الروتا تقريبا 28% وأن السلالة الجينة G2 هي السلالة السائدة بين الأطفال المحصنين ضد فيروس الروتا، بينما السلالة الجينية G1 هي السائدة بين الأطفال غير المحصنين ضد فيروس الروتا.

وخلصت هذه الدراسة الى أن الانخفاض في معدل سلالة G1 في الأطفال المحصنين يعكس مدى فعالية التحصين وظهور بعض السلالات الغير معتادة مثل سلالة G3 و G9 سوف يوفر قاعدة بيانات للدراسات المستقبلية للتحصين.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
aa	amino acid
AAH	Amphipathic -helix
AAP	American Academy of Pediatrics
ADRV	Adult Diarrheal Rotaviruses
ATPase	Adenosine Triphosphatase
BSS	Bismuth subsalicylate
cDNA	Complementary DNA
CF	Complement fixation test
DEPC	Diethyleprocarbonate
dsRNA	double-stranded RNA
DLPs	doublelayered particles
dNTPs	deoxynucleoside-5´ triphosphate
EM	Electron microscopy
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	enzyme-linked immunospot
ER	Endothelium reticulum
HA	hemagglutinin
HID	histidine triad
hpi	hours postinfection
HRV	Human rotavirus
HSP70	heat shock protein 70
IEM	Immuno-Electron Microscopy
IgA	Immunglobulin A
IgG	Immunoglbulin G
IF	Immunofluorescence

KDa	Kilo Dalton
MAbs	monoclonal antibodies
mRNA	messenger RNA
NA	Neuraminidase
NSP	non structural protein
ORS	oral rehydration salts
ssRNA	single -stranded RNA
VP	Viral protein
SLP	Single-layer particle
TLPs	Triple-Layered Particles
RNA	Ribonucleic Acid
RR	Relative Risk
NTPase	Nuraminidase Triphosphatase
PABP	poly(A) binding protein
PCR	Polymerase chain reaction
PLC	phospholipase C
RPHA	reverse passive hemagglutination assay
RT-PCR	Reverse transcriptase-PCR
RV	Rotavirus
SA	sialic acid
SiRNA	Small interfering RNA
SCID	Severe combined immunodeficiency
SPSS	Statistical package for social sciences
TMB	tetramethylbenzidine
Ts	Temperature-Sensitive
UK	United Kingdom
USA	United States of America

UV	Ultraviolet
VLPs	Virus-like particles
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

CHAPTER ONE

INTRODUCTION

1.1. Introduction

Diarrhea remains one of the leading causes of childhood mortality worldwide particularly in developing countries, causing approximately *(1.87)* million deaths each year (Valentine *et al*., 2012).

There is a universal agreement that rotaviruses are the single most important etiologic agents of severe diarrhea illnesses of infants and young children worldwide. Although repeated rotavirus infections occur throughout an individual's lifetime, symptomatic infections occur most commonly during the first two years of life (Yasutaka and Albert., 2000).

Rotavirus causes about 30-50 % of diarrheal diseases in young children and the prevalence of severe rotavirus disease has remained high despite improvements in sanitation. An explanation for this may be that improved hygienic practices and oral rehydration therapy have resulted in a greater decline of hospitalization from bacterial and parasitic diarrheal diseases than hospitalization from rotavirus disease. Vaccination is considered the most effective public health strategy to reduce rotavirus disease burden (Christabel *et al*., 2012).

Worldwide, rotaviruses account for more than (125 million) cases of severe diarrhea in infants and children under the age of (5 years). The highest incidence in children aged range of (6-24 month) and causes approximately (527,000) deaths per year world-wide, with more than (85%) of those deaths occurring in developing countries in Africa and Asia (Carla *et al*., 2007).

occurring in developing countries in Africa and Asia (Carla *et al*., 2007).

World health organization (WHO) sponsored review of rotavirus studies found that (20–70%) of all hospitalizations and (20%) of deaths from diarrhea were

attributable to rotavirus. Rota virus infection may produce a spectrum of illnesses ranging from sub clinical infection to severe and on occasion fatal Dehydrating illness. Typically the clinical presentation is of (3 days) vomiting and (5 days) of watery diarrhea with moderate fever following a (2 days) incubation period (Jeevan *et al*., 2012).

Two effective rotavirus vaccines, a single strain attenuated human rotavirus vaccine and multi-strain bovine-human reasserting vaccines are now available. WHO recommends the inclusion of rotavirus vaccines in all national routine immunization schedules. In countries where diarrheal deaths account for (≥ 10 %) of mortality among children (< 5 years) of age, the introduction of rotavirus vaccine is strongly recommended. The Efficacy of these vaccines has ranged from (80 to 98%) in industrialized countries, including Latin America, and (39 to 77%) in developing countries, such as Africa and Asia (Jeevan, *et al*., 2012).

Rotaviruses belong to the *Reoviridae* family, genus *Rotavirus* and they carry three important antigenic specificities: group, subgroup, and serotype. Based on group specificity which is conferred predominantly by (VP6 glycoprotein), rotaviruses are divided into (7) groups (A, B, C, D, E, F, and G). Human rotavirus HRV associated infections are predominantly caused by group A, and less commonly by group (B or C), The group A rotavirus genome consists of (11 segments) of double-stranded RNA (dsRNA) encoding (6) structural viral proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and (6) nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, NSP6) enclosed in a (3) layer protein capsid, consisting of a protein core, an inner protein capsid, and an outer protein capsid (Luana *et al*., 2010).

Subgroup specificity, which is also determined by VP6, has been used for characterizing the antigenic properties of various Rotavirus strains in epidemiologic surveys. Most HRVs belong to either subgroup I or subgroup II. (Yasutaka and Albert., 2000).

Rotavirus can also be classified into several serotypes using neutralization assays with panels of antisera and genotyped on the basis of two outer capsid proteins, the (glycoprotein VP7) is defining G genotypes and the (spike protein VP4) is defining P genotypes. These two structural proteins elicit neutralizing antibodies in the host and encoded by (VP7 and VP4) genes of rotavirus, respectively (Luana *et al*., 2010).

Currently, (23 G genotypes) and (32 P genotypes) have been described, based on nucleotide sequence variation; however, few genotypes are known to cause infection in humans (Isidore *et al*., 2000).

The incidence and distribution of (G and P genotypes) that cause disease in humans may vary by geographical location and by year. Epidemiological studies worldwide have documented the major human G types are (G1, G2, G3, G4, and G9), which combined with the P types (P8, P4, and P6), account for more than (80%) of rotavirus associated gastroenteritis episodes worldwide. Knowledge about the diversity and distribution of (G and P types) circulating in the population is critical for the formulation of an adequate vaccine as well as for the evaluation of protection after vaccination (Anita *et al*., 2010).

(Rotavirus G1) is the most prevalent genotype, and it has been detected in frequencies ranging from (36 to 74%) in different regions of the world. (G1P8)[strains represent approximately (65%) of rotavirus types identified globally (Luana *et al*., 2010).

1.2. Justification

Diarrhea remains the second leading cause of death due to infections among children under five years of age worldwide, The World Health Organization estimates that globally (527,000) deaths per year worldwide among children as a result of rotavirus diarrhea although the incidence of infection among children in developed and developing countries is similar, outcomes vary widely (Ornwalan *et al*., 2012).

About (85 %) of global children deaths are due to diarrhea caused by rotavirus in developing countries as Africa and Asia. The distribution of rotavirus strains that cause diarrhea in children may vary by geographical location and by year. In Sudan few studies were conducted to study rotavirus infection and to characterize the circulating strains in the country (kafi *et al*., 2013).

This study is conducted to molecularly characterize the major rotavirus strains circulating among children attended with Diarrhea to Mohammed Alamin Hamid Pediatric Hospital in Khartoum state.

1.3. Study objectives

1.3.1. General objective:

To characterize human rotavirus strains circulating among children attended with diarrhea to Mohammed Alamin Hamid Pediatric Hospital using molecular methods.

1.3.2. Specific objectives:

1. To determine the frequency of rotavirus associated diarrhea among children who attended Mohammed Alamin Hamid Pediatric Hospital.
2. To detect the major rotavirus G genotype circulating among children attended with Diarrhea to Mohammed Alamin Hamid Pediatric Hospital.
3. To detect and compare the most prevalent G-genotype among vaccinated and unvaccinated children with Diarrhea who attended Mohammed Alamin Hamid Pediatric Hospital.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1. Rotavirus classification:

Rotaviruses comprise the genus Rotavirus within the family Reoviridae, The family *Reoviridae* currently consists of fifteen genera: *Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus, Mycoreovirus, Dinovernavirus، Idnoreovirus, Seadornavirus، Cardoreovirus، Mimoreovirus and Oryzavirus* (Ergin *et al.*, 2013).

Orthoreoviruses and *rotaviruses* affect mainly vertebrates; *orbiviruses* and coltiviruses are found in vertebrates and insects, while the remaining genera contain viruses of insects and plants. Rotaviruses are the only important human pathogens in the family, and also cause diarrhea in livestock (Ramos and Sabin., 1954).

2.2. Virion Structure:

Rotavirus is a medium-sized (70 nm) non enveloped virus. The mature particle consists of a triple shelled capsid consisting of the outer, intermediate, and inner layers. The outer capsid contains two proteins (VP4 and VP7), whereas the intermediate layer is formed by VP6, and the inner by VP2 which encloses two other proteins (VP1 and VP3), as well as the viral genome consisting of (11 segments of double-stranded RNA), the latter encoding six structural and (6) nonstructural. Because of the segmented nature of the rotavirus genome, genetic reassortment occurs at high frequency during mixed infection (Yasutaka and Albert., 2000).

The naming of the rotavirus structural proteins is based on their molecular weights, with VP1, the largest at 125 kDa, VP2 (95 kDa), VP3 (88 kDa), VP4 (85 kDa), VP6 (45 kDa), and VP7 (34 kDa) (Bowman *et al*., 2000).

The outer layer of the TLP is composed of two structural proteins: (VP7 and VP4). VP7, the major constituent of the outer layer, is a glycoprotein in most rotavirus strains although glycosylation is not required for capsid assembly. Seven hundred eighty copies of VP7 are grouped as (260 trimers) at all the icosahedral and local (3-fold) axes of a (T=13 icosahedral lattice) surrounding (132 channels). The outer layer is decorated by (60 spikes), each of which is formed by a dimer of VP4. Thus each rotavirus particle has (120 copies) of VP4 (Mathieu *et al*., 2001).

The intermediate layer is formed by the (VP6 protein), and is in direct contact with the VP7 later. Particles carrying VP6 on the outside are called double layered particles (DLPs). The (VP6 layer) maintains the same icosahedral symmetry as the (VP7 layer) with (780 copies) of VP6 arranged as (260 trimers) on a (T=13 icosahedral lattice). These trimers are located right below the (VP7 trimers) such that the channels in the (VP7 and VP6 layers) are in register (Nejmeddine *et al*., 2000).

The DLP is the transcriptionally competent form of the virus during the replication cycle. VP6 is the major protein of the rotavirus particle by weight. It plays a key role in the overall organization of the rotavirus architecture by interacting with the outer layer proteins, (VP7 and VP4), and the inner most (layer protein VP2). Thus, it may integrate two principal functions of the virus: cell entry (outer layer) and endogenous transcription (inner layer) (Chen *et al*., 2010).

VP2 layer) is the innermost protein layer of the rotavirus structure. The (particle structure at this level is referred to as the single-layer particle (SLP). The SLP houses the dsRNA genome within a protein layer composed of (120 copies) of VP2 arranged in an unusual (T=1 icosahedral lattice) with two molecules in the

icosahedral asymmetric unit. All the structurally characterized members of the *Reoviridae* and of other dsRNA viruses exhibit this unique organization of the core protein (Jayaram *et al*., 2002).

(VP1 and VP3) are enclosed within (VP2 layer) and they act as RNA-dependent RNA polymerase and Guanylyl and methyl transferase capping-enzyme molecules respectively (Brian *et al*., 2010).

The morphologic appearance of rotavirus particles is distinctive, and three types of particles can be observed by electron microscopy (EM). The complete particles resemble a wheel with short spokes and a well-defined, smooth outer rim. The name rotavirus (from the Latin rota, meaning wheel) was coined based on this morphology. The complete infectious particles (virions) are also called TLPs. DLPs lacking the outer shell are described as rough particles because their periphery shows projecting trimeric subunits of the inner capsid. Single-layered particles (SLPs or cores) are seen infrequently; they usually lack genomic RNA and are aggregated (Ball *et al*., 2005).

The outer capsid is composed of structural proteins which surround a nucleocapsid core that includes enzymes for RNA synthesis and (10 reo) and (11 rota) different double stranded genomic RNA segments. Interestingly, rotaviruses resemble enveloped viruses in that: they have glycoproteins that act as the viral attachment proteins, they acquire but then lose an envelope during assembly and appear to have a fusion protein activity that promotes direct penetration of the target cell membrane (Pattrick *et al*., 2002).

The outer capsid is composed of two structural proteins, (VP4 and VP7), which define the P (protease cleaved protein) and G (glycoprotein) genotypes, respectively (Luana *et al*., 2010).

2.3. Structural proteins:

VP1 is encoded by genome segment 1, and it is one of three proteins (VP1, VP2, and VP3) that make up the rotavirus core particles. VP1 act as an RNA polymerase enzyme which produces mRNA transcripts for the synthesis of viral proteins and produces copies of the rotavirus genome RNA segments for newly produced virus particles (Marry and Jean., 1989).

VP2 is encoded by genome segment 2, is the most abundant structural protein found in core particles, VP2 forms the core layer of the virion and binds the RNA genome, and is the third most abundant protein in double-shelled particles. VP2 is the only structural protein shown to possess nucleic acid (dsRNA, ssRNA, and dsDNA)-binding activity when evaluated by an RNA overlay-protein blot assay (Settembre *et al*., 2011).

VP3 is encoded by genome segment 3, it is part of the inner core of the virion and is an enzyme called guanylyl transferase which is a capping enzyme that catalyses the formation of the 5' cap in the post-transcriptional modification of mRNA. The cap stabilizes viral mRNA by protecting it from nucleic acid degrading enzymes called nucleases (Crawford *et al*., 2001).

VP4 is the protein product of genome segment 4, and it is a nonglycosylated outer capsid protein and a hemagglutinin in many virus strains, VP4 is located on the surface of the virion that protrudes as a spike which binds to molecules on the surface of cells called receptors and drives the entry of the virus into the cell. In the presence of trypsin, VP4 is cleaved into VP5 (molecular weight approximately 60,000) and VP8 (molecular weight approximately 28,000) and this cleavage results in enhancement of viral infectivity. Cleavage of VP4 has been shown to enhance penetration (but not binding) of the virus into cells. VP4 is also associated with restriction of the growth of certain rotavirus strains in tissue culture cells and

in mice and with protease-enhanced plaque formation. VP4 protein determines how virulent the virus is and it determines the P-type of the virus (Crawford *et al*., 2001).

VP6 is encoded by genome segment 6 and is the major structural protein in virus particles located on the outer surface of single-shelled particles. It forms the bulk of the capsid. Biochemical characterization of VP6 removed from particles revealed that it is a trimer, and three-dimensional structural studies of single-shelled particles have shown trimers to be present on the surface of these particles. VP6 is both highly immunogenic and antigenic, and it is the most frequently targeted protein in diagnostic assays to detect virus particles. VP6 contains common (cross-reactive) epitopes shared by other group A viruses, and it can contain several forms of an antigen called the subgroup antigen (Cohen *et al*., 1989).

VP7 is the second most abundant capsid protein and is encoded by genome segment 9, VP7 is a glycoprotein that forms the outer surface of the virion, it determines the G-type of the strain and, along with VP4, is involved in immunity to infection. Biochemically VP7 is a glycoprotein that contains only N-linked high-mannose oligosaccharide residues, which are added co-translationally as this protein is inserted into the membrane of the endothelium reticulum VP7 is also an integral membrane protein with a luminal orientation whose oligosaccharides are modified by trimming which occurs only in the endothelium reticulum (Lawton *et al*., 1997).

2.4. Nonstructural proteins:

The rotavirus genome codes for six nonstructural proteins (NSP1-6). The proteins are involved in replication (NSP1, 3, 5, 6) and morphogenesis (NSP4) and except for NSP4, interact with nucleic acid.

NSP1 is the least conserved of the rotavirus proteins. It seems to be nonessential for virus replication, although it was recognized as a virulence factor in mice, but not in piglets or rabbits. NSP1 contains a relatively conserved zinc finger domain, but this domain is missing in some virus strains, showing that it is not essential for virus replication and genome segment reassortment (Hua and Patton., 1994).

NSP2 is an oligomeric NTPase, which is localized to viroplasms, possesses helix-destabilizing activity, and is possibly involved in RNA encapsidation and virulence (Fabbretti *et al*., 1999).

NSP3 is a sequence-specific RNA-binding protein that requires only four nucleotides at the 3' end for RNA recognition. NSP3 functions similarly to the cellular poly (A)-binding protein (PABP). It binds to eIF4G and thereby inhibits cellular RNA translation and protein synthesis, while enhancing the translation of viral mRNA (Deo *et al*., 2002).

NSP4 is the only nonstructural protein that does not bind to RNA. The nonstructural protein NSP4 encoded by gene 10 is 175 amino acids in length and has been shown to play a key role in viral morphogenesis. NSP4 is an ER glycoprotein that has been proposed as a receptor for double-layered particles and is crucial to translocate these immature viral particles across the endoplasmic reticulum membrane (Iturriza-Gomara *et al*., 2003).

NSP5 is an *O*-glycosylated phosphoprotein. It self-assembles into dimers, has autokinase activity and exists in several phosphorylated isomers in infected cells. NSP5 interacts with NSP2, and when co-expressed, NSP2 induces hyperphosphorylation of NSP5. NSP6 is encoded in an alternative ORF on gene segment 11 of most rotaviruses and is known to interact with NSP5 and accumulate in viroplasms (Choi *et al*., 2005).

2.5. Rotavirus genotypes, serotypes and antigenic composition:

Rotaviruses are classified serologically by a scheme that allows for the presence of multiple groups and of multiple serotypes within each group. A rotavirus group includes viruses that share cross-reacting antigens detectable by a number of serologic methods, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA), and IEM (Gouvea and Santos., 1999).

Based on group specificity which is conferred predominantly by VP6, rotaviruses comprise seven distinct groups (A to G). Group A, B, and C rotaviruses are found in both humans and animals, whereas rotaviruses of groups D, E, F, and G have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups (Hoshino and Kapikian., 1994).

Group A rotaviruses have clearly been established as causing significant diarrheal disease in infants and in the young of various mammalian and avian species. Group B rotaviruses have been associated with epidemics of severe diarrhea primarily in adults in China. Group C viruses have been sporadically reported in fecal specimens from children with diarrhea and in several family outbreaks (Gerna et al, 1984).

Subgroup specificity, which is also determined by VP6, has been used for characterizing the antigenic properties of various rotavirus strains in epidemiologic surveys. Most Human Rota Virus (HRV) belong to either subgroup I or subgroup II (Krishnan et al., 1999).

Rotaviruses can also be classified on the basis of two outer capsid proteins, the glycoprotein VP7 which defines G genotypes and the spike protein VP4 which defining P genotypes. These two structural proteins elicit neutralizing antibodies in

the host and encoded by VP7 and VP4 genes of rotavirus, respectively (Anita *et al*., 2010).

Currently 23 G-genotypes and 31 P-genotypes have been described, of which 12 of each type have been found in human rotavirus isolates. However, only a limited number of G/Pgenotype combinations are found frequently in humans, such as G1P8, G2P4, G3P8, G4P8, and G9P8, and, more recently, the G12 genotype in combination with P8 or P6. It has been hypothesized that the G9 and G12 genotypes have been able to successfully infect, spread, and persist in humans because of reassortment events with human Wa-like rotavirus strains, which has resulted in the G9 and G12 rotaviruses combining with P8 and the 9 remaining gene segments belonging to genotype 1(Jelle *et al*., 2010).

The incidence and distribution of G and P genotypes that cause disease in humans may vary by geographical location and by year. The major human G types are G1, G2,G3, G4, and G9, which combine with the P types P8, P4, and P6, these account for more than 80% of rotavirus associated gastroenteritis episodes worldwide (Kang *et al*., 2005).

Analyses of monoclonal antibodies (mAbs) directed against rotavirus outer-capsid proteins VP7 and VP4 of naturally-occurring or laboratory-generated rotavirus reassortants have shown that both VP7 and VP4 carry epitopes that are responsible for evoking neutralizing antibodies. Neutralizing antibodies in hyperimmune antiserum raised against a rotavirus strain are primarily directed to the major surface glycoprotein VP7. However, hyperimmune antiserum raised against reassortant rotaviruses, in general, demonstrates high neutralizing activities not only to the VP7 but also to the surface spike protein VP4(Chen *et al*., 1989).

In neutralization assays with hyperimmune antisera and the criterion of a greater than 20-fold difference between homologous and heterologous reciprocal neutralizing antibody titres, it has been possible to designate 14 rotavirus G serotypes and 11 rotavirus P serotypes (Browning *et al*., 1991).

Most serotypes are shared between humans and animals; 10 of the 14 G serotypes (G1, 2, 3, 4, 5, 6, 8, 9, 10, and 12) and 7 of the P serotypes (P1, 2A, 3, 4, 5A, 8, and 11) have been detected in humans (Kapikian and Chanock., 1996). Only G serotype 7, 11, 13, and 14 and P serotype 6, 7, 9, and 10 have been detected exclusively in animals. The neutralization assay is by definition the only method of determining neutralization specificity (serotype) of VP7or VP4 (Estes., 1996).

2.6. Genome structure:

The viral genome of 11 segments of dsRNA is contained within the virus core capsid. The segments range in size from 667 (segment 11) to 3,302 base pairs (segment 1), with the total genome containing approximately 18,522 base pairs. This number, compiled from sequence data of segments from different virus strains, agrees closely with the genome size (18,680 base pairs) determined by EM measurements(279a) (Chan *et al*., 1986).

Hydrodynamic studies of the flexibility or stiffness of isolated rotavirus RNA segments in solution have indicated that these RNA segments cannot be packaged into the rotavirus capsid unless intimate protein-RNA interactions take place. In solution, these RNA molecules possess a "wormlike" or flexible cylinder structure; as an example, RNA segment 1 (3,302 base pairs and a contour length of 928nm) theoretically cannot be bent into a capsid of 50 nm as a free molecule because the persistence length is 112.5 nm.Therefore, to obtain RNA flexibility, one has to assume that intimate protein-RNA interactions occur in the virion to induce the needed bending and packaging of the dsRNA segments into the virus capsid. The

proteins directly responsible for segment packaging remain unclear. The structural proteins present in core particles (VP1, VP2, and VP3) are obvious candidates, but nonstructural proteins may also play a scaffolding role (Kapahnke et al., 1986).

Deproteinized, purified rotavirus dsRNAs are not infectious, reflecting the fact that virus particles contain their own RNA-dependent RNA polymerase required to transcribe the individual RNA segments into active messenger RNAs (mRNAs). The genome RNA is highly ordered within the particle, with about 25% of the genome making up a dodecahedral structure, and VP2 interacts with the RNA. Several points of contact between the inwardly protruding portion of VP2 interact with the RNA surrounding each fivefold axis, and VP2 interactions with the VP1 polymerase are required for replicase activity (Chen *et al*., 1989).

Each RNA segment starts with a 5' guanidine followed by a set of conserved sequences that are part of the 5' noncoding sequences; an open reading frame coding for the protein product follows, and another set of noncoding sequences, which contains a different subset of conserved 3'-terminal sequences and ends with a 3'-terminal cytidine, is found after the stop codon (Chen *et al*., 1989). The 5'-terminal consensus sequence 5'GGC (AIU)(AIU)U(AIU)A(AIU)(AIU) is found only once (in segment 4, base pairs 2166 to 2177) in a position other than at the termini of the other known sequences. The 3'-terminal consensus sequence is U (GIU)(UIG)(GIU)(AIG)CC-3'. The complementary sequence of the 3'-terminal consensus sequence (UGUGACC-3') is also found in segment 5 (positions 1513 to 1519) (Chan *et al*., 1986).

The lengths of the 3' and 5'noncoding sequences vary for different genes. However, these lengths are conserved among strains for a given gene, with the exception of the 3' end of segment 7 (134 bases for SAl and 112 bases for bovine UK viruses) and segment 10 (from 182 to 186 bases for five different virus strains) Clark and Desselberger., 1988).

There is no preferential use of stop codons, and a polyadenylation signal is not found at the 3' end of the genes. Most of the sequenced segments (except gene 11) possess only one long open reading frame. The first initiation codon (in all genes except 7, 9, and 11) is a strong initiation codon based on Kozak's rules Clark and Desselberger., 1988).

For genes 7 and 9, the second in-phase AUG is the favored initiation sequence. For gene 7, it is unknown which AUG is used. Some evidence indicates that gene 9 is a bicistronic gene. Gene 11 contains a second in-frame open reading frame and also an out-of-frame open reading frame that are conserved in all of the segment 11 sequences found to date. The initiation codon for the second out-of-frame open reading frame is a favorable one, but it is not known whether this second reading frame is used Clark and Desselberger., 1988).

All of the rotavirus gene sequences are A+T rich (58 to 67%), and the codon usage is biased against CGN and NCC codons, as in many eucaryotic and other viral genes.The RNA segments are base paired end-to-end, and the plus-sense strand of the genomic dsRNA contains the capped 5' sequence m7GpppG(m)GPy. Similar features of the RNA termini (capped structures and 5' and 3'conserved sequences) are found in the primary structures of the genome segments of other viruses (e.g., reovirus, Cytoplasmic polyhedrosis virus, and orbivirus) in the family Reoviridae, and 5' and 3' conserved terminal sequences are also found in other virus families with segmented genomes (Orthomyxoviridae, Arenaviridae, and Bunyaviridae). Therefore, these terminal sequences are thought to contain signals important for genome transcription, replication, and possibly assembly of the viral genome segments (Clark and Desselberger., 1988).

2.7. Gene-coding assignments:

The gene-coding assignments and known properties of the proteins encoded in each of the 11 genome segments are now fairly well established. These

assignments have been determined by in vitro translation studies with mRNA or denatured dsRNA and by analyses information on simian SA11 rotavirus strain serves as the basis for comparative studies with other rotavirus strains. Such comparisons have shown that the absolute order of migration of a gene coding for a particular protein (cognate gene) may differ for different virus strains, and so gene assignments cannot be based on RNA patterns. Instead, identification of cognate genes must be based on hybridization with gene-specific probes, analysis of reassortants, or protein identification based on biochemical or immunologic identification of the protein translated in a cell-free system programmed with mRNA specific to the gene. The ability to directly obtain sequence information from dsRNAs or ssRNAs and the accumulating nucleic acid sequence data bases also make it possible to identify cognate genes solely on the basis of sequence homology (Besselaar et al., 1986).

The rotavirus genes code for structural proteins found in virus particles and for nonstructural proteins found in infected cells but not in mature particles. The consensus is that the protein products (VP1 to VP4, VP5, VP6, VP7, VP8) of six of the genome segments are structural proteins found in virus particles and that the other five genome segments code for nonstructural proteins(Dyall-Smith and Holmes., 1981).

It remains unknown whether the additional open reading frames found in genome segment 11 code for other proteins. Early studies often contained seemingly conflicting conclusions concerning the numbers and locations of the rotavirus proteins. Many of these were resolved, as reviewed elsewhere, when it was recognized that post translational modifications (glycosylation, trimming of carbohydrate residues, and proteolytic cleavages) occur following polypeptide synthesis. In addition, strain variations (such as the presence of more than one glycosylation site on VP7 in some bovine and human rotavirus strains) have been

clearly shown, and these provide explanations for other discrepancies (Furlong *et al*., 1988).

The nomenclature of the viral proteins designates structural proteins as viral protein (VP) followed by a number, with VP1 being the highest molecular-weight protein, and proteins generated by cleavage of a larger precursor being indicated by an asterisk [VP4 is cleaved to produce VP5* and VP8. Initial studies referred to the nonstructural proteins as NS followed by a number indicating the protein's molecular weight. This nomenclature has been replaced by NSP1 to NSP6 to facilitate comparisons among cognate nonstructural proteins of different molecular weights (Hundley et al., 1985).

2.8. Stages of Replication:

The natural cell tropism for rotaviruses is the differentiated enterocyte in the small intestine, suggesting these cells express specific receptor(s) for virus. However, recent recognition that extraintestinal spread of rotavirus occurs suggests a wider range of host cells than previously thought (Jourdan *et al*., 1995).

Rotavirus replication in continuous cell cultures derived from monkey kidneys is fairly rapid, with maximal yields of virus being found after 10 to 12 hours at 37Â°C or 18 hours at 33Â°C when cells are infected at high multiplicities of infection (10 to 20 plaque-forming units [pfu]/cell). Recent studies examining rotavirus replication in differentiated human intestinal cell lines (Caco-2 cells) grown on permeable filter membranes show that the virus replication cycle is slower, with maximal yields of virus being detected on the apical surface of cells 20 to 24 hours after infection (Brown *et al*,1981).

The general features of rotavirus replication (based on studies in cultures of monkey kidney cells) are as follows:

- Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This ensures activation of viral infectivity by cleaving the outer capsid protein VP4.
- Replication is totally cytoplasmic.
- Cells do not contain enzymes to replicate dsRNA; hence, the virus must supply the necessary enzymes.
- Transcripts function both to produce proteins and as a template for production of negative strand RNA. Once the complementary negative strand is synthesized, it remains associated with the positive strand.
- The dsRNA segments are formed within nascent subviral particles, and free dsRNA or free negative-stranded ssRNA is never found in infected cells.
- RNA replication occurs within cytoplasmic viroplasms.
- Subviral particles form in association with viroplasms, and these particles mature by budding through the membrane of the ER. In this process, particles acquire their outer capsid proteins.
- Levels of intracellular calcium are important for controlling virus assembly and integrity.
- Cell lysis releases particles from infected cells grown on monolayers (Clark et al., 1979).

2.8.1. **Attachment:**

VP4 and VP7 are implicated in the initial interactions with host cells. The initial step in virus infection consists of virus binding to the surface of host cells. A broad range of cells can bind rotaviruses and be infected with different efficiencies, suggesting that the initial binding (attachment) is a promiscuous interaction with postattachment receptors being critical for virus entry into the cell. Virus attachment is by VP4 or its cleavage product VP5 on Triple layer particles (TLP).

Binding to cells does not require cleaved VP4 or glycosylated VP7. The demonstration that some rotavirus strains hemagglutinate red blood cells and that neuraminidase treatment of red blood cells reduces virus binding first indicated a role of sialic acid (SA) in virus attachment. Neuraminidase (NA) treatment of cultured cells reduces the infectivity of HA-positive virus strains and SA-containing compounds, such as fetuin and mucin, inhibit virus binding to cells. (Mendez *et al*, 1999).

The most easily cultivatable animal rotaviruses bind SA and preferentially infect polarized intestinal cells apically. Most rotaviruses, including all human rotaviruses, initiate infection by an NA-independent mechanism based on their ability to bind to NA-treated cells; these viruses preferentially infect polarized intestinal cells through the basolateral surface in a previously unrecognized manner. The consequences of these differences in modes of cell entry are not fully understood at this time, but it is likely that further studies on rotavirus entry into cells will reveal other virus-host interactions that affect the outcome of infection and pathogenesis. Some NA-resistant strains are suspected to bind to NA-insensitive internal SA moieties on glycolipids or to modified SA moieties in oligosaccharide structures, such as those present in the GM1 ganglioside, that are resistant to NA treatment. These results suggest it is better to classify viruses by the sensitivity of their infectivity to NA treatment of cells than to call them SA-independent viruses as initially done, unless virus binding to SA has been determined directly (Ciarlet *et al*., 1999).

VP4 is known to be the hemagglutinin (HA) based on studies of:

a. reassortants showing that HA activity segregates with the rotavirus gene 4.
b. mutants that have lost their HA activity and were selected with VP4 monoclonal antibodies.
c. VP4 produced in insect cells showing this protein agglutinates red blood cells.

An x-ray crystallographic structure of a SA complexed with VP8 from a hemagglutinating virus strain shows the VP8 domain exhibits a sandwich fold of the galactins, a family of proteins whose natural ligands are carbohydrates, despite a lack of sequence similarity. The SA moiety binds within a shallow groove on the VP8 surface using residues that are conserved in SA-binding strains. The VP8 structure represents one of the first observed cases of a rotavirus protein taking on a fold seen among host proteins and, based on this structural result, it is proposed that VP4 arose from the insertion of a host carbohydrate-binding domain into a viral membrane-interaction protein. The structure of the VP8 core from an NA-resistant human rotavirus (DS-1 strain) shows conservation of the same galactinlike fold but other structural differences make it unlikely that the DS-1 VP8 binds an alternative carbohydrate ligand; instead, it is suggested this SA-independent spike binds a protein ligand. VP8 binding to the minimal sialoside (2-O-methyl Î±-SA) has a relatively low affinity, supporting the idea mentioned above, that the virus entry event is probably responsible for cell type and host specificity (Dormitzer *et al*, 2002).

2.8.2. Penetration and Uncoating:

Rotavirus cell entry is a coordinated, multistep process involving sequential interactions with several ligands and involves a series of conformational changes in the capsid proteins. For some virus strains, the initial interaction of VP8 with SA-containing cell receptors is thought to induce conformational changes on VP4 that allow virus to interact with integrin Î±2Î²1 through a DGE sequence in VP5 binding to the Î±2 I domain. In NA-resistant rotavirus strains, VP4 may already be in a confirmation that is suitable for directly interacting with downstream receptors. After these interactions, at least one (and up to three) additional interactions take place. These postattachment interactions involve VP5* and VP7

binding to heat shock protein 70 (HSP70) and integrins, such as Î±vÎ²3, Î±xÎ²2, and a4Î²1, depending on the cell type (Estes, 2001).

These receptors have been identified by inhibition of virus infectivity by antibodies to, or peptides mimicking, the proposed receptors and by showing binding of expressed proteins to the proposed receptors, as reviewed elsewhere. Binding of purified HSP70 to VP5 can be demonstrated in cell free assays and may modify the conformation of virus particles to help the virus enter cells. To date, however, binding affinities to the proposed post-SA binding receptor molecules have not been determined, and no structural studies have been reported of rotavirus-protein receptor complexes (Estes *et al*, 1981).

Trypsin cleavage of VP4 enhances viral penetration and infectivity and the VP4 cleavage products of VP8 (aa 1 to 247) and VP5 (aa 248 to 776), remain associated with virions. The mechanism by which trypsin cleavage of VP4 enhances virus penetration into cells is not known, but trypsinized particles enter cells more rapidly than those not trypsinized. The body of the spike (VP5) has lipophilic activity and is thought to be important for entry into cells. Before trypsin cleavage, VP4 is flexible and spikes are not visible by cryo-EM, although they are present on particles. Trypsinization of VP4 stabilizes the spike structure, inducing a disorder-to-order transition that is unique, not documented with any other virus to date, and is thought to prime VP4 for later conformational changes. In contrast with the dimeric spike detected by cryo-EM, the 3.2 Ã… crystal structure of the main part of the VP5 stalk reveals a coiled-coil stabilized trimer with substantial intersubunit interactions. Two individual monomers of VP5 clearly fit into the main body of the spike in the cryo-EM structure. It appears that each spike is indeed a trimer of VP4, but that two molecules of VP4 form the visible spike as seen in the cryo-EM reconstruction of trypsinized rotavirus particles, with the other monomer being floppy and not visible in the reconstruction. It is proposed that, during cell entry,

an unknown, entry-associated event triggers a second transition of the floppy VP5 monomer together with the other two VP5 molecules to form a tightly associated trimeric conformation, shaped like an umbrella seen in the VP5 structure. The trimeric architecture of VP4 is reminiscent of the trimeric viral spikes on many other viruses (Falconer *et al*., 1995).

Recent studies with pH-treatment of a SA-dependent virus provide support for this proposal. At elevated pH, the spike undergoes a dramatic irreversible conformational change and becomes stunted with a pronounced trilobed appearance, although the amount of VP4 on particles remains unchanged. Three Fab fragments of the VP5-specific mAb, 2G4, can then bind to these altered spikes, suggesting that VP4 has undergone a dimer-to-trimer transition. Particles with altered spikes are not able to hemagglutinate red blood cells or infect mammalian cells. They retain the ability to bind to mammalian cells, however, but in an NA-resistant manner, different from untreated particles that bind cells in an NA-sensitive manner. High-pH treatment may trigger a conformational change that mimics a post-SA attachment step. These particles appear to resemble a mutant virus of the SA-dependent rhesus rotavirus that exhibits NA-resistant cell binding in contrast to its parental strain and attaches to cells by interacting with the integrin Î±2Î²1 through a DGE motif in VP5 (Dormitzer *et al*., 2004).

Currently, rotaviruses are characterized by their NA-sensitivity for infectivity as well as their integrin usage. Integrin usage has primarily been characterized in nonpolarized monkey kidney cells. It is likely that integrin and postbinding receptor usage differs depending on the cell type(s) analyzed and that the interactions between NA-sensitive and -resistant rotaviruses with intestinal or extraintestinal cells are distinct and may affect the pathogenesis and outcome of infections. Despite demonstrated binding of VP5 and VP7 to integrins, no evidence

currently exists for signaling after this binding, leaving open the question of how virus actually penetrates the membrane (Keljo and Smith., 1988).

Internalization does not take place at 0Â°C to 4Â°C, indicating that this step requires active cellular processes. All virus is internalized by 60 to 90 minutes after binding. The mechanism of internalization (penetration) into cells remains unclear. Both morphologic and biochemical approaches have been used to investigate the mode of entry of rotaviruses into cells, and both receptor-mediated endocytosis and direct membrane penetration have been suggested as mechanisms of rotavirus entry into cells; trypsin-treated and non trypsin-treated virus may enter cells by different mechanisms as reviewed previously (Pesavento *et al*., 2005).

Use of the calcium ionophore A23187 to increase the intracellular Ca^{2+}-concentration during the early stages of replication can block uncoating. These results support early hypotheses that low Ca^{2+}- concentrations in the intracellular microenvironment may be responsible for uncoating. This idea was originally proposed because it was known that removal of the outer capsid of particles and activation of the endogenous polymerase could be accomplished by calcium chelation (Gerna *et al*., 1984).

Other viruses that initiate infection by mechanisms involving receptor-mediated endocytosis often depend on the acidification of endosomes for partial uncoating or entry into the cell. The importance of acidification of endosomes for the initiation of infection of rotaviruses has been studied by several groups. In all cases, lysosomotropic agents (ammonium chloride, chloroquine, methylamine, amantadine) do not affect rotavirus entry. Energy inhibitors (sodium azide and dinitrophenol) have a minimal effect on rotavirus infection, and this has been taken to suggest that rotaviruses do not use endocytosis to enter cells. Other endocytosis inhibitors, such as dansylcadaverine and cytochalasin D, or the vacuolar adenosine triphosphatase (ATPase) inhibitor bafilomycin A1, do not block rotavirus entry

either. These results indicate that neither endocytosis nor an intraendosomal acidic pH or a proton gradient is required for rotavirus entry into cells (Pesavento *et al*., 2005).

It seems clear that the passage of rotaviruses from endocytic vesicles to the cytoplasm does not occur by a pH-dependent fusion mechanism, but this does not rule out the possibility that rotaviruses are still taken up by endocytosis. Direct demonstration of virus fusion with membranes or hemolysis is lacking. Protease cleavage of VP4 is important for rapid entry into cells, and particles containing cleaved VP4 possess lipophilic activity and can affect release of fluorescent dyes from liposomes and isolated membrane vesicles. Rotavirus entry into cells can also be monitored by coentry of toxins, such as Î±-sarcin, into cells and by a cell-to-cell fusion from without assay. Most observations are consistent with the hypothesis that virus enters cells after direct interactions with a receptor on the plasma membrane (Falconer *et al*., 1995).

The outer capsid proteins of rotavirus that are solubilized from virus particles are able to permeabilize cellular membranes, and it has been proposed that the outer capsid proteins are solubilized within an endocytic vesicle because of low Ca^{2+} concentrations. The decrease in calcium concentrations within the endosomal vesicle might trigger conformational changes in the capsid, capsid solubilization, and vesicle lysis. In this Ca^{2+}-dependent endocytosis model, acidification of the endosome would not be needed for the infectious process. It is also possible that more than one mechanism, including endocytosis and direct entry, is operative for rotaviruses, as has been proposed for polioviruses and reoviruses. Further studies are needed to determine whether the common endocytosis-mediated entry pathway exists for rotaviruses. Studies with drugs and dominant-negative mutants suggest that virus enters cells through a clathrin-independent, caveolin-independent mechanism that depends on the presence of cholesterol on the cell membrane and

on a functional dynamin. Trypsin has been detected associated with the rotavirus outer capsid and is activated by solubilization of the outer capsid proteins. It is proposed that activated trypsin cleaves VP7 and VP4 into fragments capable of disrupting membranes and this may allow DLPs to gain access to the cytoplasm to begin actively transcribing viral mRNA to complete the next step in the viral life cycle (Gerna *et al*., 1984).

A single report that the protein product of genome segment 10 influences virus adsorption to cells further complicates the question of the mechanism of virus attachment to cells. This study of a series of reassortants suggests that the nonstructural protein NSP4 may be indirectly involved in adsorption by influencing the arrangement of outer shell proteins assembled in infectious particles. This observation remains to be confirmed but is of interest based on the new information that NSP4 can affect calcium mobilization in cells and the proposal that rotaviruses may enter cells by a Ca^{2+}-dependent endocytic mechanism (Gerna *et al*., 1984).

2.8.3. Transcription:

Synthesis of viral transcripts is mediated by an endogenous viral RNA-dependent RNA polymerase (transcriptase), which has a number of enzymatic activities. The transcriptase is a component of the virion, and this enzyme complex of VP1 and VP3, which appears as a flower-shaped feature in the icosahedrally averaged cryo-EM reconstruction of the virion, is attached to the inner surface of the VP2 layer at all the fivefold axes. Properties of the transcriptase have been inferred by studying the characteristics of products from in vitro transcription reactions and by EM analysis of transcribing particles. Rotavirus particles contain the enzymatic activities needed for synthesis of capped messenger RNA, including transcriptase, nucleotide phosphohydrolase, guanyltransferase, and methylases. These activities have either been demonstrated biochemically, or they are inferred because

rotavirus transcripts made in vitro in the presence of S-adenosyl methionine possess a methylated 5-terminal cap structure, m^7GpppGm, and transcription is inhibited by pyrophosphate. Particles also contain a poly (A) polymerase activity whose precise function remains unknown; it has been postulated to be responsible for the synthesis of oligo (A) molecules (Gorziglia *et al*, 1981).

The virus-associated transcriptase is latent in TLPs and can be activated in vitro by treatment with a chelating agent or by heat shock treatment. Such treatments result in removal of the outer capsid proteins with conversion of TLPs to DLPs. In infected cells, TLPs have been shown to be uncoated to DLPs, and it is thought that transcription in cells occurs from such particles. Consistent with this idea, cells are susceptible to infection by liposome-mediated transfection of DLPs, indicating that simple delivery of these particles into the cell cytoplasm permits transcription to proceed. Transcription is asymmetric, and all transcripts are full-length positive strands made off the dsRNA negative strand. Primary transcription may occur before viroplasms are formed. The main site of transcription (secondary transcription) within the cytoplasm at 8 to 9 hours after infection is in viroplasms.

The dynamic transcription process is beginning to be understood. Rotavirus transcription occurs within structurally intact DLPs and requires a hydrolyzable form of adenosine triphosphate (ATP). Studies with analogs that inhibit transcription suggest that ATP is required in reactions other than polymerization. ATP may be used for initiation or elongation of RNA molecules. Structural studies of transcribing particles indicate that RNA synthesis occurs within the core of the virion, near the fivefold axes, because this is the location of the transcriptional complexes composed of VP1 and VP3. Newly transcribed RNA exits the core through the type I channels in the VP2 layer, and multiple mRNA transcripts can be released simultaneously from an actively transcribing particle. Each genome segment is transcribed by a specific polymerase complex, and the resulting

transcripts exit through the channel system at the axis adjacent to its site of synthesis. This mechanism of transcription offers an explanation of why no dsRNA virus contains more than 12 genome segments (Silvestri *et al*., 2005).

Some mAb to VP6 can block transcription. Cryo-EM studies of DLPs complexed with these antibodies suggest that such antibodies, or VP7 added onto DLPs, induce a conformational change at the interface of the VP2-VP6 layers or in the VP6 trimers to inhibit sustained elongation and translocation of transcripts. It is also possible that binding of VP6 to NSP4, which serves as an intracellular receptor for particle assembly, is the key interaction that inhibits transcription. This hypothesis is consistent with the observation that knockdown of NSP4 by siRNA modulates viral mRNA synthesis (Silvestri *et al*., 2004).

Following endogenous transcription and release of mRNA, the rotavirus replication cycle can be viewed as having four subsequent stages:

a. Translation and synthesis of viral proteins
b. Secondary transcription
c. Replication, genome packaging, and dlps assembly
d. Budding of newly formed DLPs into the ER and assembly of the outer layer to form mature TLPs.

The positive-stranded RNA transcripts encode the rotavirus proteins and also function as templates for production of negative strands to make progeny dsRNA (Clark et al., 1980).

2.8.4. Translation:

Viral mRNAs are capped but not polyadenylated, and viral proteins are translated by the cellular translation machinery. Most of the rotavirus structural proteins and the nonstructural proteins are synthesized on the free ribosomes, although the nascent proteins on free ribosomes have not been analyzed. Instead, this conclusion has been drawn based on the absence of signal sequences that would indicate

targeting to the ER and lack of protection from digestion in in vitro protease protection studies. The viral glycoproteins VP7 and NSP4 are synthesized on ribosomes associated with the membrane of the ER and are cotranslationally inserted into the ER membrane as a result of signal sequences at their N-termini. VP7 has a signal sequence that is cotranslationally cleaved, whereas the signal sequence on NSP4 is not cleaved (Poncet *et al*, 1993).

Translation of the viral mRNAs that are capped but not polyadenylated is facilitated by the action of the nonstructural protein NSP3, which is one of the five nonstructural proteins (NSP1-3,5,6) that bind nucleic acid. NSP3 function parallels that of the cellular poly(A) binding protein (PABP). The N-terminus of NSP3 interacts with the 3-consensus sequence of viral mRNAs and the C-terminus of NSP3 interacts with eIF4G as does PAPB, but with higher affinity. These events lead to NSP3 evicting PABP from eIF4G, to the enhancement of translation of rotavirus mRNAs, and to the concomitant impairment of translation of cellular mRNAs (Deo *et al*, 2002).

In vivo, NSP3 stimulates the translation of mRNAs in synergy with the cap structure possibly enabling circularization of viral mRNAs and its delivery to the ribosomes for viral protein synthesis. The x-ray structures of both domains of NSP3 complexed with bound ligands indicate that both domains have novel folds and NSP3 functions as a dimer. Whereas the RNA binding domain forms a rod-shaped symmetric dimer, the N-terminal domain tightly binds to the consensus 3-end of the mRNAs inside a tunnel formed at the dimeric interface. NSP3 and eIF4G also interact with a novel cellular protein, named RoXaN, which has a role in translation regulation that remains to be understood. The binding of NSP3 to viral mRNAs has also been proposed as a possible mechanism to transport newly made mRNAs to viroplasms for subsequent replication. The cytoskeleton-binding function of NSP3 might be involved in this process directly or through interactions

with other host proteins because numerous links occur between the translational machinery and the cytoskeleton (Ericson *et al*., 1983).

2.8.5. Replication of genomic RNA:

The synthesis of positive- and negative-stranded RNAs has been studied in SA11-infected cells in a cell-free system using extracts from infected cells, and in an electrophoretic system that allows separation of the positive and negative strands of rotavirus RNAs in acid urea agarose gels. The kinetics of RNAs synthesis in infected cells show that positive- and negative-stranded RNAs are initially detected 3 hours after infection. After 3 hours, the level of transcription increases until 9 to 12 hours, at which time the concentration of positive-stranded RNAs have reached a maximum. The ratio of positive- to negative-stranded RNA synthesis changes during infection, and the maximal level of negative-stranded RNA synthesis occurs several hours before the peak of positive-stranded RNA synthesis (Chizhikov and Patton., 2000).

The delay in obtaining maximal positive-stranded RNA synthesis has been hypothesized to be caused by a requirement for the accumulation of stoichiometric amounts of a protein (e.g., VP6) that is necessary for the assembly of transcriptase particles. Both newly synthesized and preexisting positive-stranded RNA can act as templates for negative-stranded RNA synthesis throughout infection. The observation that the level of RNA replication does not increase continually in conjunction with the increasing levels of positive-stranded RNAs suggests that RNA replication is regulated by factors other than simply the level of positive-stranded RNAs in the infected cell. Based on nuclease sensitivity assays with this system, about 20% of the RNA made in vitro is double-stranded, and 80% is single-stranded. The synthesis of dsRNA in vitro has been determined to be an asymmetric process in which a nuclease-sensitive, positive-stranded RNA acts as template for the synthesis of negative-stranded RNA (Chen *et al*, 1994).

It is assumed that rotavirus RNA replication, as with that of reovirus, takes place in a conservative fashion; that is, both strands of parental dsRNA remain within partially uncoated particles. After its synthesis, dsRNA remains associated with subviral particles, suggesting that free dsRNA is not found in cells. Because of its inherent stiffness, dsRNA is not packaged. Subviral particles (complexes separable by sedimentation through sucrose gradients and by equilibrium centrifugation in CsCl gradients) in which dsRNA synthesis occurs have been characterized both in infected cells and in a cell-free system. These replicase complexes consist of the core proteins, VP1 and VP2, small amounts of the protein VP6, large amounts of the nonstructural protein NSP3, and lesser amounts of NSP1 and NSP2. These types of results indicate that some of the NSPs (NSP2, NSP3, NSP5) are involved in the RNAs replication process based on:

a. The presence of these nsps in replication complexes isolated from infected cells.
b. The nucleic acid binding properties of most of the NSP
c. Localization of most nsps to viroplasms
d. The RNA negative phenotype of temperature-sensitive (ts) mutants mapped to the genes that encode these NSPs (Chen *et al*., 1990).

According to one model, rotavirus double-layered particles are assembled by the sequential addition of VP2 and VP6 to precore replication intermediates consisting of VP1, VP3, NSP2, NSP3, NSP5, and attached RNA. Because of the inability to separate particles with transcriptase and replicase activities completely and the problem of contamination of some fractions with proteins from neighboring fractions, it has remained unclear whether the components characterized in this system are those necessary for RNA replication or simply present in these complexes for other reasons. A foreign reporter molecule was also engineered into a rotavirus genome segment and tested for expression in cells coinfected with a

helper virus. This system identified cis-acting nucleotides at the 3-end of the genome necessary for gene expression, but the lack of direct evidence of synthesis of negative strands did not permit determination of whether these signals were necessary for RNA replication. Unfortunately the efficiency of these systems was too low to ensure clear evaluation of the roles of cis- and trans-acting sequences and proteins required for replication and packaging. Instead, it seems likely that detection of an increased reporter signal was caused by enhanced translation in rotavirus-infected cells, possibly owing to the presence of the 3-translation enhancer on the expressed reporter gene (Aponte *et al*, 1994).

The role of individual proteins and specific protein complexes in RNA replication and viral morphogenesis will probably not be completely solved until they are studied in vitro with pure species of native rotavirus proteins and viral RNA. Progress toward this goal came with the development of a template-dependent replication system that uses only viral core proteins and exogenously added purified mRNAs. Virus-like particles that are expressed and self-assembled in insect cells also possess replicase activity. The conversion of exogenous mRNAs to dsRNA by subviral particles has facilitated studies assessing (a) the specificity of viral proteins in recognition and replication of rotavirus mRNAs and (b) the effect of adding exogenous synthetic RNAs containing specific mutations on replication. Cis-acting sequences required for replication have been identified (Gombold and Ramig., 1987).

This system is unique for mammalian RNAs viruses because it supports the de novo synthesis of genome-length, negative-strand RNA in the presence of only viral proteins and in the absence of host cell extracts. Adding viral mRNAs containing deletions of the 3´-consensus sequence, 5´-UGUGACC-3´, to the replication system, has shown that this sequence contains a cis-acting signal that is essential for negative-strand synthesis. Additional studies identified parts of the

5â€²-untranslated region as containing sequences that stimulate RNA replication. Similarly, sequences upstream of the 3-consensus sequence in the 3-untranslated region contribute to the efficient synthesis of dsRNA by open cores. Based on the location of the cis-acting sequences and computer modeling, it has been hypothesized that the ends of the viral mRNA interact in cis by complementary terminal sequences to form panhandle structures that promote the synthesis of dsRNA. An important feature of the predicted secondary structure of the rotavirus mRNA is that within the panhandle structure, the 3-consensus sequence is either not base-paired or only partially base-paired to the 5-terminus. It appears that the 3-consensus sequence of the mRNA must be single-stranded for efficient dsRNA synthesis.Work is proceeding to enhance the efficiency of this in vitro replication system and to develop a cell-free system to support rotavirus RNA replication and the assembly of RNA into particles. No evidence currently exists of RNA encapsidation in this system. This in vitro template-dependent replication system is surprising because it absolutely does not require the nonstructural proteins for replicase activity. The nonstructural proteins, however, may play a role in increasing the efficiency of replication or play other roles in the replication cycle.

New structural information, as well as results from knocking out the nonstructural proteins with siRNA during viral infection, are providing clues about the NSPs and RNA binding, transport, replication, and assembly. The details of RNA replication in cells remain unclear but information from siRNA experiments has led to the idea of two separate pools of mRNA for the distinct functions of translation versus RNA replication and a second round of transcription may be driven from de novo synthesized DLP (Komoto *et al*., 2006).

RNA replication, genome packaging, and the initial steps of assembly of DLPs occur in perinuclear nonmembrane-bound electron-dense, cytoplasmic inclusions called viroplasms, which first appear 2 to 3 hours after infection. Viroplasms

contain NSP2, NSP5, together with the viral polymerase VP1, the guanylyltransferase and methylase VP3, the inner core protein VP2, and NSP5, the main constituents of the replication intermediates. NSP2 and NSP5 can form viroplasmlike structures and they also are implicated in genome replication and packaging. NSP5 is a dimeric phosphoprotein rich in Ser and Thr residues that undergoes O-linked glycosylation. In cotransfection experiments with NSP5 and NSP2, NSP2 upregulates the phosphorylation of NSP5. NSP2 and NSP5 are able to form empty viroplasm-like structures when coexpressed, with the number of viroplasms initially increasing and then decreasing with time after infection, whereas the area of each viroplasm increases, suggesting fusions of viroplasms. Suppression of either NSP2 or NSP5 expression inhibits the formation of viroplasms, genome replication, and viral assembly (Kapahnke *et al*., 1986).

Because the viral mRNAs, located outside the viroplasms that are involved in translation are susceptible to siRNA-induced degradation, whereas the mRNAs in the viroplasms that undergo replication are not, a model was suggested in which the transcriptionally active progeny DLPs act as a nucleation site for viroplasm formation and mRNAs can leave viroplasms to enhance further translation but cannot reenter viroplasms. This model eliminates the necessity for transport of viral mRNAs to the viroplasms for negative strand synthesis and subsequent DLPs assembly and genome packaging. It also helps explain why an exogenously added mRNAs had not been successfully rescued into an infectious virus. It does not explain, however, how (a) a single, incoming infectious virus particle can make multiple viroplasms, (b) reassortment would occur without mRNAs exchange between viroplasms, or (c) superinfection as late as 24 hours after the first infection could yield reassortant progeny. In addition, an exogenously added mRNAs has now been successfully rescued so a more likely interpretation of these siRNA experiments, which were performed relatively late in the infectious cycle (8

to 12 hours postinfection (hpi), is that these data are most relevant to late events related to secondary transcription from DLPs formed within viroplasms, which in turn have been produced earlier in the replication process (Patton., 1986).

NSP2 is an octamer with nucleotide triphosphatase (NTPase), nonspecific ssRNA-binding and nucleic acid helix destabilizing activities. The x-ray structure shows NSP2 has two domains that are separated by a deep cleft. The C-terminal domain resembles the histidine triad (HID) family of proteins that hydrolyze nucleotides. The NTP-binding resides within the cleft between the two domains. The association of monomers results in a doughnut-shaped octamer with a 35 Ã… central hole and grooves lined by basic residues, which are predicted to be the ssRNA-binding sites. Thus, although the NTPase activity is localized in the monomeric subunit, the ability to bind RNA and other proteins, such as NSP5 and VP1, may require the formation of the octamer (Gombold and Ramig., 1987).

Based on this structure, speculation is that the replication complex may be organized around the NSP2 octamer that provides a platform or scaffold. In this model, the hydrophobic side of the octamer may bind to VP1 and the basic grooves of the octamer may bind to the acidic NSP5 protein. Although the role of NSP5 in the overall replication process remains to be elucidated, NSP5 may be an adapter, with its ability to interact with VP2, to facilitate interactions between NSP2 and VP1 and VP3 in core assembly and RNA encapsidation, modulating the role of NSP2 as a proposed molecular motor involved in packaging of viral mRNA. If the NSP2 and RNA binding sites on NSP5 overlap, the function of NSP5 may be to regulate the binding of RNA by NSP2 during replication and packaging. These ideas are consistent with the observation that NSP5 is localized to the outside of the circular NSP2 structures in cells. The role of NSP6, which is encoded by an alternative reading frame of gene segment 11 and interacts with NSP5, in the

replication process remains unclear, but NSP6 has been suggested to have a regulatory role in the self-association of NSP5 (Gombold and Ramig., 1987).

Recent siRNA experiments indicate NSP4 plays a role in regulating the levels of expression of the other viral proteins as well as mRNA synthesis and possibly genome encapsidation. New data show that a vesicular form of NSP4 is modulated by the levels of intracellular calcium and that NSP4 also forms caps on viroplasms and colocalizes with the autophagy protein LC-3. How these new functions of NSP4 regulate RNA synthesis remains to be determined (Chizhikov and Patton., 2000).

2.8.6. RNA Encapsidation (Packaging):

The selective packaging mechanism that leads to the presence of equimolar genome segments within rotaviruses, or any of the other members of the family Reoviridae, remains a challenging puzzle. Several models have been proposed based on the characterization of replication intermediates from rotavirus-infected cells, one model proposes the formation of precore replication intermediate precursor complexes composed of viral mRNA, the viral RNA-dependent RNA polymerase, and the capping enzyme that would serve as nucleation sites for the binding of the VP2 core protein and the assembly of the T = 1 core. Based on the ability of the rotavirus capsid proteins to self-assemble into empty VLPs, and on data on the dsRNA bacteriophage phi6, a second model suggests that empty cores are first made and that mRNAs would be inserted into these cores. A third model is based on structural data indicating that the core represents a collection of functionally separate pentameric units, with each unit containing its own RNA-dependent RNA polymerase activity and capping enzyme complex and being responsible for transcription of one of the genome segments. In this model, encapsidation would be concurrent with capsid assembly, and each VP1, VP3

enzymatic complex would associate with a specific mRNA and attract the VP2 core protein and assemble into pentamers (Pesavento *et al*., 2006).

RNA and RNA interactions between the mRNA of the distinct pentameric units would then drive the assembly of the icosahedral core from the pentameric units. Structural changes in the core lattice protein VP2, as a consequence of pentamer binding, may activate the RNA-dependent RNA polymerase and stimulate negative-stranded RNA synthesis to form the genome. Future work will determine which of these models, if any, may be correct (Mirazimi *et al.*, 1998).

2.8.7. Virion Assembly:

A distinctive feature of rotavirus morphogenesis is that subviral particles, which assemble in the cytoplasmic viroplasms, bud through the membrane of the ER, and maturing particles are transiently enveloped. This is one of the most interesting aspects of rotavirus replication, differing from members of other genera in the family Reoviridae and from any other virus. The envelope acquired in this process is lost as particles move toward the interior of the ER, and the envelope is replaced by a thin layer of protein that ultimately constitutes the outer capsid of mature virions. Rotavirus particle transport, maturation, and assembly remain an interesting model to understand the transport of protein complexes across the ER membrane as well as envelope particle formation (Altenburg *et al.*, 1980).

Morphologic and biochemical data are consistent with rapidly assembling DLPs serving as an intermediate stage in the formation of triple-layered virions. The sites and precise details of RNA replication are beginning to be understood, and the viroplasms are the sites of synthesis of the double-layered particles that contain RNA (see above). This conclusion is based on the localization of several of the viral proteins (VP2, NSP2, NSP5, NSP6) to viroplasms, of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER, and on the

observation that particles emerging from these viroplasms bud directly into the ER that contains the glycoproteins VP7 and NSP4 (Both *et al*., 1983).

A fraction of VP4 has recently been detected in a filamentous array and at the plasma membrane associated with microtubules. Although the function of VP4 in lipid rafts at the plasma membrane in viral morphogenesis remains unclear, it has been proposed that VP4 is added to particles as an extra-ER event. Particles lacking VP4 can be formed in cells treated with siRNA to gene 4, suggesting that VP4 is not essential for assembly or for release of DLPs from the ER. Infectious particles are found associated with lipid rafts at the cell surface, A recent study that evaluated what affects the rotavirus-raft interactions found silencing VP4 and NSP4 (but not VP7) reduced this association apparently by reducing targeting of VP4 to the rafts; these results support the idea that the primary association of VP4 with rafts occurs during the initial stages of particle assembly in the ER. Taken together, two pools of VP4 appear to exist, with one associating with particles in the ER and one being found independently at the plasma membrane (Erk *et al.*, 2003).

In the (ER of SA11-infected cells), two pools of VP7 exist that can be distinguished using two classes of antibodies. One pool, which is found only on intact particles, is detected only by a neutralizing mAb. The second pool of VP7 is unassembled, remains associated with the ER membrane, and is detected by a polyclonal antibody made to denatured VP7. Distinction of these two forms of VP7 permitted a kinetic study of the assembly of VP7 and of other structural proteins into particles. The incorporation of the inner capsid proteins into double-layered particles was found to occur rapidly, whereas (VP4 and VP7) appeared in mature TLPs with a lag time of (10 to 15 minutes). Kinetic analyses of the processing of the oligosaccharides on the two pools of VP7 show the virus-associated VP7 oligosaccharides have a (15-minute lag) compared with that of the membrane-

associated form, suggesting that the latter is the precursor to virion VP7. This lag appears to represent the time required for virus budding and outer capsid assembly. NSP4 plays a key role in the assembly process. This nonstructural glycoprotein NSP4 is the only nonstructural protein that does not bind to RNA. NSP4 has been studied extensively because it plays a role in viral morphogenesis and functions as an enterotoxin (Gonzalez *et al*., 2000).

NSP4 has multiple domains and an increasing number of functions. NSP4 is a (20K) primary translation product; it is cotranslationally glycosylated to become a (29K species), and oligosaccharide processing yields the mature 28K protein that is a transmembrane protein of the ER. The (175 aa) polypeptide backbone of NSP4 consists of an uncleaved signal sequence, three hydrophobic domains with two N-linked high mannose glycosylation sites being in the first hydrophobic domain, a predicted amphipathic Î±-helix (AAH) that overlaps a folded coiled-coil region, the H2 transmembrane domain that traverses the ER bilayer, and the C-terminus, which is hydrophilic, forms an extended cytoplasmic domain. The carbohydrate moieties remain sensitive to endoglycosidase H digestion, and processing of the Man9GlcNAc carbohydrate added to NSP4 stops at Man8GlcNAc with the mannose-9 species predominating, indicating that no further trimming occurs in the Golgi apparatus (Pesavento *et al*., 2006).

The C-terminal cytoplasmic domain aa (161 to 175) of NSP4 functions in viral morphogenesis by acting as an intracellular receptor on the ER membrane. NSP4 binds newly made DLPs and mediates the budding of these particles into the ER lumen. A receptor role for NSP4 is supported by the observation that DLPs bind to ER membranes containing only NSP4. The AAH region, distinct from the receptor domain, is predicted to adopt an (Î±-helical coiled-coil structure) and is thought to mediate oligomerization of the virus-binding domains into a homotetramer (Kaljot *et al.,* 1988).

A crystal structure of the oligomerization domain of NSP4, which spans aa residues 95 to 137 (NSP4 95 to 137), self-associates into a homotetrameric coiled-coil, with the hydrophobic core interrupted by three polar layers and two of the four (Glu120 residues) coordinating a divalent cation. Sequence analyses have identified at least five distinct genetic groups (clades) of NSP4. The highest sequence diversity of NSP4 is located in the cytoplasmic domain. It is unclear whether this sequence diversity is important and driven by interactions with specific residues on VP6 or divergent regions on VP4, or both. However, specific combinations of types of (VP6 and NSP4) have been found in natural reassortants, suggesting these interactions are biologically important (Kaljot *et al.,* 1988).

Glycosylation of NSP4 is not required for its binding activity to DLP or for oligomerization, but it is required for interaction with calnexin. NSP4 also has a binding site for VP4, and may play a role in removing the transient envelope. Heterooligomers of (NSP4, VP4, and VP7) have been detected in enveloped particles, and calcium has been shown to be important for oligomerization of these proteins in the ER as well as for proper folding of (VP7 epitopes) and outer capsid assembly. The precise mechanisms of how (a) the envelope on particles is removed, (c) the heterooligomeric complexes function in particle budding through the ER, and (c) the outer capsid is assembled onto the newly made DLPs remains poorly understood. However, siRNA experiments indicate that VP4 is assembled in the (ER and VP7) is involved in removal of the transient envelope (Mirazimi *et al.,* 1998).

Rotavirus maturation is a calcium-dependent process, and virus yields are decreased when virus is produced in cells maintained in calcium-depleted medium. Viruses produced in the absence of calcium are often found to be exclusively DLPs, and budding of virus particles into the ER is not observed. Among the viral proteins, reduced levels of VP7 are observed, caused by the preferential

degradation, not the impaired synthesis, of VP7. An interesting finding of these studies is that (unglycosylated VP7) made in the presence of tunicamycin is relatively stable in a calcium-free environment. Although the role of calcium in morphogenesis is not completely understood from these studies, it is possible that calcium stabilizes or modulates folding or compartmentalization of the newly (glycosylated VP7) for subsequent assembly into particles. The budding process occurs in the absence of calcium, but VP7 is retained within the ER. VP7 does not fold properly unless it is expressed with other rotavirus proteins, and calcium must be present in cells for correct epitope formation. Calcium deprivation may destabilize the (ER or ER proteins) required for the stable association of (glycosylated VP7) with the membrane. Outer capsid assembly also requires proper formation of disulfide bonds on VP7. Recent studies with siRNA for VP7 have found that envelope removal is blocked, suggesting that VP7 is involved in removal of the envelope. Earlier studies showed treatment of cells with various agents (tunicamycin, dithiothreitol, or calcium-blocking drugs, such as thapsigargin) results in a build-up of enveloped particles in the ER. These agents may disrupt the proper folding of VP7 that is required for removing the envelope.

NSP4 is a novel calcium agonist; it mobilizes intracellular calcium when expressed intracellularly by a phospholipase C (PLC)-independent mechanism or by a PLC-dependent mechanism when it is added to cells from the outside. Mobilization of intracellular calcium by a PLC-independent mechanism is likely important for viral assembly. Binding of (VP6 on DLPs to NSP4) that occurs when viroplasms are capped by NSP4 is thought to trigger the budding process. Recent reports indicate that siRNA silencing of (NSP4 expression) in rotavirus-infected cells affects the distribution of other viral proteins, mRNA synthesis, and the formation of viroplasms where viral RNA replicates, suggesting previously unrecognized NSP4 functions in rotavirus replication (Mirazimi *et al.*, 1998).

Other new data indicate at least three pools of (intracellular NSP4) exist in rotavirus-infected cells dependent on the level of (NSP4 protein expression). The first pool is represented by NSP4 localized in the ER membrane and is present throughout the course of infection. This pool serves as a receptor for the budding of immature viral particles into the ER, as described above, at the peak of viral infection, when all viral proteins are abundant (after 6 hours after infection). A second minor pool of (NSP4 molecules) enters the ERGIC compartment and can be recycled back to the ER or may be a part of the nonclassical secretion pathway for delivery and cleavage of a (NSP4 peptide) into the medium of infected cells at early time-points after infection when the levels of viral proteins are relatively low. The third pool of (NSP4 molecule), distributed in cytoplasmic vesicular structures associated with the autophagosomal marker LC3 and viroplasms, is regulated by calcium levels and appears at (6 hours) after infection, when there is an increase of intracellular calcium levels because of increased expression of viral proteins. NSP4 also interacts with calveolin-1 and this may be involved in the secretion of NSP4. Inhibition of (NSP4 expression) interferes with the formation of large viroplasms, affects viral protein expression and viral mRNA synthesis in rotavirus-infected cells after (6 hours) after infection. The NSP4- and autophagic marker LC3-positive vesicles may serve as a lipid membrane scaffold for the formation of large viroplasms by recruiting early viroplasms or viroplasm-like structures formed by (NSP2 and NSP5). These NSP4-positive membranes may also function to regulate packaging of the rotavirus genome and transcription through NSP4 association with (VP6 on DLPs). The calcium-dependent compartmentalization of NSP4 into an autophagosomal pathway raises questions regarding the involvement of autophagosomal membranes in rotavirus replication and release of infectious virus from cells (Kaljot *et al.*, 1988).

Understanding viral morphogenesis has been facilitated by the expression of the rotavirus structural proteins individually or in combinations in insect's cells using recombinant baculoviruses. This approach first showed that the single-layered (VP2 particle) shell self-assembles when VP2 is expressed alone, and that all of the other capsid proteins have been shown to self-assemble into virus-like particles when coexpressed in the proper combinations. Virus-like particles composed of (VP2, VP1/2, VP1/2/3, VP2/3, VP2/6, VP2/6/7, VP2/4/6/7, and VP1/2/3/6) can be made. The outer and inner capsid proteins of different virus strains have also been shown to reassociate and be able to be transcapsidated onto other virus strains. These results demonstrate that the structural proteins contain the intrinsic information required to form particles and that coexpression of mutant proteins is a feasible approach to analyze the domains responsible for the structural interactions between the proteins composing the virus particles. These particles have been useful to:

a. analyze the role of cleavage sites in the spike protein in infectivity
b. investigate the role of individual structural proteins in inducing protective immunity.
c. probe the inner structure of particles by analyzing difference maps of particles with distinct protein compositions.
d. Analyze RNA transcription and replication and RNA packaging and assembly. Future studies should address questions of which mechanisms control packaging of the viral genome and virus assembly (Kaljot *et al.*, 1988).

2.8.8. Virus Release:

Electron microscopy studies have shown that the infectious cycle ends when progeny virus is released by host cell lysis in nonpolarized cells. Extensive cytolysis late during infection and drastic alterations in the permeability of the plasma membrane of infected cells result in the release of cellular and viral

proteins. Despite cell lysis, most DLPs and many TLPs remain associated with the cellular debris, suggesting that these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested, and virus purification procedures generally use Freon extraction to release particles from cellular debris. Whether the cytoskeleton provides a means of transport of viral proteins and particles to discrete sites in the cell for assembly or acts as a stabilizing element at the assembly site and in the newly budded virions, or whether particles are simply trapped by the cytoskeleton remain to be determined. VP4 interacts with actin and lipid rafts and can remodel microfilaments and this has been suggested as a mechanism by which the brush border membrane of polarized epithelial cells is destabilized to facilitate rotavirus exit from cells (Au *et al.,* 1993).

2.9. Pathogenesis and Pathology:

Rotaviruses replicate in the nondividing, mature enterocytes near the tips of the villi, suggesting that differentiated enterocytes express factors required for efficient infection and replication. The severity and localization of intestinal infection vary among animal species; however, the pathologic changes are primarily limited to the small intestine. Rotavirus infection is associated with few visible lesions, some lesions such as enterocyte vacuolization and loss, or significant histopathologic changes such as villus blunting and crypt hyperplasia. Inflammation is mild compared with that observed for other intestinal pathogens, especially bacterial pathogens. In many cases, no clear correlation exists between the degree of histopathologic changes and the severity of diarrheal disease. Even in species where histopathology can be significant, frequently significant diarrhea occurs before the observation of intestinal pathology (Chasey., 1977).

Pathogenesis of rotavirus infection is multifactorial and both host and viral factors affect the outcome of disease. Thus, the age of inoculation of animals can result in

biliary atresia, diarrhea and some extraintestinal replication of virus, or asymptomatic infection (no diarrhea) but extraintestinal replication of virus. Analysis of different virus reassortants identified several viral proteins as being involved in virulence (VP3, VP4, NSP1, VP6, VP7, NSP2, NSP3, and NSP4). These are thought to have roles in the efficiency of virus replication (VP3, NSP2, VP6, NSP3), shut-off of host protein synthesis (NSP3) and extraintestinal spread of virus (NSP3 and VP6), virus entry into cells (VP4 and VP7), regulation of the induction of (interferon NSP1), and the induction of (diarrhea NSP4). Rotavirus diarrhea has been attributed to different mechanisms, including malabsorption secondary to destruction of enterocytes, villus ischemia, a virus-encoded toxin (NSP4), and activation of the enteric nervous system (Kapikian and Chanock., 1990).

Discovery of NSP4 as the first viral enterotoxin is of interest because it shows this protein has pleiotropic properties besides its intracellular role in viral replication and morphogenesis. In 1996, NSP4 was shown to induce age-dependent diarrhea in mice that mimics disease caused by rotavirus infection, and this has been confirmed for the (NSP4 proteins) from several (group A and non group A viruses). These results explain how NSP4 might function as a virulence factor as determined by analyzing reassortant viruses in gnotiobiotic pigs. A model for NSP4 function as an enterotoxin made several predictions that have been confirmed (Kapikian and Chanock., 1990).

The predictions that NSP4 would be released from rotavirus-infected cells and have paracrine effects on adjacent uninfected cells have been confirmed in vitro and in vivo. First, the mechanism of (NSP4 enterotoxin function) has been shown by extracellular administration of NSP4 to the intestinal mucosa or to crypt cells from mice, and to human intestinal cell lines, which results in triggering a signal

transduction pathway leading to mobilization of intracellular calcium by a PLC-dependent pathway and chloride secretion (Ball *et al*., 1996).

In vivo, in mice infected with a murine rotavirus strain, NSP4 is detected both in cells at the tips of the intestinal villi (together with other structural proteins) as well as by itself at the basolateral surface of cells and in cells in the lower part of intestinal villi. The basolateral localization of NSP4 is consistent with the observation that NSP4 interacts with (laminin Î²-3) and fibronectin. A role for the enteric nervous system in rotavirus diarrhea has been shown by the ability to attenuate rotavirus-induced diarrhea in mice and children with drugs that block this pathway, and NSP4 or other mediators released from virus-infected cells may mediate this effect directly or by stimulating responses from enterochromaffin cells. These results suggest common mechanisms between diarrheas induced by viruses and bacteria not previously appreciated. Antibody to NSP4 can also attenuate rotavirus-induced diarrhea in mice. A role for NSP4 in inducing diarrhea in children remains to be proved (Parashar *et al*., 2003).

2.10. Epidemiology of rotavirus infection:

2.10.1. Rotavirus Infections in Children:

Rotaviruses were the major etiologic agents of serious diarrheal illness in infants and young children throughout the world in both developed and developing countries (Malek *et al*., 2010).

Although rotavirus diarrhea occurs with high frequency in the developed countries, mortality is low. In the United States (USA), rotaviruses cause about (5 to 10%) of all diarrheal episodes in infants and children under (5 years) of age; however, these viruses account for (30 to 50%) of the severe diarrheal episodes. In this age group, it is estimated that, in the United States, rotaviruses are responsible annually for more than (3 million episodes) of diarrheal illness, (500,000) visits to a medical

practitioner, (60,000 to 70,000) hospitalizations (including those with nosocomial rotavirus discharge diagnoses), and (20 to 40 deaths), (Miller and McCann., 2000). Thus, the risk of rotavirus illness per child under (5 years) of age during the (first 5 years) of life in the United States ranges from practically every child experiencing rotavirus illness of varying severity, to the death of (1 in 100,000) children in this age group (Glass *et al*., 2006).

A high morbidity but low mortality rate can be attributed to the development during the 1940s of effective means for replacement of fluid and electrolytes lost during illness and their routine use in developed countries. This improvement in therapy has brought about a marked decrease in mortality from all forms of infantile diarrhea. In developed countries, the widespread distribution of rotaviruses in the community is indicated by the universal acquisition of serum antibodies to these viruses at an early age. The high prevalence of rotavirus antibodies is maintained into adult life, suggesting that subclinical rotavirus reinfection occurs commonly. A similar pattern of acquisition and maintenance of rotavirus antibody has also been observed in many other parts of the world (Maiklang *et al*., 2012).

In developing countries, rotaviruses are documented consistently as the leading cause of life-threatening diarrhea . The global burden of rotavirus diarrheal disease in infants and young children under (5 years) of age worldwide, but predominantly in developing countries, is estimated to be more than a (100 million episodes), over (20 million outpatient) visits, and a staggering more than (600,000 deaths) (Giaquinto *et al*., 2012).

2.10.2. Rotavirus Infections in Adults:

Adults appear to undergo rotavirus reinfections commonly, but characteristically with minimal or no clinical manifestations. Although subclinical rotavirus infection is the most common outcome in adults, rotavirus gastroenteritis in adults

has been described in army recruits in Finland as well as in patients and staff in hospitals in various countries. Several outbreaks in geriatric groups had a high attack rate and some fatalities. Nonetheless, these outbreaks are unusual, because of the usually persisting high level of rotavirus immunity that most adults have acquired from previous infections. Other outbreaks have been described but these are considered to be unusual, isolated events. Rotavirus infections have also been associated with traveler's diarrhea in certain settings. These viruses, however, are not considered to be important etiologic agents of this disease (Ball *et al*., 1996).

Group B (adult diarrheal rotaviruses [ADRV]) rotaviruses have been implicated in several large outbreaks of severe gastroenteritis in adults in various parts of China involving (12,000 to 20,000) individuals. Affected individuals developed cholera like, severe, watery diarrhea, and a few elderly patients died. Group B rotaviruses have also been associated with severe diarrheal illnesses in adults and in a few children in Bangladesh. They have also been detected in adults and children in India (Ball *et al*., 1996).

2.10.3. Nosocomial Infections:

Nosocomial rotavirus infections occur frequently. Approximately one of every five rotavirus infections appeared to be hospital acquired. More recently, the important contribution of nosocomial infections to the composition of hospital discharge diagnoses attributed to rotaviruses was analyzed from various studies: a median of (27%) of patients in developed countries and (32%) in developing countries discharged with a diagnosis of rotavirus diarrhea were likely to have acquired rotavirus infection while in the hospital (Chiu *et al*., 1997).

Nosocomial infections have also been described in various neonatal nurseries around the world. These are usually subclinical, although a nosocomial outbreak of rotavirus gastroenteritis was described in a newborn nursery in Italy as well as in

various neonatal intensive care units in the United States. (Chiu *et al*., 1997).

2.10.4. Transmission:

Rotaviruses are transmitted by the fecal-oral route. Oral administration of rotavirus-positive stool material induces diarrheal illness in adult volunteers. Nevertheless, speculation continues whether rotaviruses are transmitted also by the respiratory route. Evidence for this is circumstantial and rests on the following:

1. The rapid acquisition of rotavirus antibodies in the first few years of life in all settings, regardless of hygienic standards.
2. A few large outbreaks in which a fecal-oral spread could not be documented.
3. The occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis (Blutt *et al*, 2006).

The source of rotavirus infection for the young infant who is normally not in contact with other infants and young children with gastroenteritis is not well documented. Most likely, infection is acquired from an older sibling or parent with subclinical infection. Shedding of rotavirus from the intestinal tract before onset of diarrhea or following cessation of diarrhea has been well documented (Blutt *et al*, 2006).

Resistance to physical inactivation may contribute to the efficient transmission of the human rotaviruses. This inference is drawn from the observed stability of various human and animal rotaviruses at ambient temperatures and from the low infectious dose for humans. For example, calf rotavirus present in feces retained infectivity for (7 months) when kept at room temperature. Other observations that suggest environmental contamination as sources of infection include:

1. The persistence of rotavirus infection in certain newborn nurseries.
2. The high frequency of nosocomial rotavirus infection in hospitals (Kosek *et al*., 2003).

The ability of rotaviruses to survive on various surfaces under different conditions may contribute to the rapid spread of these agents. Although rotaviruses have been detected in raw or treated sewage, it is unlikely that contaminated water plays an important role in transmission of group A rotaviruses. High relative humidity (about 80%) results in a rapid loss in human rotavirus infectivity. Effective disinfection of contaminated material and careful hand washing constitute important measures to contain rotavirus infection, especially in a hospital or institutional setting (Kosek *et al*., 2003).

2.10.5. Incubation Period:

The incubation period of rotavirus diarrheal illness has been estimated to be less than (48 hours). The onset of experimentally induced rotavirus diarrhea in adult volunteers occurred (2 to 4 days) after challenge. One of the volunteers who developed diarrhea (3 days) after virus administration developed a fever and vomited on the first day after challenge, thus indicating from such studies that the incubation period of the illness under experimental conditions was (1 to 4 days) (Chiu *et al*., 1997).

2.10.6. Distribution:

2.10.6.1Geographic Distribution and Seasonal patterns:

Rotaviruses have been detected throughout the world wherever they have been sought. Furthermore, these viruses consistently constitute the major etiologic agents of severe infantile diarrhea in every country where this disease has been studied by appropriate techniques. Rotaviruses display a seasonal pattern of infection in developed countries, with epidemic peaks occurring in the cooler months of each year (Kosek *et al*., 2003).

The cause for this striking seasonal pattern is not known, but the influence of low relative humidity in the home has been suggested as a factor facilitating the survival of rotaviruses on surfaces. A correlation of relative humidity with the

temporal pattern of infection has not been observed, however, in every epidemiologic setting (kafi *et al*., 2013).

2.10.6.2. Age, Sex, Race, and Socioeconomic Status

Rotavirus gastroenteritis sufficiently severe to require hospitalization characteristically occurs most frequently in infants and young children from approximately (6 months to 2 years) of age. Infants under (6 months) of age experience the next highest frequency of such illness. In certain studies, this age group had the highest frequency. In one USA study, the age distribution of patients admitted to the hospital with gastroenteritis of any cause was different for black and nonblack patients; (59%) of all black patients admitted for gastroenteritis were younger than (6 months) of age. This difference was also reflected in admissions for rotavirus diarrhea. Because the children were predominantly from inner-city areas, these differences may have reflected the effects of crowded living conditions, which may have allowed earlier and more efficient transmission of the virus (Parashar *et al*., 2003).

Malnutrition is considered to play an important role in increasing the severity of clinical manifestations of human rotavirus infections. This phenomenon has been reproduced in an experimental mouse and a piglet model. It has also been suggested that repeated diarrheal infections may be a precipitating factor in the development of malnutrition by damaging intestinal mucosa so that absorptive cells are compromised over an extended period. It is striking that the World Health Organization (WHO) estimates of the causes of the deaths of the (10.6 million children) under (5 years) of age cited earlier (with 18% attributed to diarrhea) found undernutrition to be an underlying cause in (53% of the 10.6 million deaths (Parashar *et al*., 2003).

2.11. Immunity to Rotavirus:

The immunologic effectors that prevent rotavirus disease have been partially identified, particularly through studies with animal models, but in humans remain poorly understood. Because rotaviruses replicate in intestinal enterocytes, resulting in the associated GI symptoms, it is generally assumed that effector mechanisms must be active at the intestinal mucosa. The most obvious immunologic effector is (secretory IgA). Following infection of mice with a high dose of heterologous rotavirus, a large fraction of all (IgA cells) in the lamina propria of the intestine can be rotavirus-specific. Furthermore, protection against rotavirus infection in orally immunized mice correlates with levels of intestinal (stool) and (serum rotavirus IgA) but not serum rotavirus IgG (Patrick *et al*., 2002).

In humans, titers of serum rotavirus (IgG and IgA) as well as intestinal rotavirus IgA correlate with protection following natural infection. However, the titer of any isotype of rotavirus-specific antibody could not be consistently correlated with protection after either natural infection or vaccination ((Blutt *et al*, 2006).

Thus, the possibility remains that rotavirus antibody is merely an indicator of protection and not the actual effector. The most obvious mechanism of protection by antibody is by virus neutralization. Passive protection has been definitively linked with the consumption of neutralization antibody in both animal and human studies. Evidence that active immunity induced by oral inoculation with live rotavirus or natural rotavirus infection is due to neutralizing antibody is varied.

Most data from animal studies indicate that classic neutralization is not the only mechanism of protection. The most immunogenic protein is VP6, which does not appear to stimulate neutralizing antibody responses. Evidence, however, suggests that (IgA antibodies) directed at VP6 are protective by as yet incompletely understood mechanisms (Patrick *et al*., 2002).

Vaccination with either virus-like particles (VLPs) that lack the outer capsid proteins and thus, do not induce neutralizing antibody, or a (chimeric VP6 protein)

can also elicit protective immunity against infection in adult mice. Passive protection against murine rotavirus disease in neonatal mice has also been produced by adoptive transfer of (CD8+ T cells) from spleens of mice previously infected (orally) with either homologous or heterologous rotavirus strains. Similarly, (CD8+ splenic or intraepithelial lymphocyte) from rotavirus-infected mice can eliminate chronic rotavirus shedding in severe combined immunodeficiency (SCID) mice. Thus, at least passive protection against rotavirus disease and resolution of rotavirus shedding can be promulgated with cytotoxic T cells (Patrick *et al*., 2002).

2.12. Clinical Features of rotavirus infections:

Rotavirus infections produce a spectrum of responses that varies from subclinical infection to mild diarrhea to severe and potentially fatal dehydrating illness. A sudden onset of symptoms typically manifests in children (1 to 2 days) after infection with RV. The clinical picture is characterized by (4 to 7 days) of acute febrile illness, vomiting, and watery, nonbloody diarrhea (Parashar *et al*., 2003).

This combination can lead to rapid dehydration without appropriate intervention. Secondary infections with RV are clinically milder or asymptomatic (Soriano-Gabarro *et al*., 2006).

Adults more frequently experience asymptomatic RV infection than children; however, when symptoms are reported in adults, the most common are diarrhea, fever, headache, malaise, nausea, or cramping. Despite adults having milder symptoms of RV, they are still infectious, and thus can transmit the infection to susceptible children (Vesikari *et al*., 1981).

2.13. Laboratory diagnosis of Rotavirus infection:

The clinical manifestations of rotavirus illnesses are not sufficiently distinctive to permit diagnosis on this basis alone. The diagnosis, therefore, requires detection of virus or viral antigen, demonstration of a serologic response, or both. The

epidemiologic pattern of rotavirus disease at any one time may suggest this diagnosis, but laboratory confirmation is required (Blutt *et al*, 2006).

Many assays have been developed for the detection of rotavirus in stools. Specimens from the first to the fourth day of illness are optimal for virus detection using conventional assays (e.g., EM or ELISA); however, shedding can continue for (upto 3 weeks), depending on the duration of symptoms, and may be detected longer by highly sensitive assays (e.g., RT-PCR) (Blutt *et al*, 2003).

Viral shedding as detected by conventional techniques, such as ELISA, usually coincides with the duration of diarrhea, but diarrhea can continue for an additional (2 to 3 days), (El Assouli *et al*., 1992).

Initially, direct visualization of stool material by EM was employed for rotavirus detection. It had the advantage of high specificity because rotaviruses have a distinctive morphologic appearance. The EM continues to be important in the diagnosis of rotaviral diseases and is frequently used to resolve discrepancies in results from other techniques (Blutt *et al.*, 2003).

When only a few specimens are to be examined for rotavirus, EM is the most rapid diagnostic method because fecal specimens can be stained with phosphotungstic acid and examined directly within a few minutes of collection. Direct EM examination of stools permits detection of rotavirus in (80 to 90%) of the virus-positive specimens as detected by conventional techniques. IEM is not necessary for the detection of rotaviruses because the particle has such a distinct morphologic appearance that it can be readily identified without the use of an immune serum. Modified EM methods have been developed, however, which may prove useful under selected conditions (Fujii *et al*., 1992).

Various automated methods are available for the detection of rotaviruses in stool specimens. The method of choice in many laboratories is the confirmatory ELISA, because it is sensitive, does not require specialized equipment, and has a built-in control for nonspecific reactions. For example, a preimmunization or postimmunization (with rotavirus antigen) animal serum is used as the solid-phase precoat; the preimmunization serum acts as the control because a fecal specimen must exhibit a significantly greater reactivity in the well that is coated with the postimmunization animal serum to be considered positive (Mackow., 1995).

Some laboratories have routinely used other methods for virus detection, such as counter immunoelectroosmophoresis, gel electrophoresis of rotavirus RNA, reverse passive hemagglutination assay (RPHA), latex agglutination, or an ELISA in which polyclonal or mAb are used. Commercial kits are available for ELISA, latex agglutination, RPHA, and immunoblot enzyme assay, but assays that do not include confirmatory reagents may yield false positive results (Nakata *et al*., 1987). An enzyme immunoassay has also been developed for detection of group B or group C rotavirus and for measurement of antibodies directed against these viruses (Suzuki *et al*., 1987).

A dot hybridization assay for detection of rotaviruses has been developed based on in situ hybridization of labeled rotavirus ssRNA transcripts to heat-denatured rotavirus RNA immobilized on a nitrocellulose membrane. The method is highly specific, yielding results that are concordant with those obtained with other tests such as EM, RNA analysis, and ELISA. In a comparative study of ELISA and dot hybridization for the detection of rotavirus in various dilutions of fecal specimens, the dot hybridization method was (10 to 100 times) more sensitive than the confirmatory ELISA. The limit of detection for purified viral RNA by the dot hybridization procedure was (8 pg) in a homologous reaction (Wyatt *et al*, 1982).

A variety of other research laboratory techniques are now used for the detection of group A rotaviruses. The most important is RT-PCR, which is (100,000 times) more sensitive than the standard electropherotype method and (5,000 times) more sensitive than hybridization. For example, using an RT-PCR based assay, virus shedding was detected from (4 to 57 days) after diarrhea onset, and (30%) of the children shed virus detectable by the assay for (25 to 57 days). PCR has also been applied to the detection of (groups B and C rotaviruses). Flow cytometry is described as another sensitive method for rotavirus detection. It is possible to recover human rotaviruses from stool specimens directly in cell culture with reasonable efficiency. This method appears to be approximately (75%) as efficient as antigen detection test procedures using conventional procedures. The efficiency of recovery of virus from rectal swabs using cell culture is considerably less. Growth of rotavirus in tissue culture allows the determination of its serotype by neutralization assay. If triple layered particles are present in fecal samples, however, the serotype can be determined by ELISA (for which commercial kits are available) and less often by solid-phase IEM or conventional IEM using absorbed antisera or {serotype-specific (VP7) mAb} for each of the four major serotypes. It may be necessary to use several mAb directed at different epitopes of the same serotype because of epitope polymorphism within a serotype. Serotype-specific mAb are used in a sandwich procedure together with one or more polyclonal antisera (Armah *et al*., 2003).

Rotavirus serotype can also be predicted by nucleotide sequence analysis of the (VP7 gene) because of the high degree of conservation of sequence in variable regions among rotaviruses belonging to the same serotype. The (VP7 serotype) can also be determined by a variety of other methods, including RT-PCR, hybridization with single gene substitution (VP7 reassortants) used as probes, and hybridization with oligonucleotide probes prepared from the (VP7 sequence) of human rotavirus

serotype (1, 2, 3, or 4) or additional strains. The use of RT-PCR has enabled the genotyping of rotavirus-positive specimens that could not be typed by ELISA. Also, the (VP4 genotype) can be identified by RT-PCR or RT-PCR derived probes, and the (VP4 serotype) can be determined by neutralization or ELISA using monoclonal or other serotype specific antibodies (Dongdem *et al*., 2010).

A variety of techniques are available to measure a serologic response to rotavirus infection, such as IEM, complement fixation (CF) tests, immunofluorescence tests (IF), immune adherence hemagglutination assay, ELISA, neutralization, hemagglutination inhibition, inhibition of reverse passive hemagglutination, enzyme-linked immunospot (ELISPOT) assay, and immunocytochemical staining assays. The CF method is about as efficient as the other methods for detecting an antibody response in patients between (6 and 24 months) of age, but it is not as efficient in adults or in infants under (6 months) of age. In these age groups, (IF, ELISA IgG, ELISA IgM, and ELISA IgA) are more efficient. Because IgA does not cross the placenta, a (rotavirus IgA), ELISA has been particularly effective in demonstrating serologic responses in young infants who possess passively acquired maternal (IgG antibodies). This assay can also be used for measuring antibodies in saliva, duodenal fluids, and stools. Fecal rotavirus (IgA antibody) levels exhibit a direct relationship with duodenal (IgA antibody) levels. In addition, a rise in fecal rotavirus (IgA antibodies) measured by ELISA reflects an intestinal neutralizing antibody response. It has been suggested that (copro-IgA) conversion in feces obtained weekly would be a more sensitive indicator of rotavirus reinfection than seroconversion or detection of virus. Based on observations from a clinical study in infants and children, it was suggested that (rotavirus IgA) in serum reflects the immunologic status of the intestinal lumen with respect to this virus. Early diagnosis can also be made by ELISA, which can

detect a specific (serum IgM response) during the acute phase of illness (Cairncross *et al*., 2010).

Neutralizing antibodies are detected by plaque reduction, neutralization of IF foci, neutralization of virus as determined by quantitation of viral antigen by ELISA, or inhibition of cytopathic effect in roller-tube cultures. Neutralization assays yield the most meaningful information about the identity of the infecting rotavirus and the development of a serotype-specific antibody response. The plaque reduction neutralization assay is more sensitive than tube neutralization for detection of antibody, although the latter is slightly more efficient for detecting a seroresponse. A competition solid-phase immunoassay that measures epitope-specific immune responses to individual rotavirus serotypes has proved to be especially useful in evaluating immune responses at the level of individual epitopes. This technique uses the test serum as the blocking reagent and the individual mAb as the detecting reagent (Yolken and Wilde, 1994).

Detection of rotavirus or the demonstration of a serologic response or both to rotavirus in an individual patient does not necessarily establish an etiologic association of rotavirus with that patient's illness. This is important in all age groups but especially in newborns and adults, who commonly undergo inapparent infection (Yolken *et al*, 1988).

2.14. Treatment:

The primary aim of treatment of rotaviral gastroenteritis is to replace fluids and electrolytes lost by vomiting and diarrhea. Intravenous fluid administration has been used successfully for many years in treating dehydration from diarrhea. Because facilities for parenteral administration of fluids and electrolytes are not readily available in many parts of the world, intensive efforts have been made to evaluate the efficacy of oral fluid replacement therapy. Various formulations of oral rehydration salts (ORS) solutions have been shown to be effective in the

treatment of dehydration caused by rotavirus diarrhea, with some minor variations in their efficacy. For example, in a double-blind study, oral rehydration therapy with electrolyte solutions containing either glucose or sucrose has been shown to be highly effective for rotavirus gastroenteritis. In another study of oral rehydration of infantile diarrhea of mixed etiology, all patients who were fed glucose electrolyte solution and (92%) of those fed sucrose electrolyte solution were treated successfully. The sucrose group experienced a slower correction of electrolyte abnormalities, and a larger proportion of patients required more than (24 hours) of therapy. It was concluded that sucrose could be used as a substitute for glucose, but that glucose electrolyte solutions were preferable (Patrick *et al*., 2002).

The standard WHO oral glucose electrolyte formula is composed of the following: (sodium, 90 mmol/L); (chloride, 80 mmol/L); (potassium, 20 mmol/L); (citrate, 10 mmol/L); and (glucose, 111 mmol/L), resulting in an overall osmolarity of (310 mOsm/L. (Bicarbonate, 30 mmol/L), can be substituted for citrate, (10 mmol/L). Recently, an ORS solution containing a reduced sodium concentration was found to be more effective than the standard WHO solution. Thus, a solution containing sodium, (75 mmol/L; chloride), (65 mmol/L; potassium), (20 mmol/L; citrate), (10 mmol/L; and glucose), (75 mmol/L), resulting in an overall osmolarity of (245 mOsm/L) (reduced osmolarity ORS solution), was compared with the standard WHO ORS solution in infants with noncholera diarrhea in a multicenter trial. The proportion of infants who required unscheduled intravenous therapy was significantly lower in the group receiving the reduced osmolarity ORS (required in 10%) than among those receiving the WHO ORS solution (required in 15%). After correction of the initial calculated fluid loss by oral rehydration solution, either water or fluids without added electrolytes (e.g., breast milk or some other form of low-solute feeding) should be administered orally in addition to the

oral rehydration solution. When this regimen is used, both continued diarrheal fluid and electrolyte losses will be replaced and normal daily fluid requirements will be maintained (Osonuga *et al*., 2013).

Rice-based solutions have been compared with glucose-based oral rehydration solutions in infants and young children hospitalized with mild to moderately dehydrating diarrheal illnesses associated with various pathogens, including rotavirus. Each was found to be effective, but the rice-based solution was more effective in decreasing total stool output and increasing absorption and retention of fluid and electrolytes. Later, glucose-based and rice-based oral rehydration solutions were each found to be effective in treating mild to moderate dehydration caused by various pathogens, including rotavirus. Although a rice-based solution was associated with less stool output during the (first 6-hour period), this effect did not persist after the initial (12 hours) of therapy, and it was concluded that this difference was of minor clinical importance (Patrick *et al*., 2002).

If oral rehydration does not correct the fluid and electrolyte loss, or if the patient is severely dehydrated or in shock, intravenous fluids must be given immediately. Oral rehydration therapy should not be given to patients with depressed consciousness because of the possibility of fluid aspiration. In a practice parameter for the management of the acute diarrhea in children (1 month to 5 years) of age, the American Academy of Pediatrics (AAP) does not recommend the use of loperamide, anticholinergic agents, bismuth subsalicylate, adsorbents, or lactobacillus-containing compounds. Also, the use of opiates as well as opiate and atropine combination drugs for treatment is contraindicated by the AAP ((Patrick *et al*., 2002).

Human milk, containing rotavirus antibodies, has been used successfully for treatment of children who are immunodeficient with chronic rotavirus infection and illness. In contrast, colostrum or milk concentrate from cows immunized with

human rotavirus was not effective for the treatment of acute rotavirus gastroenteritis in children, although a decrease in the duration of virus shedding was observed. Daily oral administration of rotavirus antibody-containing bovine colostrum, however, appeared to exert a protective effect during an orphanage outbreak of rotavirus diarrhea. The effect of a single oral dose (300 mg/kg body weight) of gamma globulin in infants hospitalized for gastroenteritis (70% rotavirus associated) was evaluated in a double-blind, placebo-controlled trial. The treatment group had a significantly shorter duration of diarrhea, viral excretion, and hospital stay. In addition, in another study, the administration of human serum immunoglobulins by the alimentary route to two infants with prolonged rotavirus diarrhea of (4 or 7 months) duration was effective in clearing the virus and the associated diarrhea. Also, bovine colostrum prepared by immunizing pregnant cows with human rotavirus strains of (G1, G2, G3, or G4) specificity was given orally to patients (4 to 6 months) of age with rotavirus diarrhea and was found to significantly reduce the duration and frequency of diarrhea as well as the consumption of oral rehydration solution (Patrick *et al*., 2002).

Bismuth subsalicylate (BSS) (20 mg/kg) given orally (5-times a day) for (5 days) as an adjunct to rehydration therapy shortened the course of disease in children (4 to 28 months) of age hospitalized with acute rotavirus diarrhea in a prospective double-blind, placebo-controlled treatment trial. Because of the association between the use of salicylates and Reye syndrome, the authors reviewed the possibility of such an association with BSS or other nonacetylsalicylic acid salicylates and could find none. In addition, blood salicylate and bismuth levels attained in the treatment group were well below those considered toxic. As noted before in considering the use of various medications for symptomatic treatment of acute diarrhea in children, in its practice parameter for the management of acute diarrhea in the (1 month to (5 years) age group, the AAP, however, does not

recommend the use of bismuth subsalicylate. The efficacy of several broad-spectrum antiviral agents has been examined as inhibitors of rotavirus replication in vitro. In this survey, various adenosine analogs were found to have antirotavirus activity, and it was suggested that this activity resulted from inhibition of S-adenosyl homocysteine hydrolase, an enzyme involved in regulating methylation required for maturation of viral mRNA (Patrick *et al*., 2002).

2.15. The rotavirus vaccination:

There are two rotavirus vaccines authorized for use by the European Medicines Agency, Rotarix and RotaTeq. Both are highly effective at preventing rotavirus infection in infants. However, the vaccines are not known to be interchangeable and a course of vaccine started with one product should be completed with the same vaccine to achieve full protection. Neither vaccine contains thiomersal nor any adjuvant (Enweronu *et al*., 2012).

Rotarix is the vaccine offered as part of the UK national childhood immunisation programme. It is a live attenuated vaccine derived from a virus initially isolated from a (15-month-old child) and then attenuated by serial cell culture passage. In clinical trials Rotarix has been shown to protect against gastroenteritis due to rotavirus serotypes G1P8, G2P4, G3P8, G4P8, and G9P8; some efficacy against uncommon rotavirus genotypes G8P4 and G12P6 has also been demonstrated. The vaccine is over (85%) effective at protecting against severe rotavirus gastroenteritis in the (first 2 years) of life. The effectiveness of the vaccine in protecting against any rotavirus infection varies between the serotypes listed (Glass *et al*., 2006).

Rotarix vaccine is supplied as an oral suspension of clear colorless liquid in an oral applicator containing the suspension solution (1.5 ml) with a plunger, stopper and a protective tip cap. The full course of vaccine is composed of (2 doses), the first dose of (1.5 ml) of Rotarix vaccine at (2 months) (approximately eight weeks) of age and the second dose of (1.5 ml) at least four weeks after the first dose. It is

preferable that the full course of (2 doses) of Rotarix be completed before (16 weeks) of age, allowing at least (4 weeks) between the (1^{st} and 2^{nd} dose). This is to provide early protection and avoid temporal association between vaccination and intussusceptions (Ruiz-Palacios *et al*., 2006).

In line with recommendations from WHO, infants should only receive the first dose of Rotarix if they are younger than (15 weeks) of age. Infants who receive the 1^{st} dose before (week 15) should receive the 2^{nd} dose of vaccine by (24 weeks) of age. If the course is interrupted, it should be resumed but not repeated, provided that the second dose can be given before the (24 week) cut-off (*et al*., 2005).

Children who inadvertently receive the first dose of rotavirus vaccine at age (15 weeks) or older should still receive their 2^{nd} dose at least (4 weeks) later - providing that they will still be under (24 weeks) of age at the time. The reason for the (15 week) age limit is to minimize a potential risk of intussusceptions. No specific clinical action needs to be taken if the 1^{st} dose of vaccine is inadvertently given after (15 weeks and zero days) of age or if the second dose is given after (24 weeks) of age. For both situations, immunizers should be reminded of the age restrictions for Rotarix, even if infants are unable to start or complete the (2 dose) schedule as a consequence of these restrictions (Vesikari *et al*., 2006).

RotaTeq is a vaccine manufactured by Merck and Co. Inc., (Merck) licensed by FDA in 2006 for the prevention of rotavirus gastroenteritis in infants (6 weeks to 32 weeks) of age. RotaTeq is a live, reassortant, pentavalent human–bovine rotavirus vaccine containing 5 rotavirus reassortants: G1, G2, G3, G4 and P8 derived from human and bovine viral species (Patel *et al*., 2012).

RotaTeq significantly reduced the number of hospitalisations and emergency department visits for rotavirus gastroenteritis caused by individual serotypes G1,

G3, G4 and G9.The vaccine is administered as a (3-dose series) to infants between the ages of (6 to 32 weeks). The first dose of RotaTeq should be administered between 6 and 12 weeks of age. with the subsequent doses administered at(4- to 10-week) intervals. The third dose should not be given after (32 weeks) of age. RotaTeq should not be administered to infants with a demonstrated history of hypersensitivity to the vaccine or any component of the vaccine or Infants with Severe Combined Immunodeficiency Disease (SCID) should not receive RotaTeq. Infants with a history of intussusception should not receive RotaTeq (Cortes et al., 2011).

RV5 (RotaTeq) is a live oral vaccine manufactured by Merck and licensed by the Food and Drug Administration in February 2006. RV5 contains five reassortant rotaviruses developed from human and bovine parent rotavirus strains. Each (2-mL vial) of vaccine contains approximately (2 x 106 infectious units) of each of the five reassortant strains. The vaccine viruses are suspended in a buffer solution that contains sucrose, sodium citrate, sodium phosphate monobasic monohydrate, sodium hydroxide, polysorbate 80, and tissue culture media. Trace amounts of fetal bovine serum might be present. The vaccine contains no preservatives or thimerosal (Patel *et al*., 2012).

Fecal shedding of vaccine virus was evaluated in a subset of persons enrolled in the phase III trials. Vaccine virus was shed by (9% of 360 infants) after dose 1, but none of (249 and 385 infants) after doses 2 and 3, respectively. Shedding was observed as early as 1 day and as late as 15 days after a dose. The potential for transmission of vaccine virus was not assessed in trials. In a post-licensure evaluation in the United States, stool samples were collected from infants for 9 days following the first dose. Rotavirus antigen was detected in stool of (21% of

103 infants), as early as day 3 post vaccination and as late as day 9(Tate et al., 2011).

2.16. Previous studies:

A study conducted in New Delhi, India by Anita C, *et al*, revealed that the result of rotavirus G genotyping indicates G1 (60%) was the most predominant (VP7 type), followed by G2 (16%), G9 (8%) and G3 (3%). Two cases of (G12 genotype) were also observed. Most rotavirus positive patients (91%) were (<12 months) of age (Anita *et al*., 2010).

Study done by Andreas K, *et al*, in Greece showed that the majority of rotavirus gastroenteritis cases occurred in children aged less than 24 months (53%). rotavirus infection peaked between December and May (31.4%). The most common rotavirus G genotypes were G4 (59.6%). The median duration of rotavirus gastroenteritis hospitalization was 4 days (range 1–10 days). The median prolongation of hospitalization due to nosocomial rotavirus gastroenteritis was 5 days (range 4–7 days) (Andreas *et al.,* 2014).

Study done by Mark A, *et al*, in Eastern Mediterranean Region showed that Among patients with diarrhea, rotavirus was detected in (40%) of inpatients and (23%) of outpatients. By (3 years) of age, (75%) of children experienced a documented rotavirus infection. Circulation of rotavirus occurred year-round, and no clear relationship between the timing of the rotavirus peak with either season or latitude was observed (Mark *et al.,* 2010).

Study conducted by Magzoub A, *et al*, in Sudan revealed that the highest infection rate was seen among 75.2% of children up to 12 months of age. Children of illiterate parents were more infected with rotavirus than children of educated parents. Severe dehydration present among 70% of infected children with rotavirus (Magzoub *et al*., 2013).

Study conducted by Alaaeldeen B, et al, in Sudan revealed that Rotavirus frequency was 21% (20% vaccinated and 22% non-vaccinated) among children (<15 years). There is incidence of rotavirus infection among vaccinated children with Rotarix vaccine against rotavirus infection. The use of universal vaccine (multiple serotypes) is the most important preventive strategy (Alaaeldeen *et al.*, 2015).

Study conducted by Kafi SK, *et al*, in Sudan revealed that (33%) of the children with diarrhea were found to be positive for rotavirus infection. The highest rate of infection (33.7%) was in the age group (6 - 12 months). Rotavirus infection was found to occur all over the year but higher in November to January (kafi *et al*., 2013).

Study done by Valentine N, *et al*, in Northern Cameroon revealed that (42.8%) of the children below (5 years) had group A rotavirus infection. Children below (24 months) were most affected (44.7%), while the age group (49-60 months) had the lowest prevalence (25%). The group A rotavirus prevalence was (44.6%) in the urban and (28.9%) in the rural settings of our study. It was observed that the proportion of children with diarrhea who had rotavirus accompanied with fever and vomiting in the outpatient group and inpatient group were (13.0 and 28.6%) respectively, P=0.03 (Valentine *et al*., 2012).

Study conducted in Nigeria by Surajudeen A, *et al*, revealed that Males excreted rotavirus at a significant higher rate than females ($P < 0.05$). Rotavirus excretion was highest when all three symptoms (diarrhea, fever and vomiting) occurred in the same child and lower when 2 symptoms occurred together (diarrhea and vomiting, diarrhea and fever), and lowest when diarrhea occurred alone. Playing with toys, attending day care, distance of source of water from toilet, eating of food not requiring cooking and playing with other children may serve as predisposing factors of rotavirus disease in these children (Surajudeen *et al.,* 2011).

Study conducted in Iran by Roghayeh K, *et al*, revealed that the prevalence of nosocomial infection due to rotavirus was (26.25%), which a considerable prevalence is compared to similar studies which reported a prevalence of (27.7%), (19.4, and 14.6%). Overall, (15%) of the (21 children) with positive rotavirus antigen in their stools had acute diarrhoea during hospitalization and up to (72 hours) after discharge (symptomatic nosocomial infection), and (11.25%) of all children (n=80) studied had asymptomatic nosocomial infection (Roghayeh *et al.*, 2007).

Study done by Yati S, *et al,* in Indonesia revealed that Among children hospitalized for diarrhea, dehydration was more common among those who tested positive for rotavirus than among those who did not (91% vs 82%; *P* ! .05), as was vomiting (86% vs 67%; *P* ! .05). Children aged 6–23 months experienced 72% of all rotavirus episodes. Rotavirus prevalence increased slightly in the cool, dry season (Yati *et al.*, 2009).

CHAPTER THREE

MATERIALS AND METHODS

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3.1. Study Design

The present work is a cross-sectional, observational Hospital based study, conducted to characterize human rotavirus strains circulating among children with Diarrhea at Mohammed Alamin Hamid Pediatric Hospital using molecular methods.

3.2. Study area

Study was conducted at Mohammed Alamin Hamid Pediatric Hospital, Which was founded in 1986, located in Omdurman, Khartoum state, west of Omdurman Teaching Hospital, with an area of 6000 square meters. The hospital contains 10 medical units, as well as specialized clinics in Asthma, Gastroenterology, Diabetes and Tuberculosis. The hospital has more than 700 workers and an average annual pediatric frequency of 54,000 children per year, the hospital has 18 patient wards of 321 beds. The practical was done at the institute of endemic diseases, Khartoum University.

3.3. Study period

The study was conducted during the period from January 2015 to November 2017.

3.4. Study population

Children under the age of 5 years, who have symptoms of watery diarrhea, fever, vomiting, and abdominal pain. Diarrhea was defined as the occurrence of three or more liquid stools in a 24 hour period and presence of diarrhea at the time of admission to hospital.

3. 4.1. Inclusion criteria

All children less than 5years of age with symptoms of acute watery diarrhea, fever, vomiting, and abdominal pain admitted for treatment of acute gastroenteritis.

3. 4.2. Exclusion criteria

The exclusion criteria were as follows: Hospital-acquired diarrhea defined as onset of diarrhea more than (48) hours after hospitalization; bloody diarrhea; and chronic and/or persistent diarrhea, which defined as diarrhea that lasted for more than two weeks.

3.5. Sample size

A total of (150 children) were recruited to participate in this study all of them were (<5 years) old with acute diarrhea.

3.6. Data collection tools:

Data was collected using self-administrated pre-coded questionnaire which specifically designed to obtain information that helped in study.

3.7. Ethical Considerations

Verbal consent was taken from selected children mothers after being informed using simple language about the infection, aim and the benefits of the study.

3.8. Study procedures

3.8.1. Specimen collection:

Approximately, (5 ml) of stool was collected from each child under study in clean, wide mouth, leak proof plastic container. The collected specimens were stored at (-20°C) until they further tested.

3.8.2. ELISA for detection of Rotavirus antigen

3.8.2.1. ELISA kit contents (ProSpecT Rotavirus Microplate Assay, oxoid company, United Kingdom).

- Microtiteration plate: 12 strips (8 microwell breakapart strips) coated with a rotavirus specific rabbit polyclonal antibody.A resealable foil pouch containing desiccant is provided for storage of unused microwells. Microwells may be used for up to (16 weeks) after initial opening, provided they are stored correctly in the pouch.

- Enzyme Conjugate: One dropper bottle containing (12 ml) of rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxidase in a buffered protein solution containing antimicrobial agent and blue dye.
- Positive Control: One dropper bottle containing (4 ml) of inactivated bovine rotavirus in buffer containing antimicrobial agent.
- Negative Control: One dropper bottle containing (4 ml) of a tris buffered saline solution, antimicrobial agent and red dye.
- Specimen Dilution Buffer: One bottle containing (120 ml) of a tris buffered saline solution, antimicrobial agent and red dye.
- Wash Buffer: One bottle containing (120 ml) of a (x10) concentrated phosphate buffered solution containing antimicrobial agent and detergent. Dilute (x10) Wash Buffer concentrate to (x1) by adding 1 part concentrate to 9 parts distilled or deionised water. Diluted Wash Buffer is stable for up to (30 days) when stored at (2 to 8°C).
- Colour Substrate: One dropper bottle containing (12 ml) of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer. The Colour Substrate was stored in and used from the light protected bottle in which it is provided. If an aliquot is removed from the original bottle for any reason, do not return unused Colour Substrate to the original bottle.
- Stop Solution: One dropper bottle containing (12 ml) of (0.46 mol/L) sulphuric acid.

3.8.2.2. Principle of the test

The prospect rotavirus test utilises a polyclonal antibody in a solid phase sandwich enzyme immunoassay to detect group specific Antigen present in group rotaviruses. Break-apart microwells are coated with a rotavirus specific polyclonal antibody. Faecal Suspension and control sample were added to the microwell and

Incubated simultaneously with a rotavirus specific polyclonal antibody conjugated to horseradish peroxidase.

Rotavirus antigen present in the specimen is captured between antibody on the solid Phase and the enzyme conjugated antibody. After (60 minutes) Incubation at room temperature, the microwells were washed with working strength wash buffer to remove excess specimen and any unbound enzyme labelled antibody. A chromogen is added to the microwells and incubated for (10 minutes) at room temperature. The presence of specifically bound enzyme labelled antibody in the microwells results in a colour change. Reagents can be dispensed directly from the dropper bottles or poured out for use with multichannel pipettes. If excess reagent has been poured, the excess should be discarded. Do not pour excess reagent back into the bottle. Addition of acid. Colour intensity significantly above background Levels is indicative of the presence of rotavirus antigen in the Specimen or control.

3.8.2.3. Dilution of faecal samples

A (10%) suspension of stool specimen was prepared from each sample by adding approximately of semisolid feces (small pea sized portion) or (100 µl) of liquid sample into (1 ml) sample diluents. The mixture was thoroughly mixed, then transferred to a labeled container using a pipette. The mixed suspension was stored at (2-8°C) for up to (8 days) prior to testing.

3.8.2.4. Test Procedure

1. The reagents were removed from the refrigerator and allowed to reach the room temperature within (30 minutes).
2. The foil pouch was removed and the required number of microplate strips was taken and placed into a microplate strip holder.
3. One well was used for each of the negative and positive control and one well for each specimen.

4. 2 drops (100 µl) were added of each diluted stool specimen, and the controls were added into the corresponding microwells.
5. 2 drops (100 µl) of conjugate were added to each microwell.
6. The plate was covered and the microwells were incubated at (20-30°C) for (60 minutes).
7. The automated washer was programmed to complete (5 wash cycles). After the washers have been correctly calibrated to ensure complete filling and emptying of microwells between each wash. After the final wash, the plate was inverted and tapped on absorbent paper to remove the last traces of wash buffer.
8. 2 drops (or 100 µl) of substrate were added to each microwell.
9. The plate was covered and the microwells were incubated at (20-30°C) for (10 minutes).
10. The Substrate reaction was stopped by adding 2 drops (100 µl) of stop solution to each microwell and mixed before reading the results.
11. The developed coloured product was Read spectrophotometrically at (450 nm)

3.8.2.5. Quality control and spectrophotometric determination

- One positive and one negative control were being included each time the test is performed.
- The negative control value, or mean of the negative control values, should be less than (0.150 absorbance units). The positive control value must be greater than (0.500 absorbance=units).
- The microwells were read photometrically within (30 minutes) of addition of the stop solution.
- The contents of the microwells were mixed and read the absorbance of each microwell using a spectrophotometer set at (450 nm). Ensure the bottoms of the microwells are clean before reading. The reader should be blanked on air before the plate is scanned.

- If the spectrophotometer allows for the use of a reference wavelength (at 620 to 650 nm), dual wavelength reading should be performed.
- The cut-off value was calculated by adding (0.200 absorbance units) to the Negative Control value, or mean value when more than one Negative Control is included.
- The test results were interpreted according to manufacturer as follow:

Positive: clinical sample absorbance value > the cutoff value.

Negative: clinical sample absorbance value < the cutoff value.

Equivocal: clinical sample absorbance value within (0.010 absorbance units) of the cut-off value.These samples should be retested or the patient resampled.

3.8.3. Extraction and purification of viral RNA

Rotavirus RNA was extracted from (150 µl) of (10%) fecal suspension in phosphate buffer saline. The feacal suspension was mixed well then centrifuged at (10,000g)

for (10 minutes). The supernatant was aspirated and subjected to the viral RNA extraction and purification using Viral Gene-spin(TM) Viral DNA/RNA Extraction Kit, according to the following manufacturer's instructions (Intron Biotechnology, inc. KOREA).

4. (150 µl) of the supernatant was mixed with (250 µl) of lysis buffer, \and then mixed by vortexing for (15 seconds). The mixture is then incubated for (10 minutes) at room temperature.
5. A (350 µl) of binding buffer was added and completely mixed well by gentle vortexing. Aspin column was then placed on the provided (2 ml) collection tube.

6. The lysate was then loaded on the column and centrifuged at (13 000 rpm) for (1 minute). The solution was discarded from the collection tube and the column was placed again in the same (2 ml) collection tube.
7. A (500 µl) of washing buffer A was added and centrctifuged at (13 000 rpm) for (1 minute). The solution was discarded from the collection tube and the column was placed again in the same (2 ml) collection tube.
8. A (500 µl) of washing buffer B was added and centrifuged at (13 000 rpm) for (1 minute). The solution was discarded from the collection tube and the column was placed again in the same 2 ml collection tube and centrifuged for (1 minute) at (13000 rpm).
9. The column was placed in RNase-free (1.5 ml) microcentrifuge tube and a (60µl) of Elution buffer was added directly onto the membrane and incubated at room temperature for 1 minute and centrifuged at (13 000 rpm).
10. The purified RNA were eluted in (60µl) of RNase-and DNase- free water and immediately stored at (-70°C) prior to use.

3.8.4. Reverse transcription protocol

The following reagents were added to an RT tube on ice, 8 µl of total dsRNA, 1 µl of random hexamer (0.02 µg µl) and 3 µl of DEPC-treated water, adding up to a total volume of 12 µl per tube. The tubes were then incubated at 80°C for 3 minutes and later chilled on ice for (2 minutes) after which they were span briefly. The following reagents were then added to the tubes, 5 µl of 5× RT buffer, 2 µl of 10 mM dNTP, 1 µl of RNAse inhibitor (20 u/ µl) and 1 µl of reverse transcriptase (200 u/ µl) adding up to a volume of 20 µl per tube. The tubes were then incubated at (37°C) for (90 minutes) then heated to (94°C) for (2 minutes), after which they

were chilled on ice for (2 minutes) and layer span briefly. All cDNA samples were stored at (– 20 ℃) ready for use.

3.8.5. Amplification of cDNA by multiplex PCR

Amplification of cDNA was done using Maxime PCR preMix Kit according to manufacturer's protocol illustrated below. A master mix which constituted 4 µl 10 Mm dNTP's, 0.3 µl Tag polymerase, 4 µl ×10 Tag buffer, 2.4 µl 25Mm $MgCl_2$ and 30 µl dH_2o was prepared whereby, the volume of each reagent was multiplied by the number of samples. 40 µl of the master mix were then put into each tube containing cDNA and span down briefly before placing in PCR block. They were then ran using the following program.

Table (3.1): Thermo cycler program for amplification of cDNA

Cycling profile	No of cycles	Temperature	Duration
Denaturation	35	95°c	30 seconds
Annealing	35	50-65°c	30 seconds
Extension	35	72°c	1 minute
Final Extension	35	72	7 minutes

(Anieta *et al*, 2010)

3.8.6. Gel documentation

The *PCR* products were visualized in (2%) Agarose gel with (0.5) µg/ml Ethidium bromide staining, the gel was prepared by dissolving (0.5) g of agarose powder in (25) ml of 1X *TBE* buffer and heated at (65)°C in microwave until the agarose completely dissolved, then left to cool at room temperature and (2) µl Ethidium

bromides was added. The comb was then placed appropriately in the electrophoresis tray and then gel was slowly poured and left to set for (30) min for solidification. In a clean Eppendorf tube (10) µl of (100) bp *DNA ladder* and *PCR* product was loaded on the gel. Gel-electrophoresis was performed at (100V) and (60) Am for (30-45) minutes. Pictures were taken by gel documentation system (Gel mega, digital camera and software in a computer).

3.8.7. Rotavirus G genotyping

G genotyping was performed by seminested multiplex PCR. In the first round of PCR, a 1062 bp fragment of VP7 gene was amplified with forward primer:

Beg 9 (5'-GGC TTT AAA AGA GAG AAT TTC CGTCTG G-3') and reverse primer:
End 9 (5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3')

The second round of typing PCR incorporated the Beg 9 consensus primer and G type specific primers in order to amplify G1(746 bp), G2 (657bp), G3(582bp), and G9(306bp) types. The amplicons were separated by electrophoresis 2 % agrose gel and visualized and photographed after staining with ethidium bromide.

A master mix was prepared by adding (10 Mm dNTP's, 25 Mm $MgCl_2$), primers Beg to a clean eppendorf tube, while multiplying the volume of each reagent by the number of the samples. To ensure the quality of the results negative control where primers were not added to one eppendorf tube was used. (40 µl) of the master mix were put into each tube containing the first time amplified (VP7 cDNA) and ran for (35 cycles) in thermo cycler. The samples were then ran in (2%) agarose gel and viewed under UV light along side with (100 bp) ladder.

Table (3.2): Oligonucleotide primer for g-typing by nested PCR

Genotype	Sequence (5'-3')	Position	Primer
GI	CAA GTA CTC AAA TCA ATG ATG G	314-335	aBT1
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	411-435	aCT2
G3	CGT TTG AAG AAG TTG CAA CAG	689-709	aET3
G9	CTA GAT GTA ACT ACA ACT AC	757-776	aFT9

3.9. Data Analysis

The data collected were entered into a database created in Microsoft excel and was subjected to explanatory data analysis and all statistical analysis were done using the SPSS Statistics 20.0.

3.10. Post testing

Safe disposal for all material used in this study was done by sterilization of the contaminated materials.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

This study was conducted at Mohammed Alamin Hamid Pediatric Hospital in Khartoum State to characterize human rotavirus strains circulating among children attended with Diarrhea using molecular methods. In this study a total of (150) children were included the majority of children (62.6%) were males and (37.4%) were females **(Table 4.1).** Their mean age was (13 months) range 2(months to 5years), more than one half were within the age group (1-12 month) **(Table 4.2).** The majority of the children (83.3%) previously received rotavirus vaccine and only (16.7%) were not vaccinated **(Table 4.3).**

Rotavirus was detected in stool of 42 (28.7%) out of 150 total children. The study revealed that the percentage of (Rotavirus Ag positive) children was (29.3%) in males which is nearly equal to the percentage of Rotavirus Ag positive children in females (26.6%) but this sex difference was not found to be statistically significant **(Table 4.4).**

The current study showed that the highest percentage of rotavirus infection was in age group of (13-24 months) where (35.5%) of cases were positive and the lowest percentage was observed in age group of more than (2 years), but this age group difference was not found to be statistically significant **(table 4.5).**

Regarding vaccination only (23.4%) of vaccinated children against rotavirus were infected with rotavirus infection in comparison to (52%) of children not vaccinated were infected with rotavirus infection, this finding was found to be statistically highly significant **(table 4.6).**

The most common G-genotypes identified were G2 (45.2%), G1 (28.5%), G3 (9.5%) and G9 (4.8%) (table 4.7). G2 was the predominant type that circulates among vaccinated children, while G1 was the predominant genotype that circulates among nonvaccinated children **(table 4.8).**

Table (4.1): Distribution of study population according to gender.

Gender	Frequency	Percent %
Male	94	62.6%
Female	56	37.4%
Total	150	100%

Table (4.2): Distribution of study population according to age group.

Age group (Months)	Frequency	Percent %
1-12	85	56.6%
13-24	45	30%
More than 24	20	13.4%
Total	150	100%

Table (4.3). Distribution of study population according to vaccination status.

vaccination status	Frequency	Percent %
Vaccinated	125	83.3%
Non vaccinated	25	16.7%
Total	150	100%

Table (4.4): Distribution of Rotavirus Ag positive children < 5 year according to gender

Gender	Total tested	Rotavirus antigen positive		
		Frequency	Percent %	Sig.(2-sided)
Male	94	27	28.7%	0.7
Female	56	15	26.6%	
Total	150	42	28%	

P value= 0.05

Table (4.5): Distribution of Rotavirus Ag positive (ELISA) children < 5 year according to age group.

Age group (Months)	Total tested	Rotavirus antigen positive children		
		No	Percent %	Sig.(2-sided)
1-12	85	23	27%	0.2
13-24	45	16	35.5%	
More than 24	20	3	15%	
Total	150	42	28%	

P value= 0.05

Table (4.6): Distribution of Rotavirus Ag positive children < 5 year according to vaccination status

vaccination status	Total tested	Rotavirus antigen positive		
		Frequency	Percent %	Sig.(2-sided)
Vaccinated	125	29	23.2%	0.006
Non vaccinated	25	13	52%	
Total	150	42	28%	

P value= 0.05

Table (4.7): The pattern of Rotavirus G-genotype among rotavirus Ag positive children.

Genotype	Frequency	Percent %
G2	19	45.2%
G_1	12	28.5%
G3	4	9.5%
G9	2	4.8%
Un typable	5	12%
Total	42	100%

Table (4.8): Distribution of Rotavirus G-genotypes according to vaccination status.

Vaccination Status	Total	Rotavirus genotypes detected									
		G1	%	G2	%	G3	%	G9	%	Unty pable	%
Vaccinated	125	5	4%	16	12.8 %	4	3.2 %	1	0.8 %	3	2.4 %
Un vaccinated	25	7	28 %	3	12 %	0	0%	1	4%	2	8%
Total	150	12	8%	19	12.7 %	4	2.7 %	2	1.3 %	5	3.3 %

Table (4.9): The relative risk for development of fever and vomiting among rotavirus positive children.

Symptom	With symptom			Without symptom			Relative Risk
	Total	Rota virus +ve	Ratio	Total	Rota virus +ve	Ratio	
Fever	65	18	0.3	85	35	0.4	0.75
Vomiting	113	37	0.3	37	7	0.2	1.5

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

DISCUSSION, CONCLUSSION AND RECOMMENDATIONS

5.1. Discussion

This study was conducted to characterize human rotavirus strains circulating among children attended with diarrhea to Mohammed Alamin Hamid Pediatric Hospital using molecular methods.

In this study rotavirus was detected in stool of (28 %) of children presenting with diarrhea to Mohammed Alamin Hamid Pediatric Hospital. The frequency of rotavirus detection is lower than that obtained by (Brandt *et al*., 1983), who showed that (34.5%) of children shed rotavirus in feces. Reports from other countries have similarly shown that rotaviruses have been present in similar high rates in symptomatic children less than (5 years) old (li *et al.*, 2010). Our result is lower than those reported from other countries including India 36.9% (Chakravarti *et al*., 2010), Denmark 39.9% (Fischer *et al*., 2009), Greece 40% (Koukou *et al*., 2011), 47% China (li *et al.*, 2010), Bangladesh 40% (Paul *et al*., 2011), Thailand 43.6% (Sungkapalee *et al*., 2006), Turkey 36.1% (Kilic *et al*., 2007), Pakistan 34% (Alam *et al*., 2013), Jordan 33% (Youssef *et al*., 2000) , Kuwait 40% (Sethi *et al*., 1984) and several cities in Iran including Tehran 35% (Kargar *et al*., 2007), Jahrom 46.2% (Kargar *et al*., 2010), Zanjan 31.5% and Isfahan 30.8% (Kazemi *et al*., 2006), although higher than that figures reported from Venezuela 21.3%(Vizzi et al., 2011) . In addition, according to the WHO-coordinated global Rotavirus surveillance network, global median rotavirus detection among (48) countries was 40% (Greenberg and Estes., 2009).

In this study the frequency of rotavirus associated diarrhea is nearly equal in males (28.7%) to that of females (26.6%) which are statistically insignificant. This finding disagrees with that obtained by (Brandt *et al*., 1983), who reported that the

frequency of rotavirus associated diarrhea was higher among males (61.6 %) than females (38.4%). Different results reported from Cameroon were in agreement with our result in which there was no significant difference between the prevalence of rotavirus infection among males and females (Valentine *et al*., 2012).

The rate of rotavirus detection in stool was insignificantly higher among the age group (1-12 months). (35.5%) compared to the other age groups (P value = 0.2). This is in accordance with the result of study done by Valentine and his co. workers, who found that the highest prevalence of rotavirus was among the age group (0-12 months). (Valentine *et al*., 2012). Study conducted in 2014 reported a lower prevalence of rotavirus diarrhea (32%) among children between the age of (7-11 month) and (29.6%) among the age group (1-6 month).

The current study revealed that the majority of G-genotypes circulating among the study population were G2 (45%), G1 (29%), G3 (10%) and G9 (4%). G2 was the predominant genotype followed by G1 and the least circulating genotype was G9. These findings were in disagreement with that obtained by Luana and his colleagues, who reported that G1 was the most prevalent genotype, and it has been detected in frequencies ranging from (36 to 74%) in different regions of the world. (Luana *et al*., 2010). Santos and his colleagues reported that 79% of all samples analyzed in Salvador were G9 (Santos et al., 2005). Similar results were also reported in Goiás, where Costa and his colleagues detected 34% of G9 rotavirus infections.

In this study the prevalence of rotavirus diarrhea was found to be significantly higher among non-vaccinated children than vaccinated children (52% vs. 23%). These findings indicate that rotavirus vaccination reduced the transmission of the virus but not prevent the occurrence. A study done in USA in 2011reported a decline in rotavirus transmission after introduction of rotavirus vaccine, however

the degree of reduction in prevalence was higher than what was found in that study (Jennifer *et al*., 2011).

Common circulating G-genotypes among vaccinated children were G2 (55%), G1 (17%), G3 (13%), and G9 (3%), in comparison with non vaccinated children where common G-genotypes were G1 (54%), G2 (23%), G9 (8%), and G3 (0%). A prominent decrease in G1 type was observed among vaccinated children which reflect effective vaccination; however there is increase in G2 type among nonvaccinated children. These findings were in accordance with that obtained by (Victor *et al*., 2010) who revealed that the most prevalent G-genotype among vaccinated children was G2 genotype.

The rate of rotavirus detection in stool specimen was found to be significantly higher among children with vomiting compared to those with no vomiting (RR=1.5). Fever was not found to be significantly associated with rotavirus antigen positively (RR=0.5)

5.2. Conclusion

- This study showed that there is reduction in rate of rotavirus infection among children less than five years old, as only (28%) of cases were attributed to *rotavirus,* according to the WHO-coordinated global Rotavirus surveillance network, declared that global median rotavirus detection among (48 countries) was (40%). But still *rotavirus* remains the main causative agent of children diarrhea.

- The Rotavirus infection rate is nearly equal in males (28.7%) and females (26.6%).
- The highest rotavirus infection rate (35.5%) was seen among children of (13-24 months) of age.
- The study concluded that (G1 genotype) is the most prevalent G-genotype among non-vaccinated children (< 5 years) old and (G2) is the most prevalent (G-genotype) among vaccinated children less than five years old. Rotavirus vaccine had been introduced since august 2011 in Sudan; the vaccine is based on heterogenic protection as a result of cross-reactive antigens between genotypes. The study predicted that the rotavirus vaccine will offer protection against the most prevalent circulating strains.
- The study highlights the existence of G3 (12%) and G9 (4.8 %) rotavirus strains. These data will be useful for making an informed decision about the introduction of rotavirus vaccine in Sudan and provides baseline data for future vaccine studies.

5.3. Recommendations

- Rapid tests for rotavirus detection should be encouraged in local health centers and professional pediatric hospitals.
- Continuous surveillance is needed to inform diarrhea prevention programs as well as to provide information about the occurrence of new rotavirus strains.
- Complementing studies should be designed to directly measure vaccine effectiveness; secondary databases can be useful tools for continuous monitoring and documentation of vaccine coverage and the effect of vaccination on childhood morbidity and mortality.
- Further studies should be carried on other viruses that are known to cause gastrointestinal infection such as enteric *adenoviruses (serotypes 40 and 41), noroviruses, sapoviruses, astroviruses, and toroviruses*.
- Health education is needed to raise community awareness about rotavirus infection and rotavirus vaccination.
- Further in depth studies including large geographical areas are needed.
- Establishment of nosocomial prevention and control may be required.

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Appendix I

Molecular characterization of Human Rotavirus Strains circulating among Children less than 5 years attended with Diarrhea to Mohammed Alamin Hamid Pediatric Hospital, Khartoum.

QUESTIONNAIRE

Name: …… **No: (**……**)**

Age (in month**):** ……

Sex: **male:** ☐ **female:** ☐

State: …… **City:** ……..

Vaccination status: Vaccinated ☐ Unvaccinated ☐

Clinical features:

Fever: yes ☐ no ☐

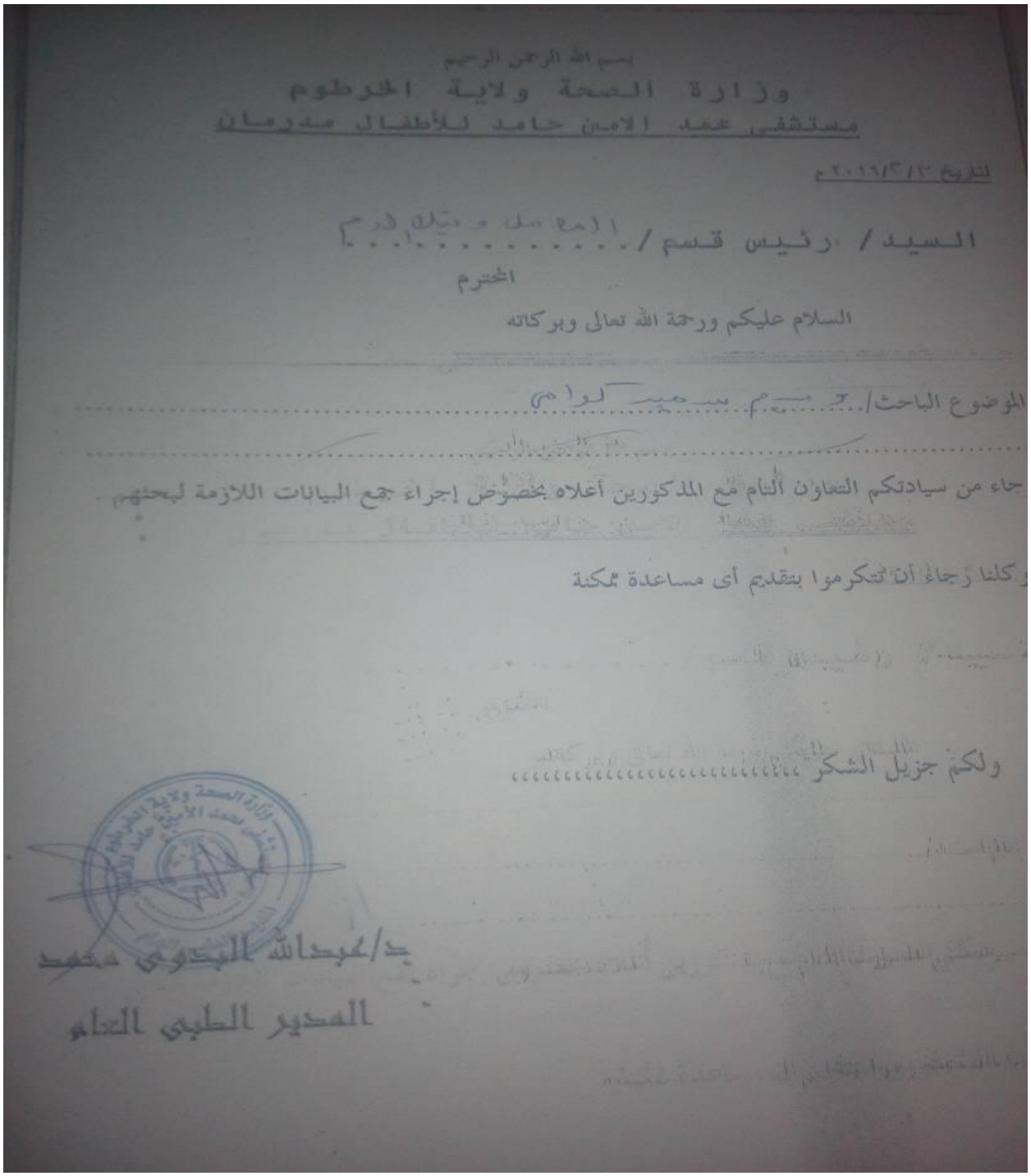
Vomiting: yes ☐ no ☐ episodes/day ☐

Diarrhea: yes ☐ no ☐ episodes/day ☐

Date of sample collection: ……

Sample result by ELISA: Positive ☐ Negative ☐

Appendix II



بسم الله الرحمن الرحيم

وزارة الصحة ولاية الخرطوم

مستشفى محمد الامين حامد للأطفال مدرمان

التاريخ ٢٠١٦/٣/١٣م

السيد / رئيس قسم /.....

المحترم

السلام عليكم ورحمة الله تعالى وبركاته

الموضوع الباحث/.....

جاء من سيادتكم التعاون التام مع المذكورين أعلاه بخصوص إجراء جمع البيانات اللازمة لبحثهم .

وكلنا رجاء أن تتكرموا بتقديم أى مساعدة ممكنة

ولكم جزيل الشكر .....

د/عبدالله البدوى محمد

المدير الطبى العام

Appendix III

ELISA WASHING MACHINE

Appendix IV

ELISA READER MACHINE

Appendix V

PCR machine