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MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES PREVALENT IN HIMACHAL PRADESH

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MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES PREVALENT IN HIMACHAL PRADESH

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IN

BIOTECHNOLOGY

By

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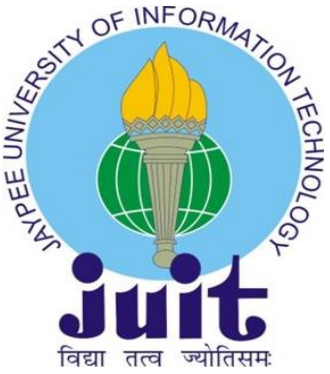

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MARCH 2017

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CERTIFICATE

This is to certify that the thesis entitled, **"Molecular characterization of group A rotaviruses prevalent in Himachal Pradesh"** which is being submitted by **Swapnil Jain (Enrollment No. 116555)** in fulfillment for the award of the degree of **Doctor of Philosophy** in **Biotechnology** by **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by him under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree celsius
µg	Microgram
µl	Microliter
aa	Amino acid
AFP	Acute flaccid paralysis
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	complementary DNA
CIEOP	Counter-immuno-electro-osmophoresis
DLP	Double layered particle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double-stranded Ribonucleic acid
EIA	Enzyme immunoassay
eIF4G	Eukaryotic translation initiation factor 4 G
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENS	Enteric nervous system
EtBr	Ethidium bromide
GEMS	Global Enteric Multicenter Study
g	Gram
Hsp	Heat shock proteins
HFMD	Hand, foot and mouth disease
IGMC	Indira Gandhi Medical College
IP3	Inositol 1,4,5-triphosphate
Kb	Kilobase
KDa	Kilodalton
Min	Minutes

mL	Milliliter
mRNA	Messenger RNA
nm	Nanometer
NSP	Non structural protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PLC	Phospholipase C
qPCR	Quantitative PCR
RCWG	Rotavirus Classification Working Group
RIA	Radioimmunoassay
RMSD	Root-mean-square deviation
RPM	Revolutions per minute
RRV-TV	Rhesus rotavirus tetravalent
RT-PCR	Reverse transcription polymerase chain reaction
Sec	Seconds
SG	Sub group
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TLP	Triple layered particle
UIP	Universal Immunization Programme
UTR	Untranslated region
UV	Ultraviolet
Vi	Viroplasm
VP	Viral protein
WHO	World Health Organization

Abstract

ABSTRACT

Diarrheal diseases are responsible for a whopping proportion of mortality and morbidity all around the globe. It is the second largest killer of children, where most of the deaths occurring in the under developed and developing nations of the world. Among different etiological agents, the contribution of viruses to gastroenteritis incidences in humans is well established. In this study, the prevalence of rotavirus, norovirus and enterovirus was studied in the diarrheagenic patients from Himachal Pradesh, India. Rotavirus was the most commonly identified viral pathogen during the study. It was detected in as high as 49% children (<5 years of age) and 14% adults (>5 years of age). Enterovirus was present in 5.6% of cases whereas norovirus had the least prevalence (1.4%). Rotavirus infections were found to be significantly associated with vomiting and dehydration whereas the trend of higher rates of fever was seen in children along with diarrhea. Seasonal distribution of rotavirus infection reveals their year round prevalence. Genotyping of the isolated rotavirus strains showed the predominance of an unusual G1P[6] genotype in the region. Phylogenetic analysis of the VP7 genes of the G1-isolates revealed belonging of the Himachal isolates to a novel sublineage within lineage 1. Also all the strains were distantly related to the vaccine strains and have 93.9%- 94.5% and 91.9%- 92.6% similarities at the amino acid level with Rotarix and RotaTeq strains, respectively. The comparative sequence and structural analysis of the Himachal strains with vaccine strains revealed differences in amino acids in epitope region of the VP7 protein especially at the antibody neutralization sites. The structures of VP7 proteins of the isolates and vaccines strains were predicted via *in silico* analysis to see whether the amino acid differences in the isolates and vaccine strains can render any effect on antibody binding to the proteins. The outcomes of the *in silico* antigen antibody binding analysis revealed no considerable effect of the mutations in the epitope region and antigen antibody binding energy remained almost similar even after multiple variations in the amino acid sequence.

This study provides a baseline data on the epidemiological characteristics of diarrheagenic viruses, especially rotavirus, in the region. The high prevalence of rotavirus during the study period is indicative of a severe public health threat and warrants immediate preventive measures against the deadly virus. The identification of unusual rotaviruses in high proportions is an important finding of the study. Also, the isolation of

rotavirus strains with mutations in the antibody binding sites attracts more extensive surveillance and molecular studies to be executed in the region.

Chapter 1

Introduction

INTRODUCTION

Diarrhea is a major healthcare concern as it is responsible for a significant proportion of morbidity and mortality around the globe. According to estimates, more than one million deaths per year are attributed to diarrhea making it the second largest killer of children across the world [1]. The major etiological agents of diarrhea include viruses, bacteria and protozoan parasites. The role of viral pathogens in diarrhea is well established and several diarrheagenic viruses have been reported to date [2, 3]. The disease burden associated with the viral gastroenteritis has been well documented in the studies from all over the world [4, 5].

Rotavirus, the RNA virus belonging to the family of *Reoviridae* is the major viral agent which is responsible for diarrhea basically in children below the age of 5 years. Globally, rotavirus is responsible for around 2 million hospitalizations [6] and approximately 0.2 million deaths annually [7]. The prodigious disease burden attributed to rotavirus can be judged by the fact that rotavirus alone is responsible for around 40% of diarrhea associated hospitalizations [8]. India is the leading nation in terms of number of rotavirus infection associated deaths and accounts for 22% of the global mortality due to rotavirus infections [7]. In a recent report from India, John and co-workers have shown that rotavirus was responsible for 78,583 deaths, 872,000 hospitalizations, 3,270,000 outpatient visits and 11.37 million episodes of diarrhea in children [9]. These figures reflect the substantial burden of rotavirus disease in the Indian population. Although rotavirus is recognized as a pathogen responsible for diarrhea associated illness among children at large, reports have also documented its ability to cause diarrhea in adults [10, 11]. However, the data related to rotavirus gastroenteritis in adults is limited.

Norovirus is also a major viral pathogen associated with diarrhea among all age groups in humans. These single stranded positive sense RNA viruses are the leading cause of gastroenteritis outbreaks and are important agents involved in sporadic diarrheal cases [12, 13]. Out of the six (GI-GVI) genogroups of noroviruses, GII is accountable for the majority of the norovirus associated gastroenteritis cases, followed by GI [14, 15].

Enteroviruses are single stranded RNA viruses, belonging to the *Picornaviridae* family and they are responsible for a number of chronic and acute diseases in humans [16]. Enteroviruses are in fact a neglected pathogen for diarrhea but recent reports have showed

its significance as a diarrheagenic virus particularly in children [17-20]. Keeping in view the mortality, morbidity and financial burden attributed to these viruses, it is imperative to study their epidemiology. This will be crucial in formulating the preventive strategies accordingly.

Among various viruses, rotavirus is the single most important etiological agent of diarrhea and requires special attention. Rotavirus is a wheel shaped virus having a segmented genome comprising of 11 segments of double stranded RNA (dsRNA) (Figure 1.1). The genome encodes for six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six non structural proteins (NSP1-NSP6). The genome is enclosed in a triple layered capsid. The inner most layer is made up of VP1, VP2 and VP3; the intermediate layer is composed of VP6 protein; and VP4 and VP7 proteins assemble to form outermost shell of the virus. Rotaviruses are classified serologically into eight groups (A-H) on the basis of VP6 reactivity [21]. Group A, B and C rotaviruses are reported to infect humans where group A rotaviruses are accountable for the majority of morbidity and mortality in humans.

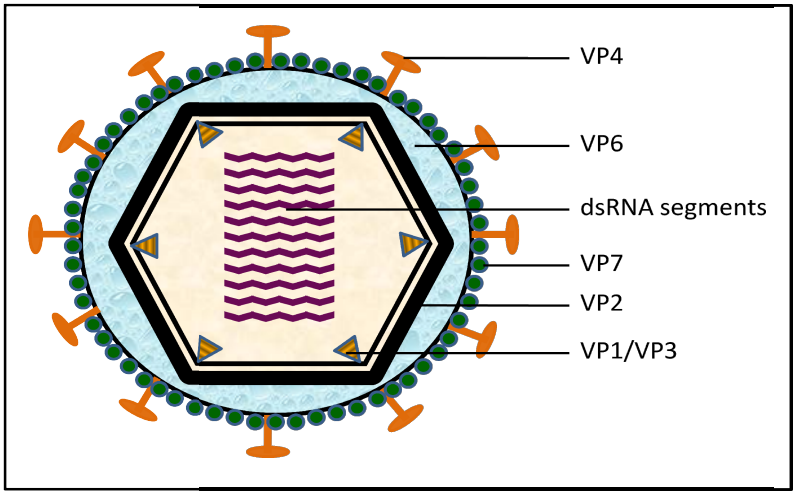

Figure 1.1: Schematic representation of Rotavirus structure

The VP4 and VP7 proteins/encoding genes are used to genotype the rotaviruses in the G/P- genotyping system. The VP7 (**G**lycoprotein) protein corresponds to G-type whereas VP4 (**P**rotease sensitive protein) protein represents the P-type [22]. According to recent reports, 32 G-types (G1-G32) and 47 P-types (P[1]-P[47]) of rotaviruses have been identified [23]. The most common genotypes isolated from humans are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] [24]. However, a number of other genotypes have also been

detected in significant fractions from time to time [25-27]. The relative frequency of the various rotavirus genotypes varies with time and geographical location on the globe [28]. The other factors such as vaccine pressure also leads to the changing pattern in diversity of circulating rotaviruses [29].

Rotaviruses are continuously evolving due to four major mechanisms: point mutation, interspecies transmission, reassortment events and gene rearrangement [29]. The mutations in the antigenic regions of VP7 and VP4 proteins lead to antigenic shift and drift mechanisms which give rise to antibody escape mutants. The VP7 glycoprotein contains 326 amino acids and spans across nine divergent regions (VR1-VR9) which are variable among the various genotypes [30]. Four of the divergent regions, VR5 (region A; aa 87–101), VR7 (region B; aa 141–151), VR8 (region C; aa 208–224) and VR9 (region F; aa 235–242) are considered to be the major antigen sites on the VP7 protein [31-33]. As reported by Aoki et al., two regions, 7-1 (includes epitopes from antigenic Regions A and C) and 7-2 (includes antigens from antigenic region C) are the sites of mutations that allow escape from neutralizing antibodies [34].

Currently, two rotavirus vaccines, Rotarix and RotaTeq are in use to protect from rotavirus associated complications globally. Rotarix is a live, attenuated vaccine having monovalent G1P[8] human rotavirus strain [35]. The pentavalent vaccine, RotaTeq contains human-bovine reassortant rotavirus strains and have G1, G2, G3, G4 and P[8] genotypes which are commonly found in humans [36]. The G1 component of the Rotarix vaccine belongs to the Lineage-2 whereas RotaTeq- G1 belongs to Lineage-3 [37]. Recently an Indian biotech company developed an indigenous rotavirus vaccine 'Rotavac' which is serially passaged attenuated human-bovine reassortant rotavirus with G9 and P[11] antigens. The phase III trials of the vaccine have shown encouraging efficacy and makes it a great candidate to be included in the Universal Immunization Programme (UIP).

India has recently introduced rotavirus vaccine in its UIP. It is crucial for the success of a vaccination programme to have a thorough knowledge of the epidemiology and strain diversity of the targeted virus in the region. The pre-vaccination data also helps in studying the post-vaccination effect on strain diversity due to vaccine pressure. A large number of reports have documented the prevalence and distribution of circulating genotypes of rotavirus from different parts of the country. But the northern, mountainous

state, Himachal Pradesh is unexplored in this context. Apart from two reports by Ramachandran et al. (1996) [38] and Jain et al. (2001) [39], there is no recent data on the prevalence and diversity of rotavirus strains circulating in Himachal Pradesh. Also, the samples screened in those studies from this region were very less (7 and 37). Therefore, this study aims to get an insight into the prevalence of rotavirus along with enterovirus and norovirus infections in this region and assess the genotypic diversity of prevalent rotavirus strains in children and adults. Also, we have carried out *in silico* analysis to study the interaction behavior of rotavirus VP7 specific antibody with the VP7 protein of the isolates and compared them with the VP7 protein of the vaccine strains.

The work was carried out with the following objectives:

Objective 1: To generate a baseline data regarding the prevalence of Rotavirus, Norovirus and Enterovirus in diarrheic patients in Himachal Pradesh.

Objective 2: To study the epidemiological, clinical and seasonal characteristics of rotavirus infection.

Objective 3: To characterize the rotavirus strains for studying their molecular epidemiology and diversity.

Objective 4: To carry out comparative analysis of the rotavirus G1 isolates and vaccine strains for studying the effect of mutations on antigen-antibody binding using *in silico* approach.

Chapter 2

Review Of Literature

REVIEW OF LITERATURE

2.1 Diarrhea

According to the World Health Organization (WHO), diarrhea is defined as passage of three or more loose or liquid stools per day (or more frequently than is normal for the individual). Diarrhea is usually a symptom associated with gastrointestinal infection by pathogenic bacteria, viruses and parasites. The infection is usually spread through contaminated food and water or from one person to another.

2.1.1 Epidemiology

Global estimates from WHO reveals that diarrheal diseases are among the top 10 causes of deaths in humans and claim upto 1.5 million lives in a year [1]. Diarrhea affects humans of all age groups but children below the age group of five years are more prone to the morbidity and mortality associated with diarrhea [12]. Diarrhea is the second most common cause of childhood mortality globally and kills about 1 million children in the first five years of their life; out of which more than 0.2 million deaths occur in India alone [1]. The most affected regions are Africa, Eastern Mediterranean and Southeast Asia where diarrhea is responsible for 11%-12% of deaths under five years [1].

2.1.2 Etiological agents

Infectious diarrhea is caused by a variety of microorganisms including bacteria, viruses and protozoan parasites. Majority of incidences of diarrhea in adults are the result of bacterial infections whereas viral gastroenteritis is more common in children. Faeco-oral is the most common mode of transmission of infectious agents of diarrhea. The overview of etiological agents of diarrhea is depicted in Figure 2.1.

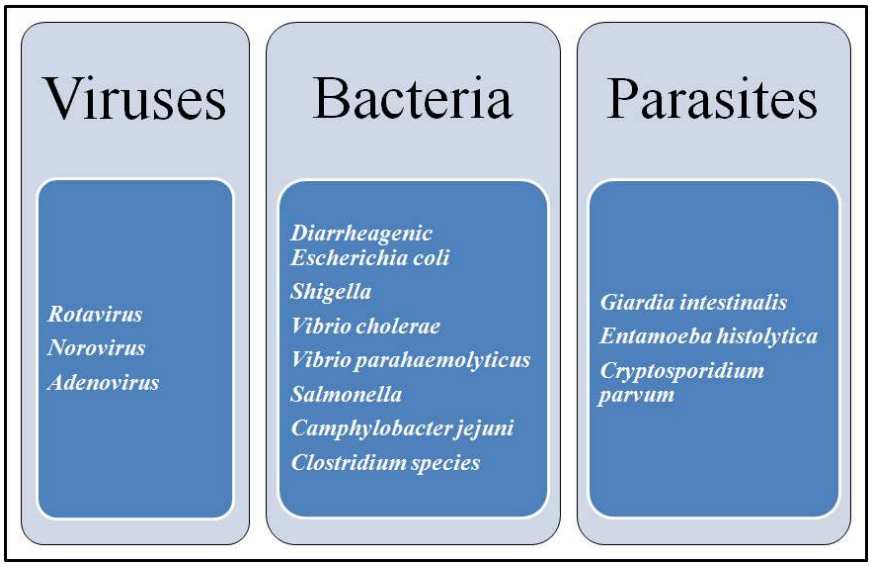

Figure 2.1: Infectious agents of diarrhea

2.2 Diarrheagenic viruses studied in this study

2.2.1 Rotavirus

2.2.1.1 Rotavirus History

Acute diarrhea has been the cause of a whopping number of child deaths for centuries. But no infectious agent could be recognized in around 80% of patients suffering from severe dehydrating diarrhea. It was in the year 1973 that Ruth Bishop and his colleagues identified 70 nm viral particles in the ultrathin sections of duodenal mucosa of children suffering from acute gastroenteritis using electron microscopy [40]. This virus was having a close resemblance with reovirus and orbivirus which were identified as a cause of diarrhea in neonatal mice and calves. The wheel like appearance of the virus leads to its name, Rotavirus (rota = Latin for wheel). After that, rotavirus has been identified as an important cause of diarrhea in the young ones of many mammals and avian species [41].

2.2.1.2 Rotavirus epidemiology and associated illness

Rotaviruses are the most important cause of diarrhea in humans where children below the age of 5 years are the most affected with this virus. This deadly virus causes 0.2 million deaths (under the age group of five years) out of 1 million total diarrhea associated child (<5 years of age) deaths each year [7]. Almost all children on the planet get infected with rotavirus at least once before they reach the age of 5 with peak incidences occurring below the age of 2 years [42]. In the recent Global Enteric Multicenter Study (GEMS),

rotavirus emerged as the leading cause of diarrhea in children [43]. This study from Asia and Africa included a cohort of over 20,000 children. Although mortality due to diarrhea has been reduced in recent times, there is no significant decline in rotavirus associated morbidity [44].

2.2.1.2.1 Rotavirus epidemiology in India

Rotavirus associated illness is a critical healthcare concern in India. Estimates suggest that in India, 1 out of every 345 children die due to rotavirus infection, 1 in every 31 children gets hospitalized due to rotavirus diarrhea and 1 in every 8 children requires outpatient visits because of rotavirus. In other words, in India, rotavirus infections in children results in 78,583 deaths, 872,315 hospitalizations and 3.2 million outpatient visits annually [9]. This reflects into a significant economic burden for the nation. The rotavirus associated hospitalizations costs INR 4.9 billion to the country each year. The country spends additional INR 5.38 billion on outpatient visits [9].

2.2.1.3 Structure

The structure of rotavirus particles have been extensively studied using cryo-electron microscopy (Cryo-EM) previously and then detailed architecture of the infectious particle was studied using X-ray crystallography (Figure 2.2) [45-48]. The virus has ~18,550 bp genome made up of 11 segments of double stranded RNA. The length of RNA segments vary from 667 to 3302 nucleotides. The genome is immediately covered by three layers of proteins generating triple layered particle (TLP), infectious in nature. The triple layered particle is around 100 nm in diameter with icosahedral symmetry. The innermost layer of the TLP surrounds the dsRNA genome and forms the core of the particle. The core shell is made up of 120 copies of VP2 protein organized in dimer conformation on a T = 1 icosahedral lattice. This 120 sub unit icosahedral organization is unique and exclusively found in dsRNA viruses. This organization is the result of different conformation adopted by each monomer unit of a dimer. In one conformation, the VP2-A protein arrange together around the five-fold vertices while VP2-B, in another conformation, remains back and resides between adjoining VP2-A molecules [47]. Each subunit of the dimer can be divided in three domains (apical, central and dimerization) with their N-terminal residues facing in the inward direction towards the genome. It has been seen in the cryo-EM structure of double layered particle (DLP) that the amino-terminus residues of VP2 protein (~1–100 of VP2-A and ~1–80 of VP2-B) have RNA binding ability and interacts

with VP1 (RNA-dependent RNA polymerase) and VP3 (Viral RNA-capping enzyme). This is further confirmed by the biochemical studies which showed that removal of amino terminal residues from the VP2 protein results in non-incorporation of VP1 and VP3 protein complexes with the protein. Thus, it can be said that there is a polymerase complex at each five-fold vertices which are

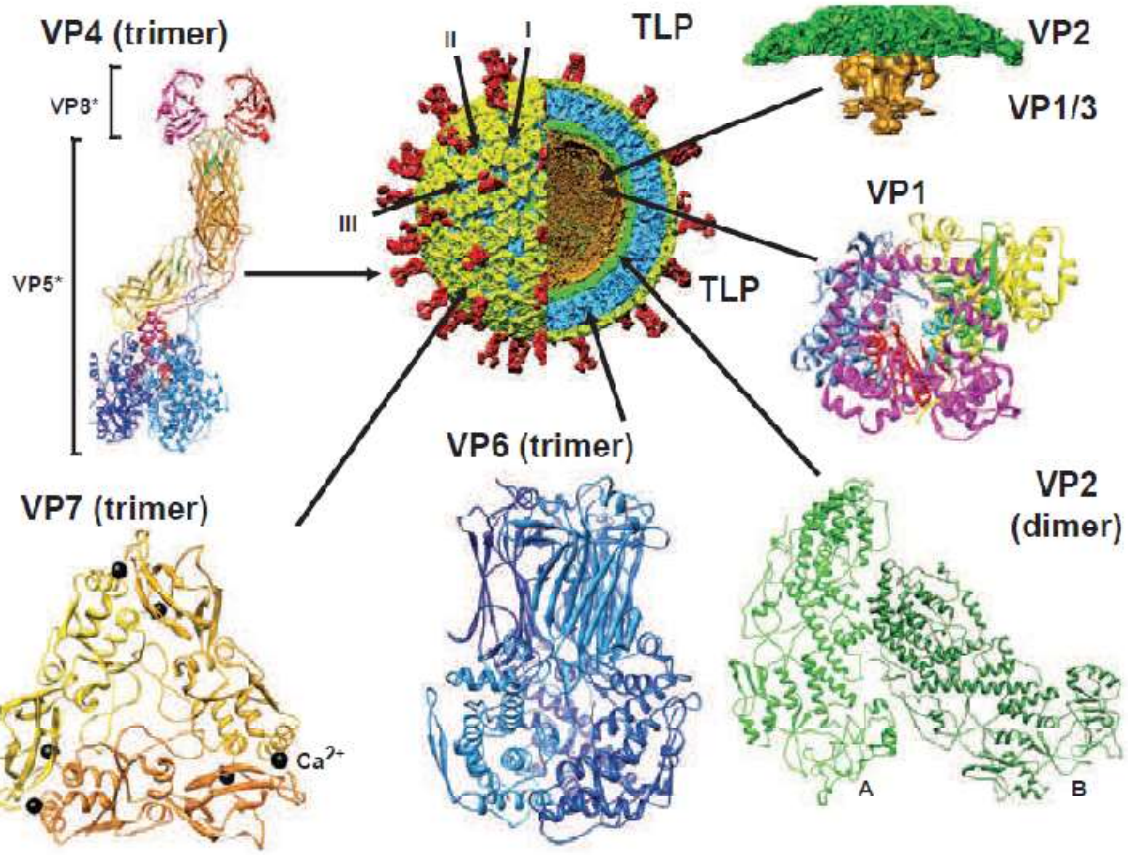

Figure 2.2: Structural organization of rotavirus (Adapted from Estes and Greenberg, 2013 [51]). Figure depicts cryo-electron microscopy (cryo-EM) reconstruction of the mature triple-layered rotavirus particle (TLP). The VP4 spikes (60 trimers) are depicted in red, the VP7 (780 trimers) surface glycoprotein in yellow, the internal (middle) VP6 (780 trimers) layer in blue, and the core VP2 (120 dimers) layer in green. Atomic structures of the viral proteins are also shown along with their locations in the virion.

held in place by interacting with the sub domains of many VP2-A and VP2-B monomers [49]. VP1 and VP3 are present in very small proportions and form the core of the TLP along with VP2. Prasad et al. revealed the exact location and orientation of the minor proteins in the core [50]. The VP1 and VP3 proteins are present as a heterodimer attached to the VP2 layer from inside at each 12 five-fold vertices. This unique organization of the innermost layer in the virus is supposed to carry out two purposes. First is to properly position the transcription enzyme complex and other one is to organize the dsRNA

genome for endogenous transcription. Outer to the inner most layer lies the intermediate layer having T = 13 icosahedral symmetry [52]. The intermediate layer is made up of 260 trimers of VP6 protein and is comparatively thick. This protein has two sub domains, the eight stranded, antiparallel β-barrel make the distal domain which is in contact with the VP7 layer and lower domain formed of α-helices which interact with the inner VP2 layer. The VP6 exists as trimers stabilized by Zn^{2+} and interact with the VP4, VP7 and VP2 proteins through the conserved residues. The base of the VP6 protein layer has negative electrostatic potential and its interaction with VP2 is mainly hydrophobic. Whereas the interaction of VP6 with the outer layer proteins (VP4 and VP7) generally involves charged amino acid residues. In solution, VP6 never forms the T=13 icosahedral conformation like in DLPs. Therefore it is believed VP2 proteins have all the information and thus forms a scaffold to arrange VP6 protein in T=13 icosahedral conformation. The VP6 layer serves a dual function by stabilizing the fragile VP2 layer by binding to the VP2 protein and to act as an adaptor for the outer layer proteins.

Cryo-EM analyses of the rotavirus particles have generated a lot of information on the outer most layer of the virus [50, 53-55]. The outer most layer of TLPs is made up of two proteins, VP4 and VP7. The VP7 protein is a glycoprotein and present in 780 copies whereas VP4 is a protease sensitive protein and exists in 120 copies. The 780 VP7 proteins oligomerize to form 260 trimers and reside at the three-fold axes of T=13 icosahedral lattice. The trimers are stabilized by the presence of two calcium ions between the subunits accounting it to six Ca^{2+} ions per trimer. The amino terminus of VP7 anchors the underneath VP6 layer [45, 46] and also remains in contact with adjacent VP7 trimers which gives integrity and stability to the outer layer. In the outer layer, 132 aqueous channels are present which are spanning the outer and intermediate layer upto a depth of around 140Å and arranged at all the five- and quasi six-coordinated axes of the lattice in T=13 conformation. These channels help in to and fro movement of aqueous material and biochemical molecules (eg. NTPs, nascent RNA) in and out of the particle. The aqueous channels in the capsid can be divided into three distinct types on the basis of their location and characteristics- Type I, Type II and Type III. The VP4 proteins are embedded in the VP7 layer as 120 Å long 60 trimeric spikes originating from the type II channels. The VP4 protein has a large globular region buried in the inner most layer which makes the total length of the spike to 200 Å [56, 57].

2.2.1.4 Genome structure and organization

Eleven segments of double stranded RNA lies within the triple layered capsid. The transcription enzyme complex is present at the inner surface of innermost capsid layer which simultaneously transcribes all the segments of genome [58-60]. Data from hydrodynamic studies have shown that RNA segments cannot be packaged into the virions until they interact intimately with virion proteins [61]. Cryo-EM analysis revealed that in dsRNA viruses, dsRNA acquires dodecahedral structure where RNA double helices interact closely with VP2 layer surrounded by transcriptional complexes present at the icosahedral vertices. The interaction of VP2 with VP1 is required for the replicase activity. According to a plausible model based on biochemical and structural data it is hypothesized that each RNA segment spools around the transcription complex (VP1 and VP3) which is further attached to the VP2 protein layer at the five-fold axis [62, 63]. The gene structure of all the species of rotavirus share some general features. The positive sense RNA segment has a guanidine at 5' end followed by a set of conserved sequences. It is followed by an open reading frame which is ending with the stop codon. Next to stop codon lies a set of conserved 3' sequences with two cytidines at 3' terminus. mRNA from all the segments have the consensus sequence UGUGACC at their 3'end, which plays an important role in replication and transcription. The genes lack polyadenylation at the 3'end. All the genes of the genome are monocistronic except gene 11 which codes for NSP5 and NSP6.

2.2.1.5 Rotavirus proteins and their functions

The rotavirus dsRNA genome encodes a total of twelve proteins, six structural (VP) and six non-structural (NSP). The proteins are named on the basis of their molecular weights. VP1 is the largest protein with the molecular weight of 125 kDa whereas the smallest protein, VP8* has the molecular weight of 28 kDa. The structural proteins form the triple layered capsid of the virus. The non structural proteins except NSP1 are responsible for virus replication and pathogenesis. The various properties and functions of the different structural and non structural proteins are depicted in Table 2.1.

Table 2.1: Rotavirus structural and non-structural proteins (Adapted from Pesavento et al, 2006 [64])

Gene segment	Protein	Mass (kDa)	Post-translational modification	Functional properties
1	VP1	125	-	RNA-dependent RNA polymerase, RNA binding, interacts with VP2 and VP3
2	VP2	95	Cleaved	RNA binding, interacts with VP1
3	VP3	88	-	Guanylyl and methyl transferase, ssRNA binding, interacts with VP1
4	VP4 (VP5*+VP8*)	85 (58+27)	Cleaved	Hemagglutinin, neutralization antigen, virulence, protease-enhanced infectivity, cell attachment, fusion region
5	NSP1	53	-	RNA binding, antagonist of interferon response
6	VP6	45	-	Hydrophobic trimer, group and subgroup antigen
7	NSP3	34	-	Important for viral mRNA translation, PABP homologue, RNA binding, interacts with eIF4G
8	NSP2	35	-	Important for genome replication/packaging, main constituent of viroplasm, NTPase, RNA binding, interacts with NSP5
9	VP7	34	Cleaved signal sequence, high mannose glycolsylation and trimming	RER integral membrane glycoprotein, neutralization antigen, Ca^{++} binding
10	NSP4	20	Uncleaved signal sequence, high mannose glycolsylation and trimming	RER transmembrane glycoprotein, role in morphogenesis, viral enterotoxin
11	NSP5	26	Phosphorylayed, O-glycosylated	Constituent of viroplasm, interacts with NSP2, RNA binding, Protein kinase
11	NSP6	11	-	Constituent of the viroplasm, interacts with NSP5

2.2.1.6 Epitopes in VP7 protein

In the present study, we have carried out the epitope analysis of the VP7 proteins of vaccines strains and isolates of rotaviruses; therefore the structural insight into the epitope of VP7 is discussed in detail here. VP7 protein forms the outer most shell of the rotavirus and is the protein which induces the immune response in the host. The protein occurs in trimeric conformation where each subunit organizes itself into two domains with disoriented N- and C-terminals and reside at the three-fold axes of T=13 icosahedral lattice (Figure 2.3). The subunits bound two Ca^{2+} ions at their interface. Aoki et al., reported the crystal structure of VP7 protein where they reported two regions, 7-1 and 7-2, on the exposed surface of the protein which have the neutralization escape mutation sites [34]. Both regions include several epitopes. Region 7-1 is immunodominant and is spread over the inter-subunit boundary. It contains mutation sites for antibody escape for 58 tested mAbs. The antibodies which bind to this region simultaneously bind to two adjacent subunits, thus stabilizing the trimers and inhibiting their disassembly [46].

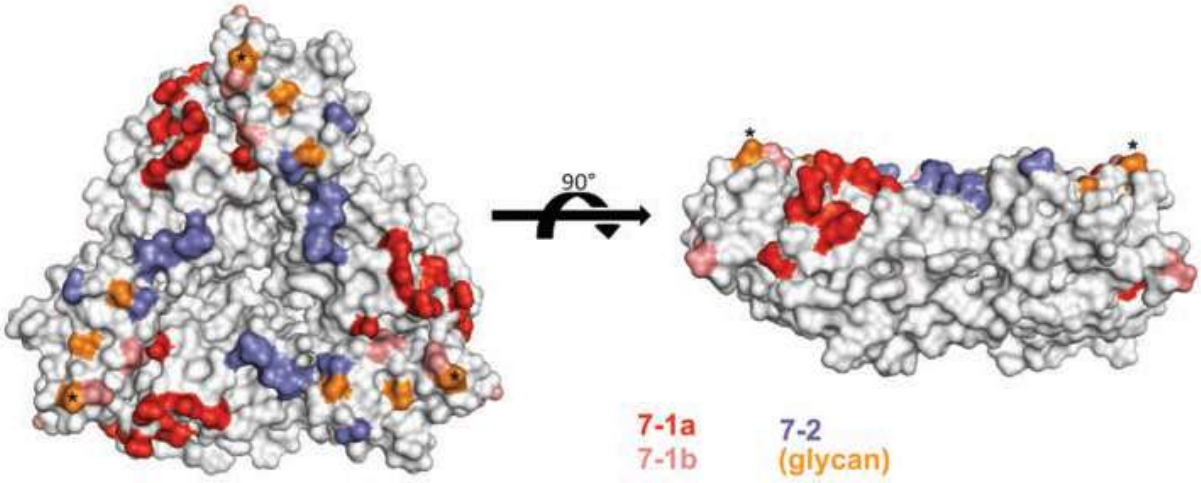

Figure 2.3: Location of neutralization escape mutation sites on VP7 protein (Adapted from Aoki et al., 2009 [34]). Region 7-1 is located at intersubunit boundary; 7-1a (red) on one side and 7-1b (pink), on the other. Residues in 7-2 are depicted in blue color. Sites at which amino acid mutations results in neutralization escape are shown in orange. Residue 211 is indicated by an asterisk.

Region 7-2 is located at the inter-domain within a VP7 subunit. The antibodies binding to this region may neutralize by a different mechanism. It was suggested that antibodies binding to region 7-2 may be cross linking the adjoining trimers and thus stabilizing the VP7 layer on the surface of the viral particle. The amino acid sequence at 87 to101 and

208 to 211 positions are conserved within a particular serotype and can be used to carry out genotyping studies [65].

2.2.1.7 Replication

Rotaviruses are transmitted through faeco-oral route and infect enterocytes in the villus of the intestine. The replication process occurs exclusively in the cytoplasm (Figure 2.4). The major steps involved in the replication cycle are depicted below:

Attachment and cell entry:

Being on the outer surface of the viral particle VP4 and VP7 protein have a crucial involvement in the entry of the rotavirus into enterocytes. A number of studies have shown a significant role of VP4 protein in attachment and cell entry [66, 67]. VP4 is a protease susceptible protein and it cleaves into its functional form before entry of rotaviruses into a cell. The cleavage is facilitated by the presence of large numbers of proteases in the small intestine. This proteolytic cleavage of VP4 protein increases the infectivity of the viral particle by several fold [68-70]. After undergoing proteolysis, VP4 protein cleaves into two fragments, VP8* (aa 1–247) and VP5* (aa 248–776). These fragments remain non-covalently associated with each other on the surface of the virion [71, 72].

The entry of viral particles into the host cell is a sequential multistep process where firstly the VP8* protein binds with the sialic acid receptors on the host cell membrane followed by interaction of the VP5* protein with heat shock cognate protein 70 (hsp70) and αvβ3, α4β1, α2β1integrins. However not all the rotaviruses, including human rotaviruses enter the cell by attaching with the sialic acid leading to sialic acid-independent entry [73]. This shows that VP5* protein is the main protein in mediating the cell entry. Structural analysis of VP4 protein reveals rigidification of the spikes post proteolysis which helps in binding of the protein with the receptor. VP7 plays a crucial role here by assisting the appropriate digestion of VP4 by trypsin. Once the virion gets attached to the cell surface, the process of entry starts by internalization of TLPs either by direct penetration or by endosome mediated pathway. The lipophilic nature of the body of VP5* protein is supposed to be the crucial feature in rotavirus entry through the plasma membrane which is assisted by the exposed three hydrophobic loops on the apex of VP5* [74]. The TLPs are trafficked to the early endosomes which has a low Ca^{2+} concentration. This leads to

the disassembly of the VP7 layer [75, 76] and activates VP5* to penetrate the endosomal membrane [77, 78]. The disassembly of the outer capsid results in the sub viral double layered particles which are released in the cytoplasm of the cell. *In vitro* studies reveal that a virus takes 60 to 90 minutes after binding to get completely internalized. Although it has been observed that different rotavirus strains employ different endocytic pathways to enter the host cell [79].

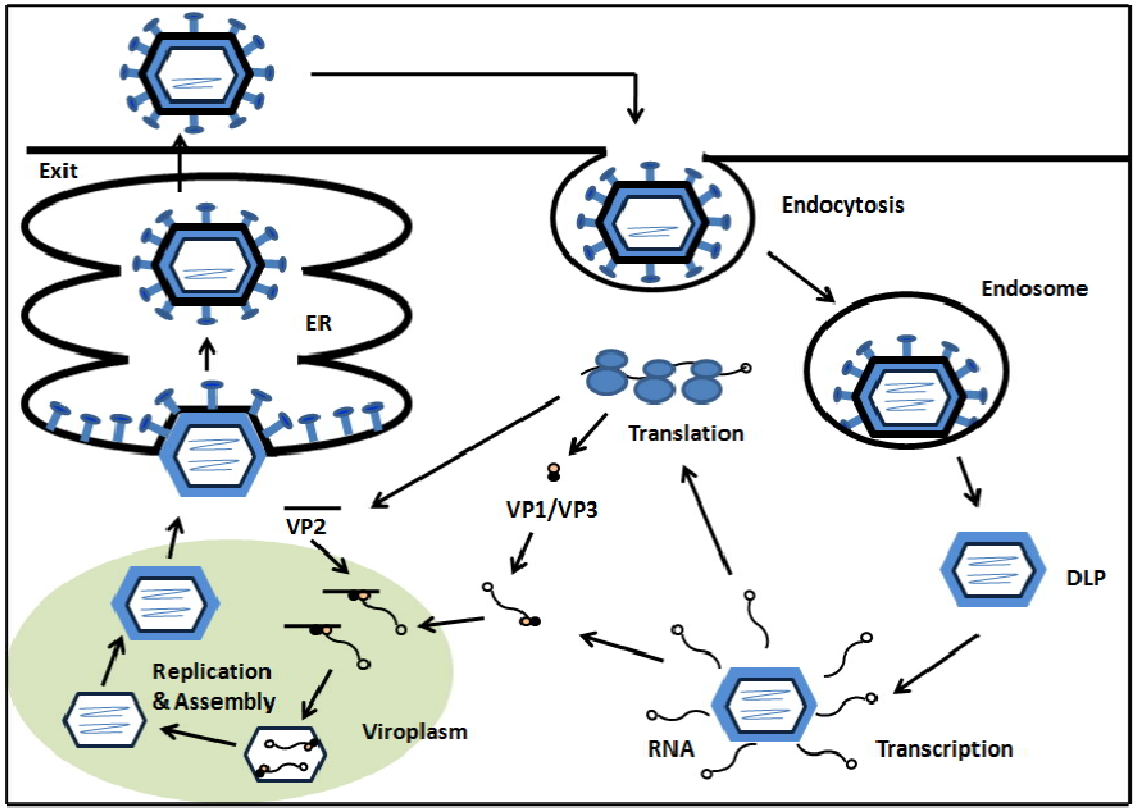

Figure 2.4: Rotavirus replication cycle. Rotavirus particle enters the enterocytes with the help of cellular receptors or by direct endocytosis. DLPs are generated in the endosomes and become transcriptionally active to yield (+) sense RNA molecules in the cytoplasm. The RNA molecules either undergoes translation to synthesize proteins required for asssembly of virion particles or replication to synthesize double stranded RNA genomes. The replication and assembly of viral components occurs in the viroplasm. This yields progeny DLPs which bud into the endoplasmic reticulum to acquire their outer most layer. In the final step, the virus particles release from the cell either by lysis or by traficking pathways.

Transcription

The transcriptionally active DLPs enter the cytoplasm to commence the next step of the replication cycle. Here the transcription machinery within DLPs transcribes the negative sense RNA of 11 segments of dsRNA into capped, positive sense RNAs [80]. The transcription machinery is made up of an RNA-dependent RNA polymerase complex which includes VP1 and VP3. VP1 is a hollow, globular protein and acts as the catalytic subunit of the complex. Similar to other members of the *Reoviridae* family, VP1 protein has a conserved, 'right handed' polymerase domain which is surrounded by amino and carboxy terminal domains [81, 82]. The catalytic centre of the protein harbors four tunnels which help in the movement of NTPs, template RNAs and newly synthesized mRNAs in and out of the enzyme. The nascent RNA leaves the DLP through type I channels that are present at the five-fold vertices [83]. But before leaving the protein complex, the nascent RNA chain acquires a 5' cap that is catalyzed by the guanylyltransferase and methyltransferase activity of VP3 [84]. Though it is not yet clear what sparks the initiation of transcription as soon as TLPs convert to DLPs. But structural studies suggest that removal of the outer layer results in dilation of the channels because of outward movement of the VP2 and VP6 proteins at five fold vertices which results in the influx of metabolites required for transcription [46, 85].

Genome replication and core assembly

After transcription, the newly synthesized positive sense RNA serves one of two functions. First is to undergo translation to synthesize viral proteins and second is to serve as template for genome replication. The role of two non-structural proteins, NSP2 and NSP5 is crucial in carrying out the replication. These proteins co-localize with the transcriptionally active DLPs and form electron dense inclusion bodies which are termed as viroplasms [86]. These viroplasms are the site of genome replication and partial packaging in the cytosol and appear after 2-3 hours of infection. NSP2 occurs as a doughnut-shaped octomer which interacts with RNA and NSP5 at the same time [87-90]. NSP5, a 22 kDa phoshoprotein rich in Ser and Thr residues binds with both RNA and NSP2 [89, 90]. A working model of replication and packaging can be elucidated on the basis of various biochemical and structural studies. Studies have identified three major regions in the template RNA for efficient negative strand synthesis. These are the 5' UTR, 3'-consensus sequence (5'-UGUGACC-3') and 3'-UTR upstream of the consensus

sequence [91-94]. A single copy of VP1 protein binds with each positive sense viral RNA at their 3' ends by recognizing the conserved UGUG sequence on the viral RNA [82, 91]. The 5'UTR and 3'UTR serves in giving a panhandle shape to the mRNA which makes the 3'consensus sequence accessible to the polymerase [95]. Also it is believed that 3'UTR contains recognition signals that lead to the binding of the viral RNA polymerase, VP1 to template mRNAs [96]. It requires a decamer of VP2 proteins to activate each VP1 protein and prepare it for replication. NSP3 is supposed to transport viral mRNA to the viroplasms for their subsequent replication. The genome is packaged within DLPs which is also carried out in viroplasms. NSP5 and NSP2 proteins play a vital role in packaging of genome and formation of DLPs as well. However an understanding of the exact mechanism for how the encapsulation occurs with such efficiency that a DLP gets only a single copy of each segment is still very limited.

Genome Translation

The translation of viral proteins occurs in the cytosol of host cell. The role of the non-structural protein, NSP3 is crucial in facilitating the translation of viral RNA. NSP3 functions as a homodimer where its N terminal binds with the 3' consensus sequence of viral mRNA whereas the C terminal interacts with eIF4G to help in the delivery of mRNA to cellular ribosomes for protein synthesis.

Maturation and release

After the accumulation of DLPs in the viroplasms, they move towards the endoplasmic reticulum to mature into TLPs after achieving their outmost layer. NSP4 is an integral membrane protein which acts as a receptor for DLPs and helps in their budding within the endoplasmic reticulum. NSP4 recruits both DLP and the VP4 protein to the cytosolic face of the endoplasmic reticulum and attaches the VP4 to the VP6 layer by some unknown mechanism [97, 98]. The NSP4-DLP-VP4 complex buds into the endoplasmic reticulum followed by assembling of VP7 proteins on the surface of the DLP. The fully assembled TLPs exit the host cell by more than one mechanism. Some *in vitro* studies have suggested the release of viral particle by direct lysis [99] whereas others have proposed the involvement of secretion pathways for viral release [100].

2.2.1.8 Pathogenesis

Rotaviruses enter the human body through an oral route and infect the enterocytes on the small intestine. The enterocytes are non-proliferating epithelial cells which perform digestive and absorptive functions. The prominent symptom associated with rotavirus infection is diarrhea. A number of studies have studied the mechanism of diarrhea induction post infection [72, 101-105]. From these studies it become evident that rotavirus associated diarrhea is multifactoral and induced by multiple mechanisms (Figure 2.5).

Malabsorption

Rotavirus infection in enterocytes leads to diarrhea because of many factors including loss of absorption capability of intestinal cells. This is known as malabsorption. Once the virus infects the enterocytes, they cause an increase in the intracellular concentration of Ca^{2+} ions likely because of the action of NSP4 [106]. This disruption of Ca^{2+} homeostasis within the cell leads to a series of events which leads to reduction in absorption by enterocytes. Increased Ca^{2+} leads to disruption of the cellular skeleton because of damage to Ca^{2+} sensitive proteins including F-actin, villin, and tubulin and subsequent lysis of cells [107, 108]. The NSP4 proteins release from the infected cells and have a paracrine effect on nearby cells. They bind to an unknown receptor on healthy and uninfected cells and trigger a phospholipase C–inositol 1,3,5-triphosphate (PLCIP3) cascade that induces the release of Ca^{2+} from the endoplasmic reticulum and thus leads to an increase in intracellular Ca^{2+} concentration. NSP4 also causes disruption of tight junctions located between cells which results in paracellular leakage [109, 110].

Rotavirus infection also causes disruption in Na^{+} and K^{+} balance in the cell which impairs NaCl and nutrient absorption resulting in fluid loss [111]. The expression of various essential digestive enzymes also gets affected following rotavirus infection [112].

Secretion

NSP4 acts on the crypt cells to increase Cl^{-} secretion and increased outflow of water. For this, NSP4 is supposed to itself form a channel or it may activate a Ca^{2+} activated anion channel [113]. However it is not well established but NSP4 protein also stimulates the enteric nervous system (ENS) to induce secretion of water and electrolytes which results in diarrhea. The secretion of prostaglandins and chemokines from infected cells could

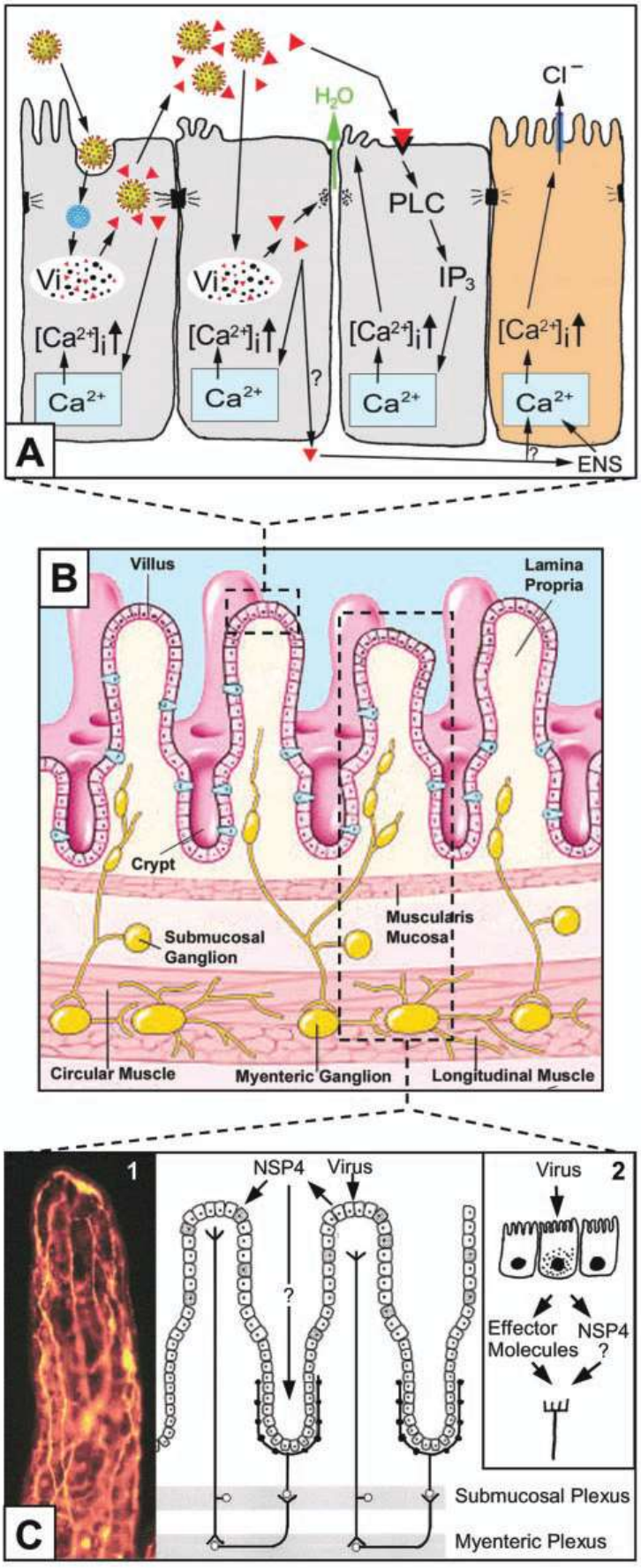

Figure 2.5: Different mechanisms by which rotavirus causes diarrhea. (Adapted from Ramig et al. 2004 [116]) (A) Viral particles infect the enterocytes and form viroplasms (Vi) followed by release of NSP4 (red triangles). (i) Intracellular NSP4 induces release of Ca^{2+} from the endoplasmic reticulum (blue), increasing intracellular Ca^{2+} concentration. (ii) The viral particles from the initial cell releases and infect other cells. NSP4 disrupts the tight junctions which lead to paracellular flow of water and electrolytes (green arrow). (iii) Binding of NSP4 to a specific receptor on a cell triggers a signalling cascade through PLC and IP3 that results in release of Ca^{2+} and thus an increase in Ca^{2+} concentation. The increase in Ca^{2+} concentation results in disruption of the microvillar cytoskeleton. (iv) The brown cell in the figure represents a crypt cell. NSP4 stimulates ENS to increase the intracellular Ca^{2+} concentration which leads to increased Cl^- secretion. (B) Normal architecture of the small intestine. This panel depicts the ENS and its ganglia in the different submucosal levels. (C) Depiction of a reflex arc in the ENS that receives signals from the enterocytes and activate the crypt epithelium.

also contribute in stimulation of ENS and thus contribute to the secretory component of rotavirus diarrhea.

Villus ischemia

In some studies, along with other physiological symptoms, villus ischemia has been observed in rotavirus infected mice [114, 115]. This could be due to the release of some unknown vasoactive agent from infected cells which could functionally damage the enterocytes.

Intestinal motility

Rotavirus infection results in increased intestinal motility owing to the activity of ENS or other rotavirus induced factors [116].

2.2.1.9 Rotavirus classification and strain diversity

Rotaviruses belong to the genus *Rotavirus* in the family *Reoviridae* [116]. The employment of Immunoflourescence and RNA fingerprinting techniques have helped in differentiating rotaviruses into different species or group [117, 118]. The basis of classification of rotaviruses in species is based on the serological characterization of intermediate capsid protein, VP6 [119]. On this basis, rotaviruses are classified into eight groups or species (RVA-RVH) [21]. Among eight groups, RVA, RVB and RVC are found to infect both humans and animals whereas group D, E, F, G and H are identified exclusively in animals. Group A rotaviruses are responsible for almost 90% of infections in humans and they are important cause of diarrhea in the young ones of mammalian and avian species. RVBs are commonly found to infect adults and a major rotavirus species involved in the rotavirus associated epidemics particularly in Asia. RVCs are also found to cause diarrhea outbreaks particularly in infants and children. Because of the segmented nature, the genome of rotaviruses belonging to the same group undergoes frequent reassortment [120, 121]. However exchange of gene segments among different groups has not been observed. Within group A, rotaviruses are further differentiated into four subgroups (SGI, SGII, SGI1II, and SG nonI-nonII) based on the reactivity with subgroup specific mAbs directed towards VP6 [122-125]. Additionally, within RVA, rotaviruses are classified into different serotypes on the basis of reactivity of surface proteins in plaque reduction neutralization assays where hyperimmune serum from antibody negative animals are used to neutralize the VP4 and VP7 proteins on the virus. Accordingly, 27

VP7 types have been identified but the lack of diverse typing serum or mAbs has hampered VP4 classification. The advancement in the molecular techniques such as reverse transcription-Polymerase chain reaction (RT-PCR) and cDNA sequencing have gave birth to an advanced classification system where VP7 and VP4 are classified on the basis of their gene sequence. This is G/P-genotyping system where G represents VP7 gene (**G**lycoprotein) and P stands for VP4 gene (**P**rotease sensitive protein). To date 32 G-types (G1-G32) and 47 P-types (P[1]-P[47]) of group A rotaviruses have been identified [23].

Table 2.2: Group A rotavirus genotypes identified till date [23]

S.No.	Protein	Genotype	Number of genotypes identified
1	VP7	G	32
2	VP4	P	47
3	VP6	I	24
4	VP1	R	18
5	VP2	C	17
6	VP3	M	17
7	NSP1	A	28
8	NSP2	N	18
9	NSP3	T	19
10	NSP4	E	24
11	NSP5	H	19

In 2008, Rotavirus Classification Working Group (RCWG) developed a complete genome classification system on the basis of nucleotide sequence of each of the segment of rotavirus genome [126]. According to this system, a specific genotype was assigned to each of the 11 gene segments. The VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of rotavirus strains are described using the abbreviations Gx-P[x]-Ix- Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x = Arabic numbers starting from 1), respectively [126]. The number of each genotype identified to date have been depicted in the Table 2.2. In 2011, RCWG proposed a nomenclature system for rotavirus strains which depicts

rotavirus as '*RV group/species of origin/country of identification/common name/year of identification/G- and P-type*' [22].

2.2.1.10 Detection of rotavirus

Although rotavirus infection is associated with a number of clinical symptoms, they are not distinctive enough to manifest the presence of rotavirus as an etiological agent. Therefore, for accurate diagnosis, it becomes necessary to detect virus or virus antigen in the biological samples of the patient. The preferred choice of sample is the fecal specimen however rectal swabs can also be used. Since 1973, a number of methods have been developed to detect rotavirus, such as:

1. Electron microscopy (EM)
2. Complement fixation and counter immunoelectrophoresis (CIEOP)
3. Radioimmunoassay (RIA)
4. Enzyme immunoassays (EIA)
5. Polyacrylamide gel electrophoresis (PAGE)
6. Latex agglutination
7. Cell culture based detection
8. Reverse transcription-Polymerase chain reaction (RT-PCR)

These methods are discussed one by one:

1. Electron microscopy (EM)

Electron microscopy is the oldest method to detect rotavirus in the stool specimens [127-130]. In this method, the rotaviruses were negatively stained before visualization under an electron microscope. This method enjoys the advantage of high specificity because of the distinctive morphology of rotaviruses. However, the method has been continuously modified over the period of time to increase its efficiency. The advancement of this technique leads to immune electron microscopy which employs antibodies specific to the virus. This technique has increased sensitivity than the orthodox method. Moreover it can differentiate between morphologically similar groups; i.e. A, B and C rotaviruses. Further advancement in immune EM gave rise to solid-phase immune electron microscopy. In this method, grids are coated with protein A followed by antibodies. After a rinse, the viral particles are added to them before negative staining. This technique showed 30 times more sensitivity than direct EM [131].

2. Complement fixation and counterimmunoelectrophoresis (CIEOP)

Complement fixation and counterimmunoelectrophoresis (CIEOP) were the first serological tests employed for the detection of rotavirus. Complement fixation assays are cheap, reliable and assists simultaneous screening of large number of samples [132, 133]. The sensitivity of these assays was comparable to EM but less than solid-phase immunoassays.

A number of groups have contributed in the development of CIEOP for the detection of rotaviruses [134-136]. This method became popular because of its quick protocol and high specificity. But this method suffers from the limitation of low specificity which is even less than electron microscopy [137, 138].

3. Radioimmunoassay (RIA)

Radioimmunoassays were the next techniques to be developed for detection of rotaviruses [139-142]. This technique was very sensitive but suffers from some limitations including short shelf life of the radio labelled antibodies and complex disposal requirement for the radioisotopes. Thus they were soon replaced by enzyme immunoassays which were similar in performance but lack the limitations associated with radioimmunoassay.

4. Enzyme immunoassays (EIA)

Enzyme immunoassays (EIA) are the most widely used method for diagnosis of rotaviruses. These are based on the detection of antigens present on rotaviruses in stool specimens. First ELISA developed for rotavirus detection was of the direct type [143, 144]. These were advantageous because of the requirement of only one antiserum. Also these were faster in comparison with indirect immuno sorbent assays. But the large scale rotavirus surveillance studies now a days prefer EIA in which rotavirus specific antibodies are attached to the plastic plates and antigens in the sample are captured over them. The detection of antigen is done through a colorimetric reaction where a second rotavirus specific antibody coupled with an enzyme is allowed to bind to the antigen. This EIA format has high sensitivity and specificity and can detect a large number of samples simultaneously in the 96-well plate format. The results can be either visualized or can be recorded on a colorimeter.

A large number of ELISA or EIA rotavirus detection kits are commercially available. As studied by a number of studies, all the kits have variable sensitivity and specificity. The World Health Organization recommends two EIA kits to be used in large scale surveillance programmes: Premier Rotaclone (Meridian Biosciences; Cincinnati, Ohio) and IDEIA Rotavirus (Oxoid (Ely) Limited Thermo Fisher Scientific, Cambridgeshire, United Kingdom). Along with group A rotaviruses, a number of EIA kits have been developed for the detection of group B and group C rotaviruses and for measurement of antibodies against these rotaviruses. *In the present study, we have used ELISA for the preliminary screening of rotaviruses in the stool samples of diarrheic patients.*

5. Polyacrylamide gel electrophoresis (PAGE)

The dsRNA segments of the rotavirus genome can be detected by running on polyacrylamide gel followed by silver staining. The different segments of the genome have different molecular sizes. On electrophoresis, the negatively charge dsRNA segments travel through the gel and get separated on the basis of their size. The pattern of dsRNA migration on the gel can be visualized by staining with silver nitrate. Silver staining is a sensitive technique used to detect nucleic acids in a polyacrylamide gel and can detect even small amounts of nucleic acid. The dsRNA isolated from Group A rotaviruses can be categorized into four classes: four large high molecular weight segments (1 to 4), two medium-sized segments (5 and 6), three small segments (7 to 9), and the two smallest segments (10 and 11). Rotaviruses exhibit two types of RNA migration patterns ‘long’ and ‘short’ on the basis of the relative migration of segments 10 and 11. The short electrophoretic pattern has a larger segment 11 that migrates between gene segments 9 and 10 [145]. Although most of the group A rotaviruses have long or short migration pattern, a super short electropherotype has also been reported. This technique is a great tool for detection of rotaviruses but also serves for studying the molecular epidemiology of the virus. Though this method is relatively time consuming and requires a trained professional, it has the advantage of lack of ambiguity in the results.

6. Latex agglutination

In this method, latex particles are coated with anti rotavirus IgG which reacts with the rotavirus particles in the stool specimens, resulting in agglutination. One drop of clear stool suspension is mixed with test latex reagent and other drop is mixed with control

latex reagent. Agglutination reaction with test latex reagent represents rotavirus positivity in the sample. This method is very rapid and takes only 2 to 5 minutes. Although these tests have excellent specificity, sensitivity is 4-10 times less in comparison with ELISA, electron microscopy and PAGE [146-149].

7. Cell culture based detection

Both human and animal rotaviruses have been successfully cultivated in monkey kidney cells by proteolytic activation of the virus in the presence of trypsin [150-152]. The cell culture of rotavirus particles followed by immunofluorescence microscopy can be used as a method to detect rotaviruses in stool specimens. However, this method is cumbersome, time consuming and requires extensive laboratory facilities and expert handling. Also, the sensitivity of this culture method for detection of rotaviruses is less widely used than ELISA [153]. These limitations make this method unsuitable for large scale surveillance studies.

8. Reverse transcription-Polymerase chain reaction (RT-PCR)

Since the introduction of PCR in 1983, it has been extensively employed for diagnostic applications. RT-PCR method is the most sensitive method for rotavirus detection. The majority of PCR are developed for the detection of Group A rotaviruses but group B and C detection PCRs are also available. For the detection of rotaviruses, the primers are designed targeting the VP6 gene whereas for genotyping, the primers are directed towards the VP7 and VP4 coding genes. Along with detection, the characterization of rotaviruses can also be done using RT-PCR. The genotyping is done by semi-nested RT-PCR where viral RNA is reverse transcribed and amplified in the presence of consensus primers for genes specific for G or P serotypes [154-156]. The designed primers are homologous to different serotypes and can amplify most of the rotavirus strains. The amplicon from the first round PCR are used as a template in the second PCR where one consensus primer and a mixture of genotype specific primers yields product of different sizes on the basis of a particular genotype. The genotypes can be determined on the basis of the size of amplified product by subjecting them to agarose gel electrophoresis.

The advancements of PCR technique lead to invention of real time-PCR (qPCR). Along with conventional PCR, the use of real time PCR as a diagnostic method is increasing exponentially. It has been reported that the sensitivity of real time RT-PCR is 2-4 logs

greater than conventional RT-PCR [157]. This technique is finding great applications in studying the viral load and gauging the severity of the disease [158-160].

2.2.1.11 Rotavirus strain diversity

Human rotaviruses make a highly diverse group. Until now, studies have reported 32 G genotypes (G1–G32) and 47 P genotypes (P[1] – P[47]) [23]. Most commonly isolated G and P types are G1, G2, G3, G4, G9, G12 and P[4], P[8] and P[6] respectively [24]. The genes encoding the VP7 and VP4 proteins segregate independently and give rise to a large number of G-P combinations. Studies from different parts of the globe disclose the existence of more than 70 diverse G-P combinations. Out of these, G1P[8] (25.3%), G2P[4] (12.5%), G12P[8] (10.0%), G3P[8] (7.6%), G4P[8] (6.3%) and G9P[8] (5.7%) are globally predominant and account for approximately 74% of rotavirus infections globally [161, 162]. Along with the common and usual strains, a number of surveillance studies from different parts of the world have reported the existence of many rare and uncommon strains in humans. With the advancement of molecular techniques such as RT-PCR and sequencing analysis, there is an exponential increase in the population of uncommon and newly detected novel strains. The evolution of rotaviruses is the result of four mechanisms: point mutation, interspecies transmission of partial or whole virus, reassortment events during co-infection of two different viruses in a common host and gene rearrangement that preferably targets non-structural protein (NSP) coding segment of the genome. These mechanisms occur separately or in combination with each other and result in an elevated diversity of rotaviruses.

1. Point mutation

Point mutations (resulting in genetic drift) are one of the major mechanisms responsible for rotavirus diversity. Rotavirus genome suffers from frequent point mutations which occur at the rate of approximately one mutation per genome replication [163]. These mutations accumulate and give rise to genetic lineages [164] and neutralizing antibody escape mutants.

2. Genetic reassortment

Reassortment (antigenic shift) is a well studied and established phenomenon responsible for continuous evolution of human rotaviruses. It occurs when two or more strains co-infect a single cell. The segmented nature of the rotavirus genome facilitates the

occurrence of reassortment events (Figure 2.6A). Evolutionary studies suggests that Group A rotaviruses that infect humans belong to two major (Wa-like and DS-1-like) and one minor (AU-1) genotype constellations which are designated as I1-R1-C1-M1-A1-N1-T1-E1-H1, I2-R2-C2-M2-A2-N2-T2-E2-H2 and I3-R3-C3-M3-A3-N3-T3-E3-H3, respectively [165]. The human Wa-like rotavirus strains have a common origin with porcine rotaviruses whereas human DS-1 like rotavirus strains share a common origin with bovine rotaviruses [126]. Multigenic reassortment occur frequently within a genogroup (genotype constellation), however reports of intergenogroup exchange of genes are not widely reported. Although, the role of the latter is significant in the evolution of human rotaviruses [166].

3. Interspecies transmission

Along with point mutations and reassortment events, the mechanism responsible for evolution of rotaviruses and their subsequent diversity is through transfer of whole animal virus or their genes in humans (Figure 2.6B). Although transmission of animal origin rotaviruses in humans is not observed frequently, still it has been documented to cause asymptomatic to severe diarrhea in humans [29]. The unusual strains of bovine and porcine origin are generally identified in humans living in rural settings where they are involved in cattle rearing and hence are in close proximity to animals. The evidence of animal to human whole virus transmission comes from the genomic analysis of some rotaviruses isolated from humans where they have all 11 segments from animal rotaviruses [167, 168]. In comparison to transmission of whole virus, transfer of gene segment from other species to humans is a frequent event. During co-infection, the gene segments of animal rotaviruses transfers to human rotaviruses and give rise to human infecting reassortants with a part of the genome derived from animals [169].

4. Genome rearrangement

Genome rearrangements such as deletions, duplications and insertions also results in diversification of the rotaviruses. These rearrangements bring in mutations in the genome and thus results in mutated protein structures and leads to viruses having novel characteristics. NSP5 and NSP6 encoding gene segment 11 of the rotavirus genome is most prone to rearrangement. However, segments 5-10 are also reported to have rearrangements [170]. The first report of such rearranged gene segments came in 1980s

when genome analysis of the rotaviruses from immune deficient children was carried out [171].

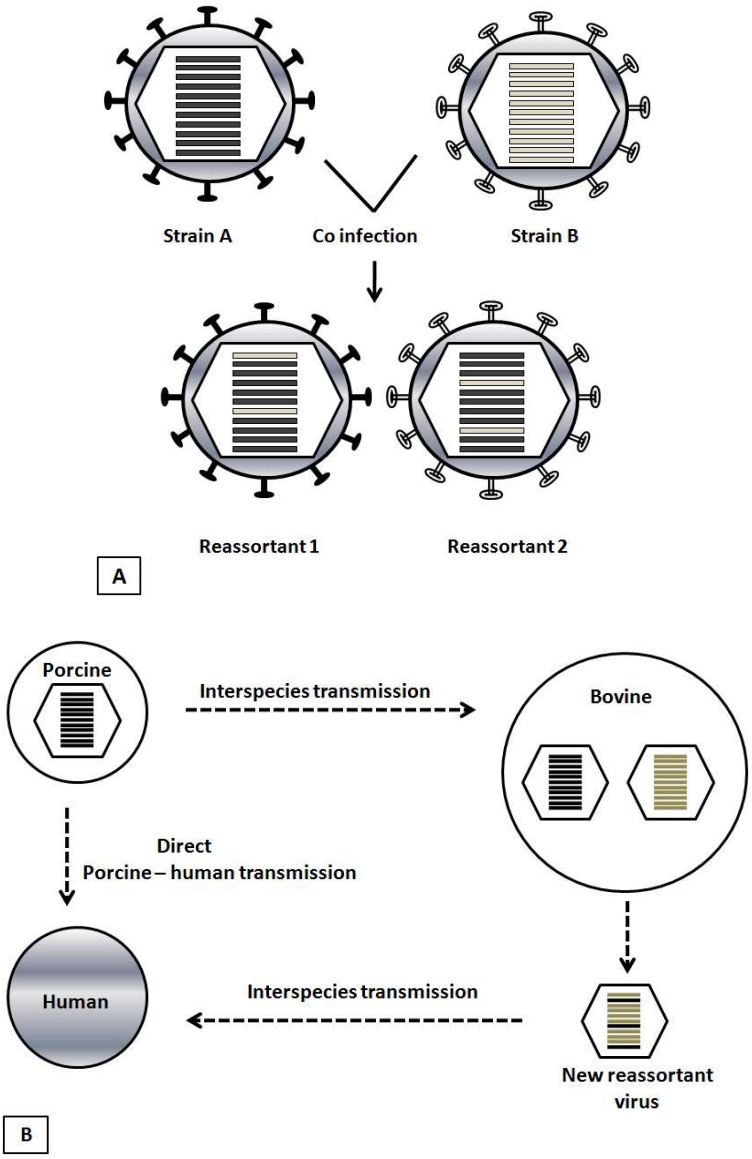

Figure 2.6: Mechanisms of rotavirus evolution (A) Reassortment in rotavirus. Co-infection of two different rotavirus strains (A and B) results in the generation/appearance of reassortants due to exchange of gene segments. The resulting reassortants acquire the gene segments from both the host. Reassortant 1 carries two gene segments from strain B and the rest of the genome from strain A. Reassortant 2 harbors VP4 and VP7 encoding genes from strain B and thus bearing surface proteins identical to Strain B. **(B) Interspecies transmission.** Transmission of whole virus or gene segments from non-humans result in evolution and detection of novel rotavirus strains in humans. The novel strains detected in human may be a result of either direct transmission of an animal strain or it could be a reassortant virus which itself is evolved because of intergenogroup transmission between non-human species.

2.2.1.12 Geographical distribution of rotavirus

Rotaviruses are responsible for substantial disease burden in almost all the countries of the world. Although, the under developed and developing nations are most affected. In the recent Global Rotavirus Information and Surveillance Bulletin, The World Health organization depicted the distribution of rotavirus genotypes in different regions of the world (Figure 2.7) [162]. The distribution of genotypes varied widely among the different WHO regions at a given time period. G1P[8] was the predominant genotype in the African (15%), South East Asian (29%) and Western Pacific (51%) regions and the second most common genotype in the Eastern Mediterranean (20%) and European regions (24%). This makes G1P[8] as the most frequently detected genotype globally [162]. In the American region, G12P[8] emerged as the leading genotype during the course of study whereas European countries showed the highest prevalence of G4P[8] strains. Interestingly, 42% of rotavirus disease in the Eastern Mediterranean Region was due to mixed infections and these were the leading cause of disease. The second most globally common genotype G2P[4] also contributes significantly to the rotavirus disease burden in all the WHO regions. However, G3P[8] was found in notable fractions in the American (11%), European (9%), South East Asian (18%) and Western Pacific (10%) regions. Although, it was negligible in African (0.5%) and Eastern Mediterranean region (1%). The emerging genotype G9P[8] was also isolated in considerable numbers in all of the WHO regions. Interestingly, the uncommon and unusual genotypes such as G1P[6], G2P[6], G3P[6], G1P[4], G9P[4], G12P[6] and G2P[8] also leads to significant proportion of infections especially in the African region [162].

2.2.1.12.1 Unusual combinations and rare strains

Inter species transmission and reassortment results in evolution of novel and uncommon genotypes in humans and thus contribute appreciably to the diversity of rotaviruses. This results in either the creation of genotypes with unusual combinations or appearance of absolutely new genotypes. Following is the compilation of the unusual G and P combinations along with newly detected strains (Table 2.3 and 2.4):

1. G1

G1 strains belongs to Wa-like genogroup and is usually found to be associated with P[8] VP4 type. Continuous reassortment and rearrangement of different gene fragments has

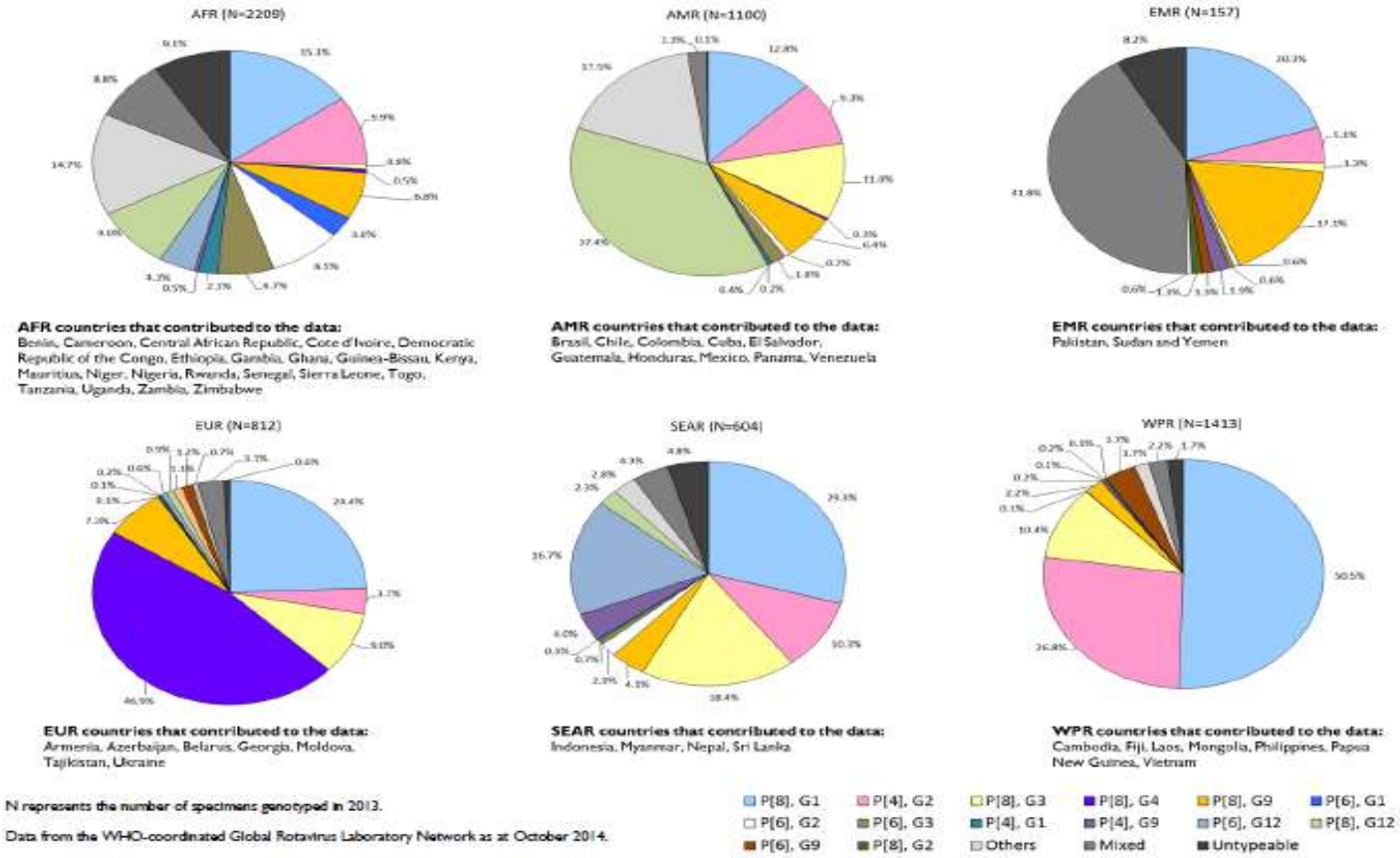

Figure 2.7: Distribution of rotavirus genotypes in WHO regions [28]

lead to evolution of diverse unusual and rare human rotavirus strains. For instance, a rare combination of G1 with P[19] was reported in India which was the result of human-porcine reassortment where the VP7 and VP6 genes were of human origin whereas VP4 and NSP4 genes belonged to porcine species [172]. In other unusual combinations, G1 genotype has also been found with other P-types such as P[4] and P[6] [173, 174].

2. G2

G2 rotaviruses commonly found to have P[4] as their VP4 counterpart. However unusual combination of G2 with P[6] was reported for the first time in Nigeria in 2001 [175]. Later, in an unusual outbreak in Philadelphia, this strain was reported again which accounted for 86% of G2 associated rotavirus infections [176]. G2 was also found to be in rare association with P[8] in Denmark [177].

3. G3

G3 is found in common association with P[8] in humans. However in 2011, a new G3 variant was reported where instead of P[8] genotype, P[2] was present. Molecular and phylogenetic studies reveal that this novel strain was a result of reassortment between simian like and group A rotavirus strains belonging to unknown animal and its subsequent human infection [178]. Along with G3P[2], a number of studies have shown the emergence of a novel HRV strain G3P[3] resulted because of interspecies transmission and reassortment. In a similar study from Thailand, G3P[3] strain has been isolated which was reported to carry simian-like VP7 and caprine-like VP4 genes [179]. Another instance of interspecies transmission of rotavirus strains was recognized in 2012; where a canine origin rotavirus G3P[3] strain was found to infect human [180]. The role of reassortment events in evolution of unusual G-P combinations was recognized after the isolation of G3P[3] and G3P[9] strains in humans in a number of studies [181-183]. The emergence of a novel G3P[9] strain was documented in 2011 and again in 2013 which was a result of reassortment between canine and feline rotavirus strains and subsequent transmission in humans [184, 185]. As discussed earlier, point mutations in the gene segments of rotaviruses also contributes towards their diversity. Such a case was observed in Japan where multiple substitutions in amino acid sequences gave rise to a new variant [186]. The same study documented the unusual combination of G3 with P[4]. Mukherjee et al. (2012) reported the unusual genotype combination of G3P[10] for the first time in eastern India. Evolutionary analysis of the virus revealed derivation of its genes from the

rotaviruses of human DS-1-like and AU-1-like strains of simian and caprine host species [187]. G3P[10] was also identified in Thailand where the virus possessed the gene segments from distinct species [188]. The rotaviruses having G3 have been also found in unusual association with other VP4 proteins such as P[25] and P[19] which were the result of human-porcine reassortment [189, 190].

4. G4

G4 is among the common G-type responsible for gastroenteritis in humans and is usually associated with P[8]. However, two novel combinations in form of G4P[4] and G4P[6] were also found in humans, which were evolved as a result of interspecies transmission and multiple reassortment [191].

5. G5

Rotaviruses belonging to the G5 genotype are rarely found to infect humans and therefore are responsible for a negligible fraction of rotavirus infection worldwide. However, epidemiological studies from Brazil have identified this strain in 1994 and 1996 [192, 193], in Argentina in 2001 [194], in Paraguay in 2002 [195] and in Cameroon in 2004 [196]. G5 has been identified to cause infection in humans of Asia for the first time in 2008 where its G5P[6] variant was a result of human porcine reassortment [197]. Similarly a G5P[6] strain was reported in Bulgaria in 2012 and is supposed to be another human porcine reassortant [198].

6. G6

G6 is not a very common G-type identified in humans, but a survey of the literature reveals a wide geographical distribution of G6 in humans around the world. A number of studies have documented the occurrence of G6 in association with a range of P genotypes such as P[6] and P[14] [199, 200]. These strains were evolved through multigenic reassortment followed by transmission to humans.

7. G8

G8 genotype rotaviruses are frequent causes of gastroenteritis in cattle [201]. However, this strain has demonstrated increased prevalence in humans in the African and European regions because of zoonotic transmission [202, 203]. Recently, G8 has been found to be associated with human gastroenteritis in the United States as well [204]. This unusual

genotype has also been isolated from humans in other parts of the globe such as India and Korea [205, 206].

Table 2.3: Usual G-types with unusual combinations [29]

G-Type	Commonly associated P-Type	Unusual P-type association
G1	P[8]	**P[4]** **P[6]** **P[19]**
G2	P[4]	**P[6]. P[8]**
G3	P[8]	**P[2]** **P[3]** **P[4]** **P[9]** **P[10]** **P[19]** **P[25]**
G4	P[8]	**P[4], P[6]**
G9	P[8]	**P[4]** **P[6]** **P[19]**

Table 2.4: Unusual G genotypes of rotavirus [29]

Genotype	Associated P-Types
G5	P[6], P[8]
G6	P[6], P[14], P[9]
G8	P[1], P[4], P[6], P[14], P[8]
G10	P[8], P[6], P[11], P[4]
G11	P[8], P[6], P[4], P[25]
G12	P[4], P[9], P[6], P[8]

8. G9

The first detection of G9 in humans was in 1987 [207], after which it disappeared for a period of time to reappear again in mid-1990s [208]. According to molecular genotyping data from different regions of the world, G9 emerged as the fifth most common genotype responsible for gastroenteritis in humans [161]. In many regional studies, G9 was found to be the predominant genotype in humans. For instance, in 2006, this particular genotype accounted for as high as 91.6% of total rotavirus infections [209]. Countries belonging to other geographical locations such as Cuba (78.5%), Argentina (61.5%), Italy (53.3%), and Spain (87.7%) also have a substantial burden of G9 associated gastroenteritis cases [210-213]. The most common association of G9 is with P[8] but other VP4 types such as P[4], P[6], P[11] and P[19] are also found with G9. These strains were originated due to interspecies transmission and reassortment events [214-216].

9. G10

G10 is a common G type responsible for infections in cattle, however it has also been found to be associated with human gastrointestinal infections [217]. The G10 strains isolated from humans revealed its molecular identity with animal rotaviruses indicating its zoonotic transmission [218]. Recently, G10 rotavirus infections in humans have surfaced in Africa and India [219, 220]. The VP4 types commonly found with G10 are P[8], P[4], P[11] and P[6] [191, 218, 220, 221].

10. G11

After its first detection in humans in 2005 [222], G11 has been infrequently detected in humans in different time periods [223, 224]. The G11 genotype isolated from humans reveals a significant similarity with those of animal origin, which advocate its zoonotic transmission [167].

11. G12

G12 rotaviruses are among the most rapidly emerging gastroenteritis agents in humans. G12 was reported for the first time in humans in the Philippines in 1987 when they were isolated from diarrheic children admitted to hospital [225]. Subsequently, it was detected in Thailand in 1998. It was again detected in the next year itself in the United States, which was followed by its heralded appearance in all the parts of the world [226, 227].

Studies from different parts of the world indicate that the G12 strains isolated from humans are usually associated with P[6] and P[8] [165]. However in some countries feline origin P[9] in association with G12 has been reported to infect humans [228, 229]. The G12 rotavirus has remarkable diversity because of its very frequent reassortment activity.

2.2.1.13 Impact of vaccination on strain distribution

Selective vaccine pressure may have a significant impact on the distribution of rotavirus strains. Post-vaccination surveillance studies from various countries including USA, Australia, Belgium and Brazil have reported a substantial increment in the prevalence of some genotypes (G2 and G3) in contrast to the pre-vaccination era [230-234]. Data from the United States, where RotaTeq was used for vaccination, depicted substantial increase in the prevalence of G3 genotypes after the introduction of the rotavirus vaccine; the G3P[8] genotype percentage remained the same (~2.8) during the first year after its introduction and increased to 35.7% during the second year. Similarly, the G9P[8] incidence decreased and then increased in the next 2 consecutive years post-vaccination. The percentage of G1P[8] in the pre-vaccination era from 1996–2005 to 2005–2006, respectively was 78.5% and 23.4%, while it increased to 69.6 and came down to 29.6 during the next 2 consecutive years [235].

In Australia, where both Rotateq and Rotarix were used, G2 was detected as prominent genotype as a whole but in the regions where Rotarix was administered, a higher percentage of G3 strains was observed. G9P[8] percentage decreased continuously during the years following vaccination, while G1P[8] and G2[4] showed no continuous trend but showed fluctuations in their occurrence with increased prevalence during the first year post-vaccination. The laboratory based surveillance performed in Brazil reported an increase in G2 detection events and a significant decline in G9 genotypes after the introduction of Rotarix vaccine in 2006 [236]. However, a noteworthy observation from the study is the decline in G2 genotype levels in 2009. During this year, G2 was reported in only 37.5% of cases in comparison to 49%, 66% and 85% in the preceding years. G3P[8] and G4[8] disappeared respectively during post-vaccination years 2007 and 2006 and emerged again during 2009 and 2008. The G1P[8] genotype against which the vaccine was used, decreased in its prevalence continuously during the next three consecutive years and increased again to 20.4% in 4th year. One of the reasons for these

changes could be that selective vaccine pressure leads to the emergence of vaccine resistant strains which were expressed during 2009.

Based on the data available so far regarding circulating rotavirus genotypes, it is tempting to speculate that vaccine-induced selection pressure has significant impact on the distribution of rotaviruses in a particular region and also, acts as driving force in the emergence of new rotavirus strains which are less susceptible to vaccine. But there are reports documenting the similar fluctuations in genotypes prevalence and also, the re-emergence of disappeared genotypes during the pre-vaccination era suggesting the role of natural fluctuations/ environmental factors in addition to vaccines. But before assigning it to after vaccine affects, continued surveillance data for extended years is needed to monitor strain changes after vaccination.

In addition, some other studies showed the emergence and circulation of various uncommon strains in a significant proportion after the introduction of vaccination [204, 237]. Furthermore, the regions where Rotarix was introduced have shown the increased prevalence of G3 and the RotaTeq states have shown the predominance of G2 strains [238, 239].

2.2.1.14 Clinical features

The clinical presentation of rotavirus gastroenteritis is similar to other pathogens that cause gastroenteritis in children although they can be more severe [240]. The infection leads to a spectrum of responses varying from subclinical infection to a mild diarrhea, to a severe and potentially lethal dehydrating sickness. The incubation period for rotavirus illness is approximately 48 hours. The first symptom to appear is vomiting followed by watery diarrhea, fever and severe dehydration, which can occur individually or in combination [241]. The symptoms may last for 3-8 days. According to studies, 10% of the first episodes of rotavirus infections are severe enough to lead to a clinic visit and require aggressive treatment [242, 243]. In 1-3% cases, the severity of infection is so high that it requires intravenous rehydration to maintain the balance of fluid and electrolytes. The children suffering from rotavirus infections tend to suffer more from vomiting and dehydration in comparison with the children having gastroenteritis due to other causes [244].

The clinical symptoms associated with rotavirus infections vary by age group. It has been observed that the first infection after the age of 3 months results in the most severe illness [240]. Infants are generally asymptomatic or suffer only mild symptoms in comparison with children from higher age groups [245, 246]. According to a study, rotavirus infection may be responsible for 30% to 40% of cases of necrotizing enterocolitis [246]. Rotavirus infection in adults remains either asymptomatic or shows mild symptoms. This may be because of the protection owing to previous infections. Although studies have reported that rotavirus infection in adults is responsible for 3-5% of gastroenteritis associated admissions [10].

In low income settings, the rotavirus associated illness can be more severe because of malnutrition, poor hygiene and concurrent infections [247]. The virus infection is not confined to the gut but viremia and antigenemia has also been found in the blood [248, 249]. In the rare case of severe infections, rotavirus have also been identified to cause extraintestinal infections in the cerebrospinal fluids [250]. The immunocompromised children act as potent reservoirs of the virus where virus shedding has been witnessed for more than a year [251, 252].

2.2.1.15 Rotavirus vaccines

Though improvements in sanitation and personal hygiene have led to significant reduction in bacterial and parasitic gastroenteritis infections, but they have proved to be of no effect on rotavirus associated morbidity and mortality [253-256]. It has been established that previous rotavirus infection renders a strong immune response against subsequent infections [257], and vaccination mimics this first infection without causing the disease, thus vaccines can be a good option to control rotavirus associated morbidity and mortality.

2.2.1.15.1 RotaShield

RotaShield (a rhesus rotavirus tetravalent [RRV-TV] vaccine) is the first multivalent, live reassortant vaccine. It comprises of four commonly found rotavirus strains G1, G2, G3 and G4; out of which G1, G2 and G4 are from three rhesus-human reassortant strains and G3 is from rhesus rotavirus [41]. The field trials of this vaccine in the United States, Finland and Venezuela have revealed its high efficacy in these settings [258-261]. Due to good efficacy, this vaccine was licensed in United States in 1998 and included in the

immunization schedule. But after the first nine months of the program, a large number of cases of vaccine associated intussusception were reported. The estimated rate of vaccine associated intussusception was 1 in 10,000 vaccinated children [262]. The greatest risk was 3 to 10 days after the first dose [263, 264]. Due to this adverse effect, RotaShield was withdrawn from the market less than a year after its licensure.

2.2.1.15.2 RotaTeq

With the increase in knowledge regarding the importance of VP4 protein in protection, the new rotavirus vaccines included a VP4 component in them. RotaTeq is a pentavalent, live attenuated, human-bovine reassortant oral vaccine developed by Merck Research Co. This vaccine is composed of five live reassortant rotaviruses: four rotaviruses express the globally common VP7 proteins (G1, G2, G3 and G4) and P7[5] from parent bovine virus WC3 (Figure 2.8). The fifth reassortant has P1A[8] from the human strain and G6 from the WC3 strain. The administration of RotaTeq occurs in three doses beginning from 6 to 12 weeks of age.

The phase III trial of RotaTeq was conducted in 11 countries which included more than 70,000 children [265]. The trial focused on evaluating the vaccine safety, immunogenicity and efficacy. The risk of intussusception was monitored for 42 days after each dose. In the trial, six children who were administered vaccine, suffered from intussusception whereas five cases of intussusception were observed in placebo group. The outcomes of the study did not suggest any increased risk of intussusception in vaccine group and the vaccine was marked safe as far as intussusception is concerned.

As far as clinical symptoms are concerned, the rate of fever and irritability were similar in the vaccine and placebo groups. Diarrhea and vomiting were more frequent in vaccine group than placebo group. The seroconversion rate was drastically higher in vaccinated children (95%) than in the placebo treated children (14%).

The efficacy studies in the clinical trials have revealed high efficacy against any severity of rotavirus infection. The efficacy of RotaTeq against rotavirus infection of any severity was 74% and against severe infection was 98%. RotaTeq showed high efficacy against all the commonly prevalent serotypes (G1, 75%; G2, 63%; G3, 83%; G4, 48% and G9, 65%). The efficacy of RotaTeq was evaluated in the second rotavirus season following vaccination and it was 65% against infection of any severity and 88% against severe

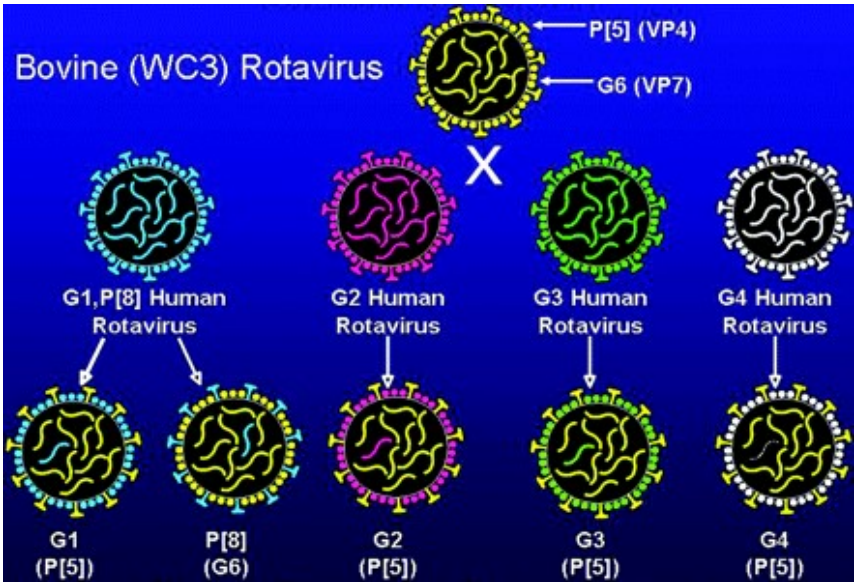

Figure 2.8: RotaTeq (Adapted from Dennehy et al., 2008 [267])

rotavirus infection [265]. The phase III trial results of RotaTeq were satisfactory enough to get it licensed in the United States in 2006. It was recommended to be administered in three doses at 2, 4, and 6 months of age [266].

2.2.1.15.3 Rotarix

Rotarix is the live attenuated vaccine developed from strain 89-12 in Cincinnati by tissue culture passage [268]. The vaccine strain has the G1P[8] genotype which represents the most common VP7 and VP4 genotypes found in humans. Further development of the vaccine was done by Avant Immunotherapeutics before its acquisition by GlaxoSmithKline Biologicals (Rixensart, Belgium). Here the vaccine strain was cloned and underwent passage 12 more times and renamed RIX4414. After licensure, it was named as Rotarix. The underlying principle behind Rotarix vaccine was to stimulate serotype-specific immunity against the most prevalent human G-type and P-type rotaviruses.

To evaluate the risk of intussusception associated with the vaccine, a double blind, placebo-controlled trial was carried out in 11 Latin American countries and Finland which included 63,000 infants. The study revealed that the vaccine did not cause intussusception [269]. Two doses of vaccine were administered at 2 and 4 months of age and during a period of 31 days after each dose, the vaccinated children showed no increase in intussusception as compared with placebo. During the study, six of the immunized children and seven children in the placebo group suffered from

intussusception showing no significant association. The vaccine group showed similar rates of diarrhea, fever and vomiting as the placebo group.

To assess the efficacy of the vaccine, a large trial was conducted which included 20,000 children [269]. The efficacy of the vaccine came out to be tremendous with 85% against severe rotavirus diarrhea and 100% against very severe rotaviral gastroenteritis. The vaccine was very efficacious against G1 (92%), G3, G4 and G9 serotypes but the efficacy was not significant against G2 (48%) serotype. Although the vaccine efficacy against G2 was lower in the trials but different studies have documented it to be very effective again non-G1 and non-P[8] strains. In another study in Europe, the vaccine efficacy of Rotarix was as high as 81% against G2P[4] strains. The vaccine efficacy against G1P[8] was found to be 96% and against non-G1P[8] strains showed 88% efficacy [270]. Mexico and the Dominican Republic were the first countries to license Rotarix vaccine in 2004 [270].

2.2.1.15.4 Rotavac

Rotavac is developed under Indo-US Vaccine Action Program (National Institutes of Health 2013) and is made from 116E rotavirus strain which is a naturally occurring reassortant strain with genotypes G9P[11] [271]. It contains VP4 gene of bovine origin and rest of the ten genes from human rotavirus. The 116E strain was found to be asymptomatic and naturally attenuated [272, 273]. The strain was cultivated on Vero cells and found to be safe and immunogenic [274]. Clinical trials revealed that the vaccine has an efficacy of 56% which is comparable to the other licensed rotavirus vaccines [271]. Rotavac has shown sustained efficacy of 49% in the second year of life, which much better than the other available vaccines [271]. The vaccine was effective against a large variety of the strains and hence has good cross protection. The most important aspect of this vaccine is its low cost of around 1USD which is ~ 20 times less than its pre-existing competitors.

2.2.1.16 Rotavirus in adults

Rotavirus is a widely recognized gastroenteritis agent in children. The role of rotavirus infection in adults is not well recognized as mostly the rotavirus infections in this case are asymptomatic or lead to mild symptoms. However, rotavirus infections in adults present a wide spectrum of severity and manifestations. In studies including adult volunteers, rotavirus ingestion results in many symptoms including diarrhea, fever, malaise,

headache, cramping and nausea [275-277]. As far as, epidemiology of rotavirus infections in adults is concerned, a number of studies from different parts of the world have held rotavirus responsible for significant incidences of rotavirus gastroenteritis in adults. Although, the infection rate varies with the geographic location and characteristics of patients. In some studies, rotavirus infections in the adult population were very low. In a study from the UK, rotavirus was isolated from 4.1% of adult patients suffering from diarrhea [278]. Similarly, rotavirus infection rate of 3% was observed in Switzerland, 3% in Sweden, 5% in Thailand, 2-4% in Netherlands and around 4% in Michigan [279-282].

In other geographical locations, the rates of rotavirus infections were even higher. In a recent study from southern India, rotavirus was found in 8.4% of diarrheal patients. In a study from Japan, rotavirus was responsible for 14% of gastroenteritis incidences in adults [11]. In an Australian study, rotavirus was the second most common cause of diarrhea in adults with 17% infection rate [283]. In Indonesia, as high as 42% adult gastroenteritis patients have rotavirus in their stool samples [284]. The Mexican study further reveals the astonishing figure of rotavirus associated diarrhea in adults where rotavirus was isolated from 63% of adult samples [285]. Along with endemic diarrhea, rotavirus is also responsible for a number of gastroenteritis outbreaks [286-288].

Rotavirus is also the important pathogen responsible for travellers' diarrhea among adults. Studies show that people travelling to Central America and the Caribbean show higher rates of rotavirus infection. In such an incidence, 9% of travellers returning from Jamaica have rotavirus diarrhea (Steffen 1999). Interestingly, in this case, rotavirus was the second most prominent cause of diarrhea in adults. In other studies, rotavirus was found in as high as 25-35% patients suffering from traveller's diarrhea [289-293].

2.2.2 Norovirus

Norovirus was first detected in the stool sample during a gastroenteritis outbreak in Norwalk, OH and hence was previously known as Norwalk virus. This was the first viral agent recognized to cause gastroenteritis in humans [294]. Initially, the illness due to Norovirus was described as 'winter vomiting disease' because of its frequent occurrence in winter season and vomiting as a primary symptom [295].

Human noroviruses are the prominent cause of gastroenteritis epidemics and outbreaks in hospitals, nursing homes, cruise ships, and the military [296, 297]. Norovirus is

responsible for 685 million annual cases all around the globe. Out of these, 200 million cases were reported in children below the age of five years. According to recent estimates, this virus is responsible for around 50,000 deaths of children, almost all of them occurring in developing countries [298].

2.2.2.1 Virology

Noroviruses, as visualized by electron microscopy are particles of 27-30 nm in diameter. These are non-enveloped viruses. The nucleocapsid is rounded and has an icosahedral symmetry. The genome of human Norovirus is made up of a linear, positive sense RNA which is around 7.6 kb in length (Figure 2.9) [299]. The 5' end of the genome is covalently attached to the viral protein genome (VPg), which has an important role in virus infectivity and initial translation. The RNA genome is polyadenylated at the 3' end [300]. The three open reading frames, ORF-1, ORF-2, ORF-3 encodes eight viral proteins. ORF-1 encodes a 194 kDa protein complex which is proteolytically cleaved into six proteins by the viral protease 3C. These proteins are the non-structural proteins and are involved in replication and transcription [301]. The second and third ORFs encodes structural proteins, VP1 and VP2, respectively. VP1 is a 60 kDa capsid protein and plays a major role in replication [302]. The VP2 is the 23 kDa protein which interacts with genomic RNA [303].

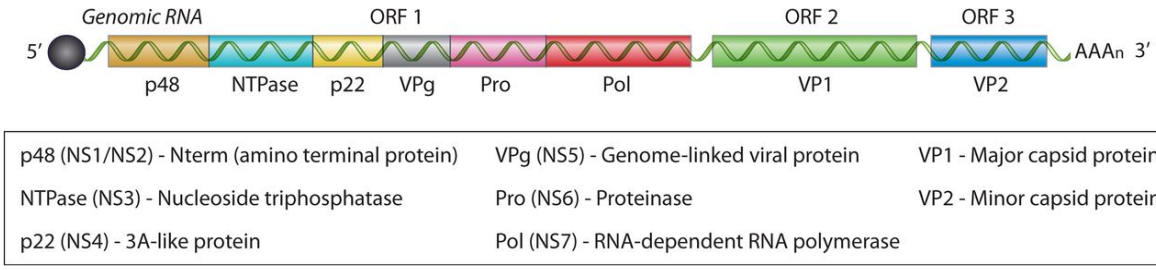

Figure 2.9: Structural representation of human norovirus genome (Adapted from Robilotti et al., 2015 [304]). The genome is a linear, positive-sense RNA, ~7.6 kb in length, linked to the viral protein genome (VPg) (solid black circle) at the 5' end and polyadenylated at the 3' end. Three open reading frames, ORF-1, ORF-2, and ORF-3, encode 8 viral proteins. Six nonstructural (NS) proteins are encoded by ORF-1 by proteolytic cleavage by the virally encoded cysteine proteinase (Pro). The structural proteins VP1 and VP2 are encoded by ORF-2 and ORF-3, respectively.

The capsid of the virion is made up of 90 dimers of the VP1 protein arranged in icosahedral symmetry. The arrangement of VP1 dimers is such that it results in cup-like structures on the surface of virus.

2.2.2.2 Nomenclature and classification

According to the new classification system, noroviruses are classified into five genogroups, GI, GII, GIII, GIV and GV. These genogroups share between them a total of 31 genetic clusters or genotypes: 8 genotypes in GI genogroup, 17 in GII, 2 in GIII, 1 in GIV, and 1 in GV [305]. Studies have documented that norovirus genotype GII.4 is responsible for 70% of norovirus outbreaks [306, 307].

2.2.2.3 Clinical and epidemiological features

Norovirus infection in humans occurs at all age groups. Though Norovirus outbreaks occur all around the year, they increase in the colder months [308]. The incubation period is generally around 1-2 days which is followed by vomiting, nausea, abdominal cramps, myalgia and diarrhea. The symptoms usually resolve within 2-3 days but according to recent studies, they can persist for longer periods (4-6 days) in children and hospital outbreaks [309, 310]. Diarrhea is the prominent symptom to develop in children below 5 years of age whereas other patients experience vomiting more frequently [309, 311]. 37-45% patients show the sign of fever that generally resolves within 2 days [312, 313]. Though, the clinical severity of the disease caused by norovirus infection in children appears to be milder in comparison to rotavirus [309] but the severity scores of the children hospitalized due to Norovirus are similar to the rotavirus infections [314]. The acute symptoms and viral shedding tends to be prolonged in the elderly and immune compromised patients.

2.2.2.4 Transmission

Noroviruses are transmitted from food or through faeco-oral route [315]. In some cases, the transmission can be through air by aerosol of infectious vomit [316]. Contaminated water can be also inferred as a source of transmission of norovirus [317]. The virus is highly contagious and is responsible for sporadic or diarrhea outbreaks in hospitals, schools, colleges, restaurants, cruises and hotels [318]. Quality control of food basically focuses on bacterial contamination and thus norovirus contaminations are not reported often [319]. Aquatic food such as oysters, living in contaminated waters and eaten raw are major routes of transmission of the virus [320]. Some characteristics of noroviruses which facilitate their rapid transmission are: (i) low infectious dose (<10 viral particles) [321]; (ii) Prolonged virus shedding; (iii) stability of the virus at broad range of temperatures

and even in high concentration of chlorine [322]; and (iv) high rate of re-infections due to lack of long term immunity and inadequate cross protection against diverse strains.

2.2.2.5 Prevention and treatment

The first and foremost strategy for prevention of norovirus infection is to stop its transmission. Washing of hands after coming in contact with the patient and its objects is very essential. Norovirus can survive on dry inanimate surfaces for 8 hours to 7 days; therefore all the surfaces are required to be cleaned by 2% hypochlorite [3, 323]. Appropriate safety measure while preparing and handling of food should be taken to prevent it from getting contaminated. Once infected with norovirus, food handlers should be refrained from handling food during the disease and for 48-72 hours after the recovery [324]. As there is no antiviral agent to treat norovirus infection, the preferred treatment which can be given is rehydration therapy. Although not very common, hospitalization in some cases is required.

2.2.2.6 Laboratory diagnosis

Electron microscopy is the classical method of detecting noroviruses. This method is employed in many diagnostic laboratories but it requires highly qualified professional and very expensive equipment.

Enzyme immunoassays (EIA) are the preferred method of choice for large scale surveillance studies. The EIAs have the antibodies raised against baculovirus expressed viral antigens. But EIAs are specific for only some noroviruses and suffer from limited sensitivity with a wide range of noroviruses [325, 326]. Although, a new generation of kits have been developed which have relatively high sensitivity and specificity and are very useful for norovirus diagnosis in outbreaks [327].

The RT-PCR technique is the most advanced technique to detect noroviruses in the clinical and environmental samples. They have the capability of detecting virus even in samples with low viral load [328]. The RT-PCR method is sensitive and has high specificity. The sequencing results after PCR can be used for phylogenetic analysis to study the evolution of the virus and to infer their genotypes. Real-time quantitative PCR are more sensitive and faster than conventional PCR and allow real time monitoring. Recently real time PCR has gained popularity for its application in large scale surveillance studies [329].

2.2.3 Enterovirus

Enterovirus is the largest genus belonging to the family *Picornaviridae* and includes around 200 serotypes [330]. This genus contains 10 species, out of which enterovirus A-D, human rhinovirus A-C, coxsackievirus A & B, Echovirus, rhinovirus, poliovirus and relatively newer enteroviruses are found to be pathogenic to humans. Enterovirus infects approximately 1 billion people each year globally and causes a large spectrum of disease in humans [330]. The diseases caused by enteroviruses include acute flaccid paralysis (AFP), aseptic meningitis, acute encephalitis, type-1-diabetes, uveitis, hand, foot and mouth disease (HFMD), diarrhea, brainstem disease, herpangina, exanthema, pleurodynia, cardiomyopathy, coagulopathy, atherosclerotic arterial disease, multi-system hemorrhagic disease, sudden deftness, fatal illness with pulmonary hypertension in neonates, transverse myelitis, aplastic anemia etc [330]. Recent reports have also documented the role of enteroviruses in gastroenteritis also. However, most of enterovirus infections are asymptomatic and only 1% of the infections cause disease severe enough to cause disease [331].

2.2.3.1 Virology

The enterovirus genome is made up of a single-stranded positive-sense RNA which is approximately 7.5 kb in length (Figure 2.10). At the 5' end, the RNA is attached to the viral protein VPg and is polyadenylated at the 3' end. The genome has one open reading frame which encodes a single protein which is proteolytically cleaved by two proteases 2A and

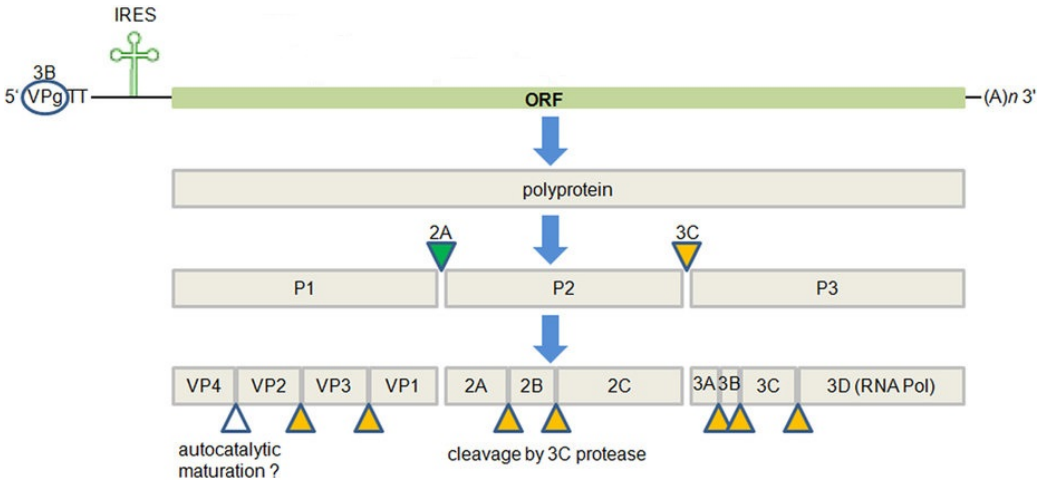

Figure 2.10: Structural organization of enterovirus genomic RNA and maturation of peptides from polyprotein (Adapted from Huang et al., 2015 [332])

3C into 11 individually competent proteins (4 structural and 7 non-structural). The structural proteins (VP1-VP4) are components of the capsid where VP1 is the most abundant protein and is the basis for differentiating the virus into various serotypes.

2.2.3.2 Enteroviruses in diarrhea

Although, enteroviruses are the cause of a large spectrum of severe diseases, their role in diarrheal disease is not very well established. However, recent reports have shown that enteroviruses are the important etiological agents of diarrhea in a number of cases. In a recent study by Rao et al. in 2014 which included children of less than 2 years of age, non-polio enterovirus was isolated from 38% of total diarrheal episodes [333]. In a different study by the same group, where the prevalence of enterovirus was studied for five consecutive years, the enterovirus infection rate in diarrheal patients ranged from 9%-19% [19]. The studies from other parts of the globe also documented the significance of enterovirus as an etiological agent of diarrhea.

Sequence analysis of VP1 gene of enterovirus strains isolated from the diarrheal patients revealed the involvement of a total of 37 serotypes in the disease. Out of these, E1, E7, E11, E13, E14, E30 and E33 were found to be responsible for majority of enterovirus associated diarrhea [19].

Chapter 3

Materials & Methods

MATERIALS AND METHODS

3.1 Sample collection and transportation

A total of 607 stool samples were collected from four hospitals located in Himachal Pradesh: Regional Hospital, Solan; Indira Gandhi Medical College (IGMC), Shimla; Regional Hospital, Nalagarh and Regional Hospital, Bilaspur from 2012-2015. Out of these samples, 247 were collected from the children (≤5 years), 50 were from older children (6-17 years) and 310 were from adult (≥18 years) patients. The hospitalized patients included in the study had diarrhea (defined as passage of three or more loose stools per day) as the common symptom along with vomiting, dehydration, fever and abdominal pain. The demographic details of the patients were documented and they or their legal representatives gave their consent. None of the subjects included in the study had been vaccinated for rotavirus. The stool samples were collected in sterile, screw cap containers and transferred to laboratory immediately under proper cold chain conditions.

3.2 Processing of stool specimens

10% stool suspension was prepared by dissolving 1 g/ml of stool in 10 ml of phosphate buffer saline (PBS) in a tube. The contents of the tube were vortexed for 1 min and then centrifuged at 10,000 rpm for 10 min to remove the coarse debris. The supernatant was then transferred to a fresh tube and stored at 4°C for short term and -80°C for long term.

3.3 Enzyme linked immunosorbent assay (ELISA)

Detection of rotavirus in the stool samples was done using commercially available enzyme linked immunosorbant assay kit (Premier Rotaclone, Meridian Biosciences Inc., Cincinnati, OH, USA). The procedure followed for the detection of rotavirus is following:

1. Sufficient number of wells for samples and the controls were snapped off and inserted into the microtiter well holder. The sample positions were recorded appropriately.
2. Two drops (100 µl) of diluted faecal sample, positive control and negative control were added to the bottom of separate wells.
3. Two drops (100 µl) of enzyme conjugate were added to each well. Mixed by gentle swirling.
4. Incubated at room temperature for 60 min.

5. The liquid in the wells was poured out into a discard vessel. The microtiter well holder was tapped upside down vigorously against absorbent paper to ensure complete removal of liquids from the wells.
6. All the wells were filled with deionized water and the liquid was poured out as in step 5.
7. The washing procedure (steps 5 and 6) was repeated for a total of 5 times.
8. Two drops (100 µl) of substrate A solution was added to each well.
9. Two drops (100 µl) of substrate B solution was added to each well.
10. Incubated for 10 min at room temperature.
11. Visual determinations were made by observing the blue colour and for spectrophotometric determinations, two drops (100 µl) of stop solution (sulphuric acid) were added to each wall and absorbance was read at 450 nm.

3.4 RNA isolation with TRIzol method

Viral RNA was isolated from stool suspensions using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The protocol for RNA isolation is following:

1. 500 µl of stool suspension was taken in a fresh 2 µl microcentifuge tube and 500 µl of TRIzol reagent was added to it.
2. The contents of the tube were vortexed thoroughly and incubated at room temperature for 15 min.
3. 200 µl of chloroform was added to the tube and vortexed thoroughly for 10 sec.
4. Incubated at room temperature for 10 min.
5. Samples were centrifuged at 12,000 x g for 15 min at 4°C.
6. The aqueous phase of the sample was removed by angling the tube at 45° and pipetting out the solution.
7. The aqueous phase was placed into a new tube.
8. 500 µl of 100% isopropanol was added to the aqueous phase and incubated at room temperature for 10 min.
9. Centrifuged the contents at 12,000 x g for 10 min at 4°C.
10. The supernatant was discarded leaving the RNA pellet at the bottom of the tube.
11. The RNA pellet was washed by mixing with 1 ml of 75% ethanol.
12. Centrifuged at 7500 x g for 10 min and supernatant was discarded.
13. The RNA pellet was air dried for 5-10 min, resuspended in RNase-free water and stored at -80°C until further use.

3.5 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was prepared by reverse transcription of isolated RNA using Verso cDNA synthesis kit (ThermoFisher scientific, Waltham, Massachusetts, USA). The components used for cDNA synthesis are:

Total RNA	7.0 µl
DMSO	1.0 µl
Random hexamer	1.0 µl
Nuclease-free water	4.0 µl

The reaction mixture was incubated at 95°C for 5 min followed by snap chilling on ice. The following components were added to the reaction mixture

5X cDNA synthesis buffer	4.0 µl
dNTP mix	2.0 µl
Enzyme mix	1.0 µl

The reaction mixture was incubated at 42°C for 50 min followed by enzyme inactivation step at 95°C for 2 min. The cDNA formed was stored at -20°C until further use.

3.6 Polymerase chain reaction

3.6.1 Detection of rotavirus, norovirus and enterovirus

Three diarrhea causing RNA viruses, group A rotavirus, norovirus (GI & GII) and enterovirus were detected using PCR based method. The primers used for detection of various viruses were VP6(F) & VP6(R) for group A rotavirus; Mon432 & Mon434 for norovirus GI; Mon431 & Mon433 for norovirus GII; and F1 & R1 for enterovirus [334-336] (Table 3.1).

Table 3.1: Primers used for the detection of viruses

Virus and Primer	Sequence	Amplicon Size (bp)
Group A Rotavirus VP6 (F) VP6 (R)	 TTTGATCACTAAYTATTCACC GGTCACATCCTCTCACTA	227
Norovirus GI Mon432 Mon434	 TGGACICGYGGICCYAAYCA GAASCGCATCCARCGGAACAT	213
Norovirus GII Mon431 Mon433	 TGGACIAGRGGICCYAAYCA GAAYCTCATCCAYCTGAACAT	213
Enterovirus F1 R1	 CAAGCACTTCTGTTTCCCCGG ATTGTCACCATAAGCAGCCA	440

The PCR reaction mixture included 2.5 µl of 10X Standard *Taq* Reaction Buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM forward and reverse primers each, 0.6U of *Taq* DNA Polymerase (New England Biolabs, Massachusetts, USA) and 3 µl of cDNA template. The volume of the mixture was made to 25 µl using nuclease free water (ThermoFisher Scientific, Waltham, Massachusetts, USA). The thermo cycler conditions employed for the detection of aforementioned viruses are shown below:

Virus	T_{ID}		T_C		T_A		T_E		T_{FE}		No. of cycles
	Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
Rotavirus	95	5	95	30	48	30	68	30	68	10	35
Norovirus GI & GII	95	5	95	30	50	30	68	30	68	10	35
Enterovirus	95	5	95	30	48	30	68	45	68	10	35

T_{ID} = Initial Denaturation
T_C = Cyclic Denaturation
T_A = Annealing Temperature
T_E = Cyclic Extension
T_{FE} = Final Extension
min = Minutes
sec = Seconds

3.6.2 Rotavirus genotyping

The cDNA samples positive for VP6 PCR were carried forward for genotyping by semi-nested PCR. The G and P typing was done using previously published primers recommended by World Health Organization (Figure 3.1, Table 3.2) [155, 156].

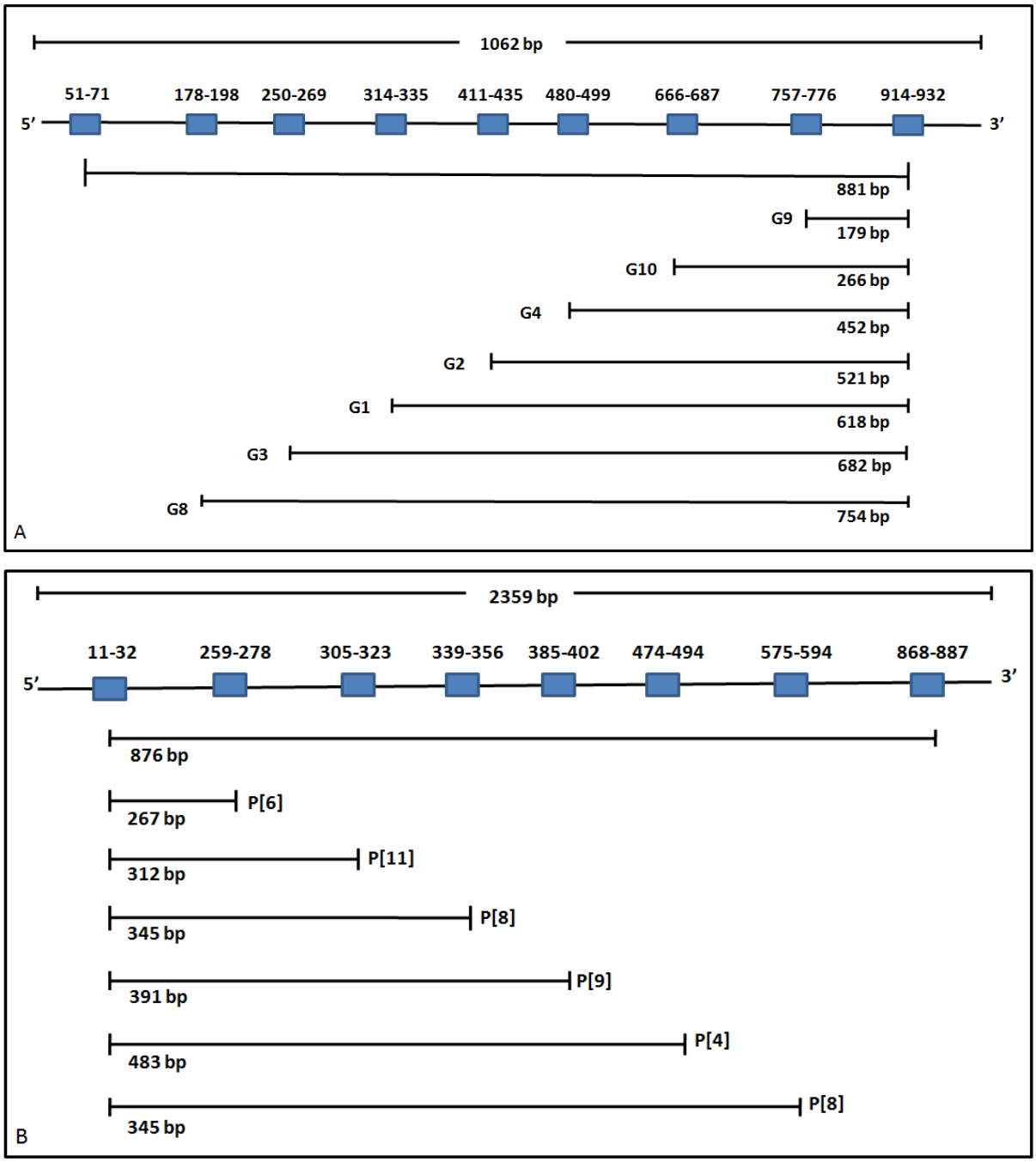

Figure 3.1: Schematic illustration of (A) VP7 and (B) VP4 genotyping PCR

Table 3.2: Primers used for genotyping of rotavirus

Primers	Sequence	Amplicon size (bp)
Primers for VP7 genotyping		
VP7F(+)	ATGTATGGTATTGAATATACCAC	
VP7R (-)	AACTTGCCACCATTTTTTCC	881
G1 (+)	CAAGTACTCAAATCAATGATGG	618
G2 (+)	CAATGATATTAACACATTTTCTGTG	521
G3 (+)	ACGAACTCAACACGAGAGG	682
G4 (+)	CGTTTCTGGTGAGGAGTTG	452
G8 (+)	GTCACACCATTTGTAAATTCG	754
G9 (+)	CTTGATGTGACTACAAATAC	179
G10 (+)	ATGTCAGACTACARATACTGG	266
G12 (-)	TAACGCTAATGAATTTTGGTACTG	450
Primers for VP4 genotyping		
VP4F (+)	TGGCTTCGCTCATTTTATAGACA	
VP4R (-)	ATTTCGGACCATTTATAACC	876
P[4] (-)	CTATTGTTAGAGGTTAGAGTC	483
P[6] (-)	TGTTGATTAGTTGGATTCAA	267
P[8] (-)	TCTACTGGRTTRACNTGC	345
P[9] (-)	TGAGACATGCAATTGGAC	391
P[10] (-)	ATCATAGTTAGTAGTCGG	583
P[11] (-)	GTAAACATCCAGAATGTG	312

First round

For G genotyping, in the first round, a 881 bp region of VP7 gene was amplified using VP7-F and VP7-R primers whereas for P genotyping, a region of 876 bp of VP4 gene was amplified using con2 and con3 primers in the first round. The reaction mixture included:

Component	20 µl reaction	Final Concentration
5X Phusion HF or GC Buffer	4 µl	1X
10 mM dNTPs	0.4 µl	200 µM
10 µM Forward Primer	1 µl	0.5 µM
10 µM Reverse Primer	1 µl	0.5 µM
cDNA	4 µl	≤ 250 ng
DMSO	0.6 µl	3%
Phusion DNA Polymerase	0.2 µl	1.0 units/50 µl PCR
Nuclease-free water	8.8 µl	

The thermocycling conditions for the first round PCR were:

T_{ID}		T_C		T_A		T_E		T_{FE}		**No. of cycles**
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	46	30	72	80	72	10	35

Second round PCR for G-genotyping

The first round product was subjected to the second round PCR using VP7-R as the reverse primer and a mixture of forward primers specific for G1, G2, G3, G4, G8, G9 and G10 genotypes. The components included in the PCR reaction mixture were:

Component	25 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer (G-type specific primers)	3.5 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	0.2 µM
Template DNA	2 µl	≤ 250 ng
DMSO	0.6 µl	3%
Taq DNA Polymerase	0.13 µl	1.25 units/50 µl PCR
Nuclease-free water	15.27 µl	

The thermocycling conditions for second round PCR for G-genotyping are following:

T_{ID}		T_C		T_A		T_E		T_{FE}		**No. of cycles**
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	42	60	68	60	68	10	35

G12-specific second round PCR

The second round PCR for the detection of G12 genotype was carried out separately. In this PCR, the VP7F (+) was used as the forward primer whereas G12 specific primer was used in reverse orientation. The components and reaction mixture used in this PCR were similar to the second round PCR except the primers. The thermocycling conditions employed were:

T_{ID}		T_C		T_A		T_E		T_{FE}		No. of cycles
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	48	30	68	40	68	10	35

Second round PCR for P-genotyping

For P-genotyping of rotavirus, the first round products of VP4 specific PCR were subjected to the second round PCR where con3 was used as the forward primer and a mixture of P[4], P[6], P[8], P[9], P[10] and P[11] specific primers were used as reverse primers. The components used in the reaction mixture were:

Component	25 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	0.5 µl	0.2 µM
10 µM Reverse Primer (P-type specific primers)	3 µl	0.2 µM
Template DNA	2 µl	≤ 250 ng
DMSO	0.6 µl	3%
Taq DNA Polymerase	0.13 µl	1.25 units/50 µl PCR
Nuclease-free water	15.77 µl	

The thermocycling conditions employed for second round PCR for P-genotyping were similar to the second round PCR for G-genotyping.

3.7 Agarose gel electrophoresis

The amplified products of all the PCRs were subjected to agarose gel electrophoresis. 2% agarose gel was formed by adding 1 g of agarose powder in 50 ml 1X TAE buffer. The agarose was mixed in the buffer by heating in microwave oven for 1 min. The solution was allowed to cool until luke warm and then ethidium bromide (EtBr) was added to the solution at a concentration of 0.5 µg/ml. The EtBr was mixed gently in the solution and

the solution was poured in the gel tray prefitted with a comb. The gel was allowed to cool and solidify in the tray for about 15-20 min. Then the comb was removed cautiously and the gel was placed in electrophoresis tank filled with 1X TAE buffer. 5 µl of the PCR product was mixed with 1 µl of 6X loading dye and loaded in the well. A DNA marker was included with each run. The electrophoresis was carried out at 100V for 30 min. Then the gel was kept under a UV transilluminator to visualize the bands and then photographed.

3.8 Sequencing and Phylogenetic analysis

The amplified 881 bp VP7- first round RT-PCR products were sequenced, aligned and analysed with the corresponding VP7 sequences of rotavirus G1 strains available in GenBank using ClustalW. Similarly, the first round PCR products of VP4 genotyping PCR were purified and sequenced. The nucleotide and amino acid similarity search was done using NCBI-BLAST algorithm [337]. The VP4 genes belonging to the P[6] genotype were analyzed with the corresponding VP4 sequences of rotavirus P[6] strains available in GenBank using ClustalW. The phylogenetic analysis was carried out with MEGA software, version 6.0 [338]. The phylogenetic tree was constructed using the neighbor-joining method. The evolutionary distances were computed using the Kimura 2-parameter method. The statistical significance of the different phylogenetic groupings was estimated by bootstrap analysis with 1,000 pseudoreplicates.

3.9 Nucleotide sequence accession numbers

The VP7 nucleotide sequences of the rotavirus strains obtained in this study were deposited into GenBank under accession numbers KM880063 (JU-SOL-173), KP938512 (JU-SHI-14), KP938513 (JU-SOL-5), KP938514 (JU-SOL-58) and KP938515 (JU-SOL-77). The VP4 nucleotide sequences used in this study were submitted in GenBank under accession numbers KP938516 (JU-SHI-14), KP938517 (JU-SOL-5), KP938518 (JU-SOL-58), KP938519 (JU-SOL-77) and KM880064 (JU-SOL-173). The accession numbers of rotavirus reference strains used in VP7 analysis were JN849114 (Rotarix-A41CB052A), GU565057 (RotaTeq-W179-9), L24165 (C95), M92651 (T449), L24164 (C60), AF426162 (SW20/21), AB018697 (AU19), U26376 (Israel-56), U26374 (Egy-8), D16343 (KU), D16323 (K2), EF079065 (7206/JP), EF079064 (7014/JP), DQ377596 (PA5/03), DQ377598 (PA2/04), AB081799 (AU007), DQ377589 (PA378), U26387 (Oh-64), U26370 (Cos-70), S83903 (1407), DQ377572 (PA78/89), DQ377587 (PA10/90),

U26378 (Kor-64), AB081795 (88H249), U26373 (Egypt-7), DQ377602 (PA17c), DQ377566 (PA3c), U26366 (Ban-59), AF183857 (CH631), DQ508167 (VN-281), DQ512979 (Thai-804), AY098670 (ISO-4), AY631049 (Dhaka8-02), EF079067 (6916/JP), DQ512986 (J-4689), AF480293 (Mvd9816), AF260945J (97S6), FJ948848 (6590), KJ870802 (KisB501), HM130926 (Seoul-045), KJ870787 (KisB104), JN258390 (2008747323), GU392988 (GER109-08), HG917358 (E88997), HG917362 (E9606), AB796443 (OH3385), AB796444 (OH3493), KF112996 (JS10-1), JX411969 (UK-HLD). The accession numbers of rotavirus reference strains used in VP4 analysis were KJ752817 (MRC-DPRU4090), LC019055 (A23), KP882231 (Bang-129), KM008690 (KOL-30-08), KM008689 (KOL-26-08), KM008688 (KOL-19-08), KJ751996 (MRC-DPRU2130-05), AF079356 (US1205), L20877 (M37), EF672612 (ST3), AY955299 (134/04-10), AY955300 (134/04-11), AY955303 (221/04-7), AY955302 (221/04-13), AY955305 (51/03), AY955306 (51/04), AY955304 (51/02), AJ621507 (BP1338/99), AJ621502 (BP271/00), AJ621504 (BP1198/98), AB770153 (AU19), AB176685 (JP3-6), AY955310 (134/04-7), AJ621503 (BP720/93), AJ621505 (BP1227/02), KR052749 (Gottfried).

3.10 Statistical analysis

The prevalence of different viruses was estimated by the proportion of positive samples. Bivariate analysis of differences in clinical characteristics of rotavirus positive and rotavirus negative patients was done using Chi-square test. Difference in rotavirus infection in male and female patients was analysed by Fisher's exact test. Differences in the rotavirus positivity by ELISA and RT-PCR; and genotype distribution in different age groups were analyzed by chi-square test. *P* values <0.05 were considered statistically significant.

3.11 *In silico* analysis

The work plan followed for the *in silico* analysis is depicted in the flow chart in Figure 3.2:

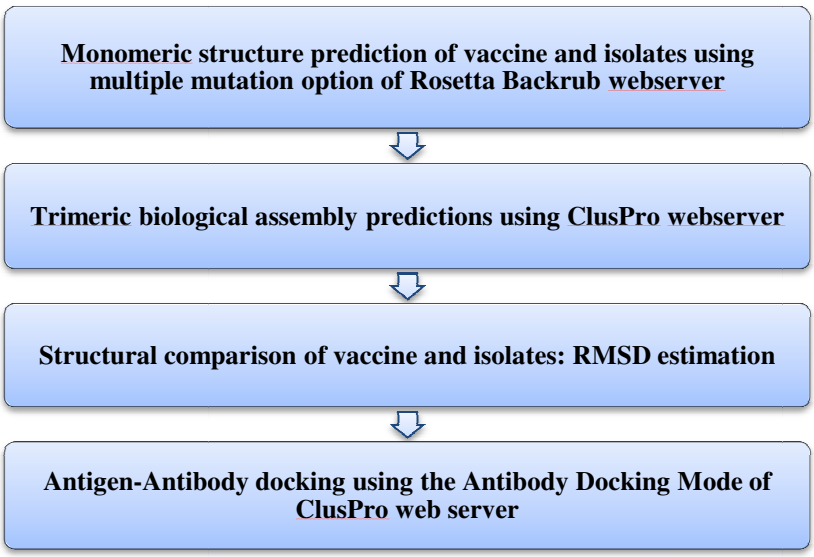

Figure 3.2: Work plan for *in silico* analysis

3.11.1 Protein modeling and comparative analysis

The three dimensional protein structure of VP7 monomer was predicted using multiple point mutation feature of RosettaBackrub server [339]. Suitable template was found by carrying out the similarity search in Protein Data Bank using BLASTP. For template, the crystal structure of VP7 protein was downloaded from Protein Data Bank by entering searching for '3FMG'. The RosettaBackrub server yielded 10 structures with different scores. The structure with least score was carried forward for oligomerization. The monomeric structures were oligomerized into trimeric form using ClusPro web server [340]. The trimeric structures were validated using RAMPAGE server and the Ramachandran plot was generated of the proteins using SAVES server. The structures of the isolates and vaccines strains were compared by superimposing them in UCSF Chimera-Molecular Modeling System which gave the deviations by calculating RMSD values [341].

3.11.2 Antigen-antibody docking analysis

The trimeric form of the VP7 protein of the isolates and vaccines were docked with the crystal structure of the anti-VP7 antibody available in association with the VP7 protein (3FMG) in the Protein Data Bank. The docking study was performed in the antibody mode of ClusPro server [342].

Chapter 4

Results

RESULTS

4.1 Sample collection

The stool samples of the diarrheic patients were collected from four hospitals across Himachal Pradesh. A total of 607 samples were collected during the study. Out of the total of 607 samples, 247 were collected from the children (≤5 years), 50 were from older children (6-17 years) and 310 were from adult (≥18 years) patients. 299 samples were obtained from the male patients and 308 samples were from female patients. The demographic details of the patients included in the study are given in the Table 4.1.

Table 4.1: Demographic details of the patients included in the study

Medical centre	Total number of samples collected	Gender		Age Group	
		Male	Female	<5 years	>5 years
Regional Hospital, Solan	502	244	258	177	325
IGMC, Shimla	82	44	38	67	15
Regional Hospital, Nalagarh	12	7	5	1	11
Regional Hospital, Bilaspur	11	4	7	2	9
Total	**607**	**299**	**308**	**247**	**360**

4.2 Incidences of diarrheagenic viruses in the sample population

In the first phase of study, a total of 287 stool samples were screened for group A rotavirus, norovirus (GI & GII) and enterovirus. In this phase, rotavirus emerged as the predominant virus isolated in 79 samples whereas norovirus and enterovirus were detected in only a small fraction of the samples. Therefore, the further study (including screening and molecular characterization) was performed on rotaviruses only.

In the first phase, 99 (34.5%) out of 287 patients were positive for the various viral pathogens (Table 4.2). Out of these, 97 (33.8%) patients were positive for a single virus whereas a mixed infection was found in only 2 (0.6%) cases. Among single infections, rotavirus was the most prevalent viruses with 27.5% (79/287) positivity among all cases.

Enterovirus was the second most predominant viruses (4.9%, 14/287) followed by norovirus GII (1.0%). Norovirus GI was the least detected virus with presence in only one (0.3%) sample. Two patients showing mixed infection had a coinfection of rotavirus and enterovirus in their stool samples.

Table 4.2: Frequency of diarrheagenic viruses in the sample population

Age Group	Rotavirus	Enterovirus	Norovirus GI	Norovirus GII	Coinfection (RV+EV)
<5 years (N=111)	55 (49.5%)	6 (5.4%)	1 (0.9%)	3 (2.7%)	–
>5 years (N=176)	24 (13.6%)	8 (4.5%)	0 (0.0%)	0 (0.0%)	2 (1.1%)
Total (N=287)	79 (27.5%)	14 (4.9%)	1 (0.3%)	3 (1.0%)	2 (0.7%)

4.3 Rotavirus prevalence: ELISA vs RT-PCR

A total of 607 samples (including 287 from the first phase) were investigated for the presence of rotavirus. 25.2% (153/607) of the samples were positive for rotavirus in ELISA whereas the PCR-based method revealed positivity in 28.3% (172/607) of the samples (P>0.05) (Table 4.3). Considering PCR as the reference, the sensitivity of ELISA came out to be 84.5% and the specificity was found to be 98.6%. The positive predictive value and negative predictive value of 96.1% and 94.1% were obtained for the ELISA test (Table 4.4).

Table 4.3: Comparison of ELISA and RT-PCR for detection of rotavirus

	Frequency		***P* value**
	ELISA	**RT-PCR**	
Positive	153 (25.21%)	172 (28.34%)	>0.05
Negative	454 (74.79%)	435 (71.66%)	
Total	607 (100%)	607 (100%)	

Table 4.4: Characteristics of ELISA using RT-PCR as reference

Statistic	Value	95% CI
Sensitivity	84.48%	78.23% to 89.52%
Specificity	98.61%	97.01% to 99.49%
Disease Prevalence	28.67%	25.10% to 32.44%
Positive Predictive Value	96.08%	91.66% to 98.55%
Negative Predictive Value	94.05%	91.46% to 96.04%

4.4 Distribution of diarrheagenic viruses in different age groups

(a) First Phase

Rotavirus was the most commonly identified virus in all age groups (Figure 4.1). A major fraction of rotavirus infections were observed in children up to 2 years of age. In this group, 50.0% (49/98) of the diarrheic patients were positive for rotavirus. The children belonging to the age group of 3-5 years showed 46.2% (6/13) positivity for rotavirus. Among the age groups of 6-17 years, 20.0% samples were positive for rotavirus. Rotavirus infections were lowest in adults and elderly belonging to the age groups of 18-65 and >65 years (15.4% and 5.0%, respectively). The distribution of enterovirus in different age groups was 5.1% in <2 years, 7.7% in 3-5 years, 15.0% in 6-17 years and 5.1% in 18-65 years of age. The elderly patients >65 years of age revealed no enterovirus infection. It is to be noted that among 6-17 and 18-65 age groups each had one case of rotavirus and enterovirus mixed infection. As far as norovirus infection is concerned, all four cases were reported in children under the age of 2 years.

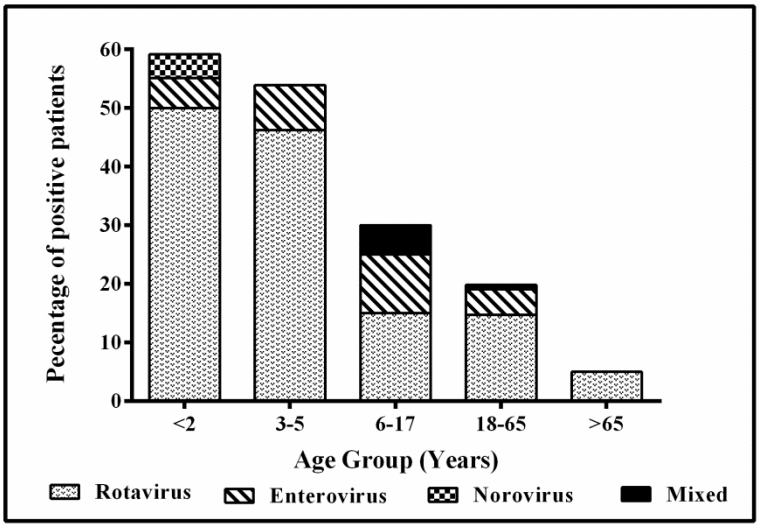

Figure 4.1: Distribution of diarrheagenic viruses in the patients of different age groups

(b) Distribution of rotavirus in different age groups

The distribution of rotavirus in different age groups followed similar pattern as the first phase study. In the children up to 2 years of age, 51.2% (106/207) of the diarrheic patients were positive for rotavirus. The rotavirus positivity in the age group of 3-5 years showed 37.5% (15/40) positivity for rotavirus. In the samples from older children (6-17 years), 16.0% (8/50) were having rotavirus in them. Adults (18-65 years) and elderly (>65 years) experienced the lowest prevalence of rotavirus with 14.0% (39/279) and 12.9% (4/31) positives, respectively (Figure 4.2).

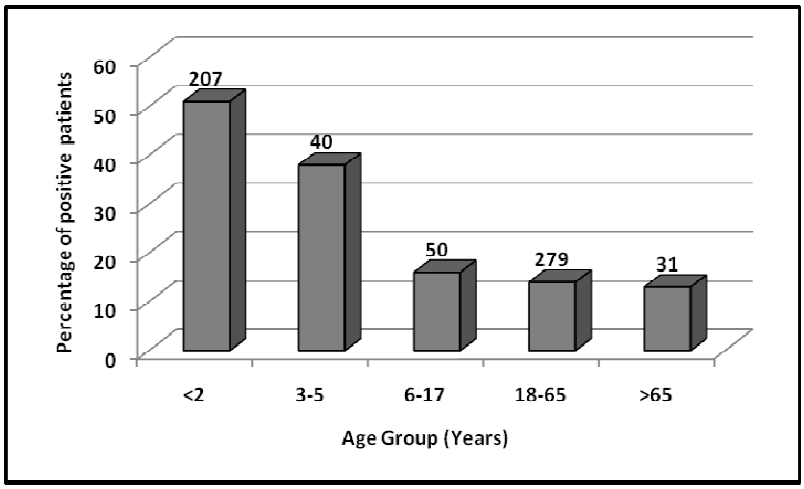

Figure 4.2: Distribution of rotavirus in patients of different age groups. The data labels represent number of samples screened.

4.5 Association of clinical features with rotavirus infection

As rotavirus was the predominant virus detected in both children and adult patients, and it is extensively reported that rotavirus infections are commonly and significantly associated with additional clinical symptoms along with diarrhea [15, 28, 30], clinical features of the rotavirus positive and rotavirus negative patients were compared to ascertain the association of rotavirus infection with the clinical features of vomiting, fever (body temperature above 100.4 F) and dehydration (Table 4.5). In children <5 years of age, 35.5% of rotavirus positive patients suffered from vomiting in contrast to 23.0% of the non-rotavirus positive patients. These data showed a statistically significant association of vomiting with rotavirus infection (P=0.0304). The statistically significant association of dehydration with rotavirus infection was also observed during the study (P=0.0240).

Similarly, the rate of fever in the rotavirus positive cases were higher compared to rotavirus negative cases, but these differences showed no statistical significance. On the contrary, in the age group >5 years, the rates of vomiting and fever in rotavirus infected individuals were lower in comparison with the patients having non-rotavirus diarrhea. In this age group, the rate of dehydration in rotavirus positive and rotavirus negative patients was almost similar (these results were not statistically significant).

Table 4.5: Comparison of clinical characteristics of Rotavirus positive and Rotavirus negative patients

Age Group	Clinical Characteristics	Rotavirus Positive (%)	Rotavirus Negative (%)	P value
		N = 121	**N = 126**	
< 5 years	Vomiting	43 (35.5)	29 (23.0)	**0.030**
	Fever	31 (25.6)	26 (20.6)	0.353
	Dehydration	37 (30.6)	23 (18.3)	**0.024**
		N = 51	**N = 309**	
>5 years	Vomiting	7 (13.7)	63 (20.4)	0.265
	Fever	2 (3.9)	32 (10.4)	0.146
	Dehydration	10 (19.6)	59 (19.1)	0.931

4.6 Seasonal distribution of rotavirus infection

The infection rate of the gastroenteritis viruses in different seasons was studied. Among all the viruses under investigation, rotavirus infections were observed throughout the year with no particular seasonal peaks (Figure 4.3 (A) and (B)). The highest prevalence of rotavirus was seen in the cold months of winter (november-february) where the overall (both children and adults) rotavirus positivity was observed in 38.5% samples. The season with second highest prevalence was summer (may-june) which experienced 29.4% rotavirus infectivity. The samples from autumn months (july-oct) revealed least rotavirus positivity. The rotavirus infection in children was around 50% (± 5%) in all the seasons except autumn where only 39% of children were having rotavirus in their stool. Enterovirus and norovirus infections were found prominently in the winter and autumn seasons.

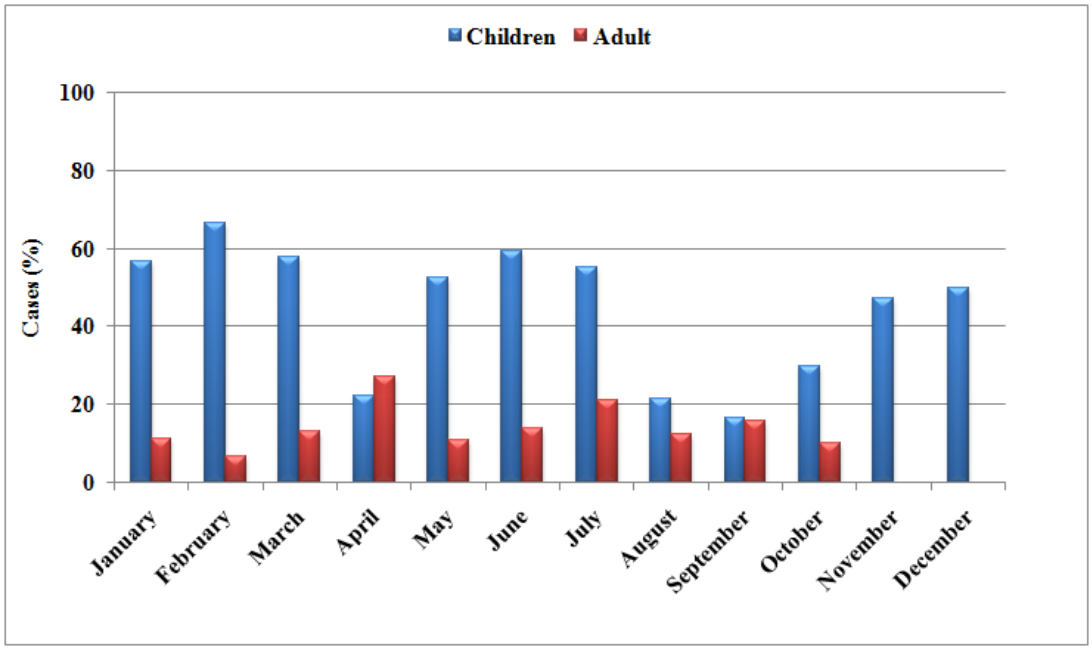

Figure 4.3 (A): Monthly distribution of rotavirus infections

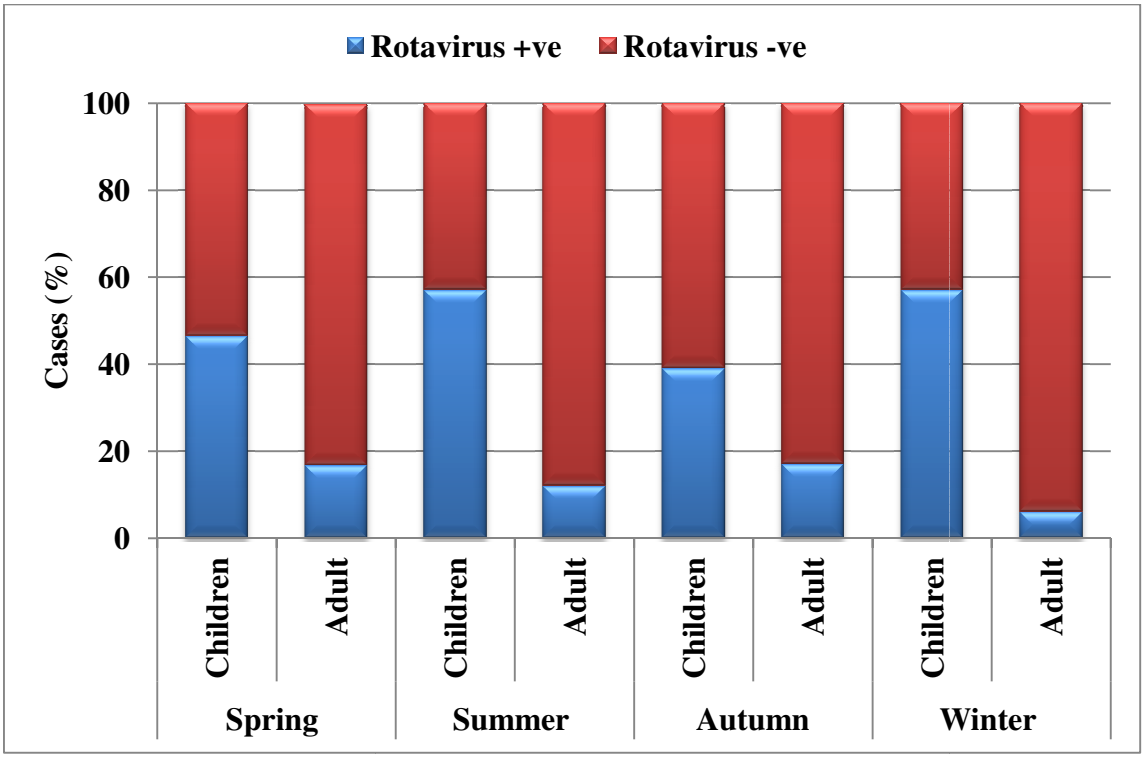

Figure 4.3 (B): Seasonal distribution of rotavirus infections

4.7 Rotavirus genotypes

Out of the positive samples, 156 were available in sufficient quantity to carry out genotyping. Out of these samples, G genotypes were obtained in 128 (82.1%) samples whereas P-types were identified in 129 (82.7%) stool specimens. Seven (4.5%) and eight (5.1%) samples could be genotyped either for G and P-type, respectively. Both G and P-types were identified for 121 (77.6%) samples. Twenty samples (12.8%) were untypable for both types. The representative gel images of the G and P genotypes identified in the study are shown in Figure 4.4 (A) and (B).

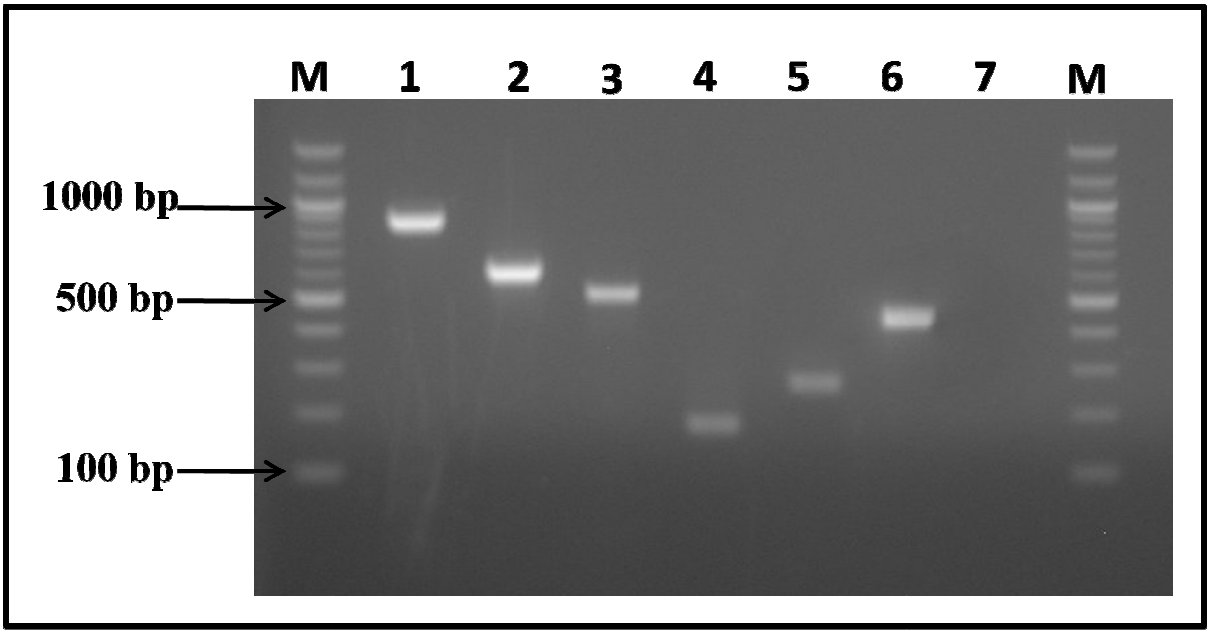

Figure 4.4 (A). Representative gel image of the G-types identified in the study. Lanes M represents 100 bp DNA ladder. Lane 1 is the VP7 first round product. Lane 2, 3, 4, 5 and 6 represents G1, G2, G9, G10 and G12. Lane 7 is the negative control.

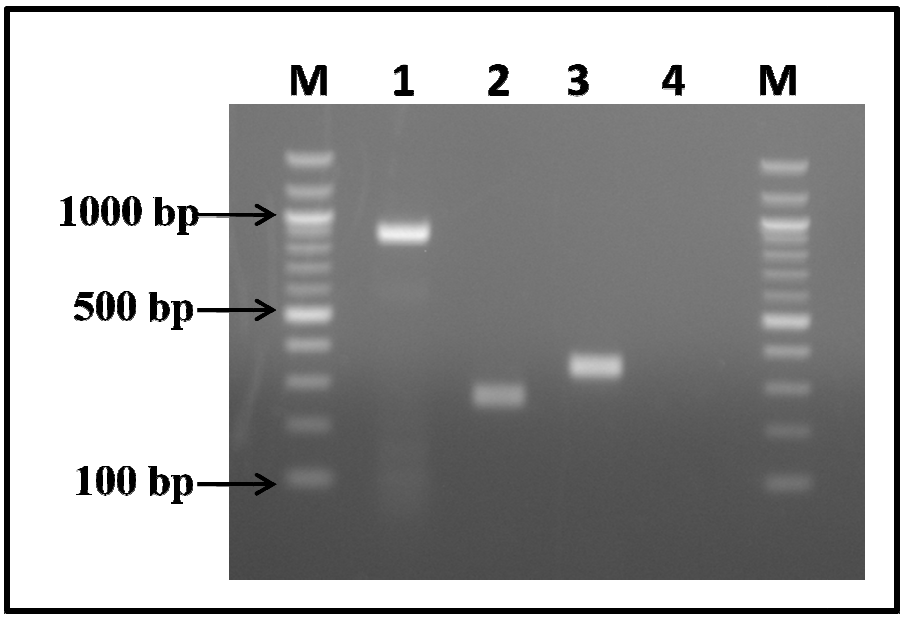

Figure 4.4 (B). Representative gel image of the P-types identified in the study. Lanes M represents 100 bp DNA ladder. Lane 1 is the VP4 first round product. Lane 2 and 3 represents P[6] and P[8]. Lane 4 is the negative control.

Among G-types, G1 emerged as the predominant VP7 type with 66.0% (103/156) positivity (Figure 4.5A). G9 (11/156; 7.1%) was the second most isolated genotype followed by G12 (6/156; 3.9%), G2 (1/156; 0.6%) and G10 (1/156; 0.6%). Mixed infections of G1/G9 and G9/G10 was observed in 3.2% (5/156) and 0.6% (1/156) samples, respectively. Among P-types identified, P[6] (104/156; 66.7%) was the most commonly identified type followed by P[8] (21/156; 13.5%) (Figure 4.5B). Coinfection of P[6] and P[8] was seen in 2.6% (4/156) of the samples. As far as G and P combinations are concerned, interestingly, G1P[6] (76/121; 62.8%) was the most abundantly circulating rotavirus strain (Figure 4.5C). The globally and nationally common G1P[8] (20/121; 16.5%) was the second most frequently detected genotype. Among other strains, G9P[6] (9/121;7.4%), G12P[6] (6/121; 5.0%) and G2P[6] (1/121; 0.8%) were detected at significant frequency. Mixed genotypes accounted for 7.4% (9/121) of all G-P combinations (Table 4.6).

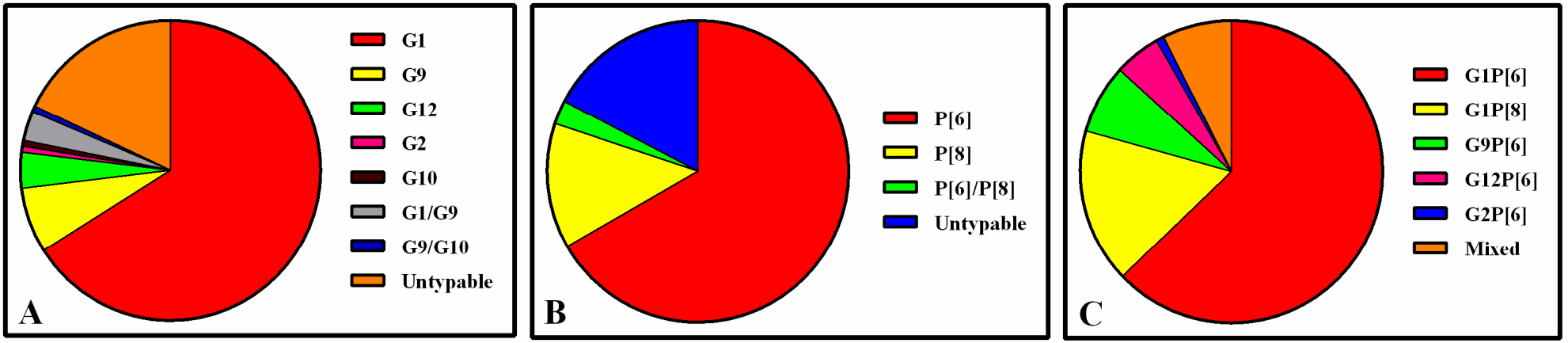

Figure 4.5: Distribution of rotavirus genotypes (A) G-types (B) P-types (C) G-P types

Table 4.6: Characterization of rotavirus strains detected in the study

Genotype	P[6]	P[8]	P[6] & P[8]	P untyped	Total
G1	76	20	4	3	103
G2	1	0	0	0	1
G9	9	0	0	2	11
G10	0	0	0	1	1
G12	6	0	0	0	6
G1 & G9	4	1	0	0	5
G9 & G10	0	0	0	1	1
G untyped	8	0	0	20	28
Total	104	21	4	27	156

4.7.1 Distribution of genotypes in different age groups

The distribution of rotavirus genotypes within children (≤5 years), older children (6-17 years) and adult (≥18 years) sample population was also studied (Figure 4.6). The frequency pattern of circulating genotypes in different age groups was similar to the overall trend. G1P[6] remained the leading genotype in all age groups. This particular genotype was identified in 56.7% (51/90) and 73.9% (17/23) of all genotyped samples in children and adults, respectively. In the 6-17 years group, all the samples (8/8; 100.0%) showed the presence of G1P[6] rotavirus strains. G1P[8] and G9P[6] were observed in both children and adult samples in notable fractions whereas G12P[6] and G2P[6] were detected only in the children samples. Mixed infections were evenly distributed in children and adult patients where each group had 8%-9% mixed infection cases. Although, the differences of genotypes with age groups showed no statistical significance (P>0.05).

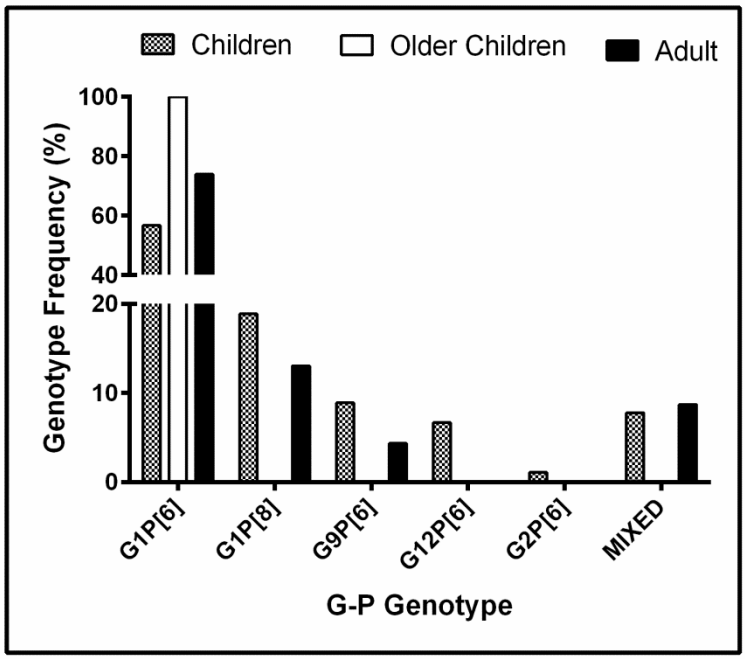

Figure 4.6: Distribution of genotypes in different age groups

4.8 Phylogenetic and sequence analysis of the VP7 sequence of G1 rotavirus

Phylogenetic analysis of VP7 gene sequences showed that the G1 strains isolated from Himachal Pradesh clustered into G1-Lineage 1 (Figure 4.7). But within G1-Lineage 1, these strains do not cluster with any of the reported five (A-E) sublineages and lack signature sequences of any of these. These results suggest that the isolated strains may belong to a novel sublineage other than the previously reported sublineages. Moreover, none of the isolates clustered with Rotarix (A41CB052A) and RotaTeq (W-179-9) vaccine strains which belong to the G1-Lineage 2 and G1-lineage 3, respectively.

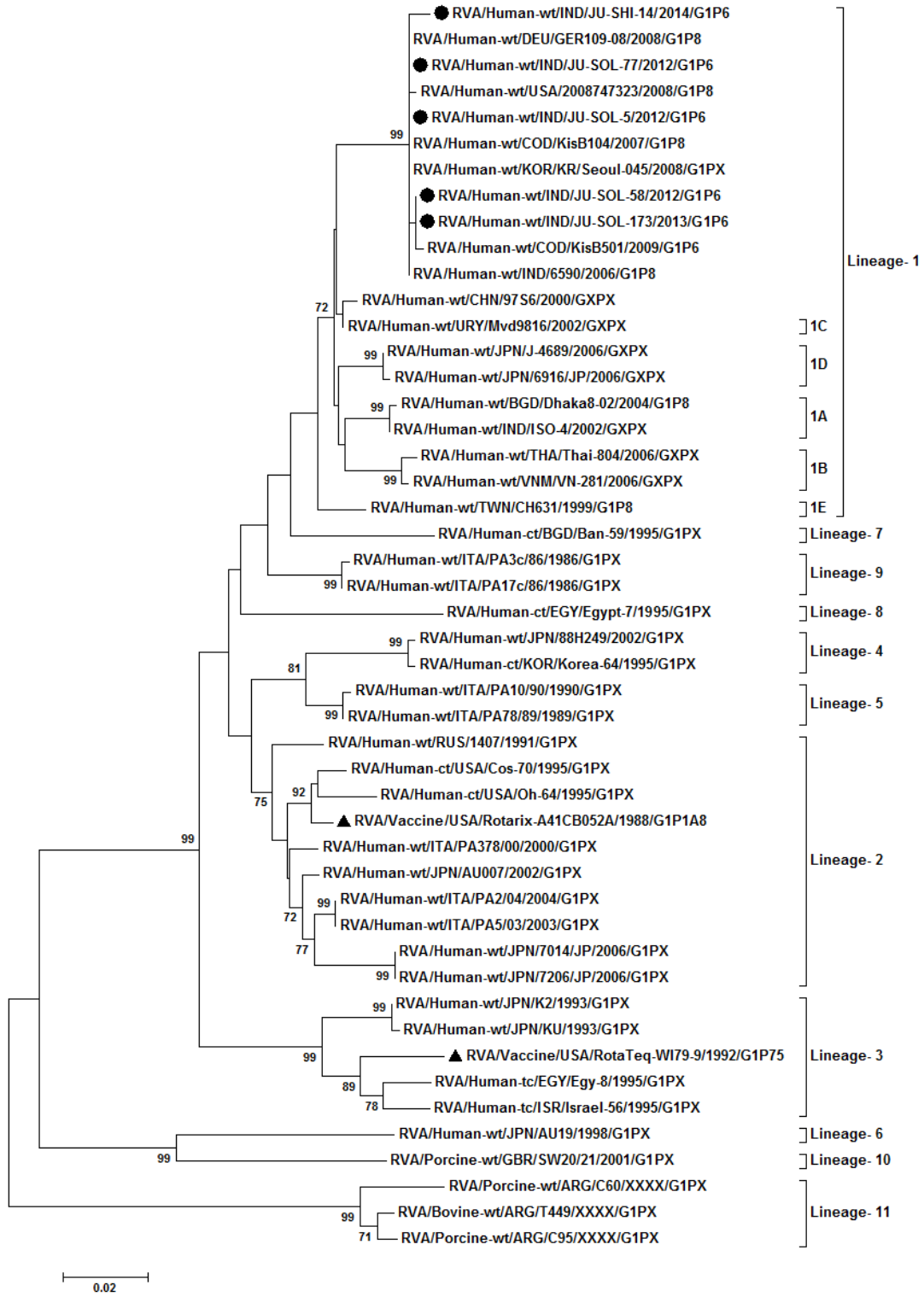

Figure 4.7: Phylogenetic tree of VP7 gene sequences of rotavirus G1 strains. The phylogenetic tree was constructed using neighbour-joining method. The values at the branch nodes represent the bootstrap values. The Himachal isolates are indicated with black dots whereas vaccine sequences are indicated with black triangles. The accession numbers of the sequences used in the tree are mentioned in the text.

To assess the molecular basis of rotavirus diversity, the nucleotide and amino acid sequences of VP7 protein were compared. The isolates JU-SHI-14, JU-SOL-5, JU-SOL-58, JU-SOL-77, JU-SOL-173 were 99% identical to each other at nucleotide level. All the five isolates showed 96.1% to 97.6% and 96.4% to 98.8% similarity with the other lineage 1 strains on the nucleotide and amino acid levels, respectively. Within lineage 1, all the five isolates revealed the maximum identity (97.4%-97.8%) with the Uruguay strain, Mvd9816 at the nucleotide level. However, on the amino acid level, the isolated strains shared maximum similarity with three strains, ISO-4 (98.2%-98.8%), VN-281 (98.2%-98.8%) and Mvd9816 (98.2%-98.8%), from India, Vietnam and Uruguay, respectively (Table 4.7).

As reported by Phan et al. [343], there is a unique identification code for defining the lineages. It comprises of fourteen amino acids at positions 29, 34, 35, 37, 42, 43, 50, 55, 57, 65, 68, 72, 74 and 75 that define a signature sequence. The alignment of translated amino acid sequences of our strains revealed the signature code specific for lineage 1. Within lineage 1, the amino acid substitutions at positions 72 and 74 defines sublineage 1A, 16 defines 1B, 268 defines 1C and 91 defines 1D. Sublineage 1E has no sublineage specific mutations [343]. Interestingly, none of the isolates absolutely have the mutations specific for any of the sublineages within lineage 1 (Table 4.8). All the isolated strains showed a substitution of amino acid G with E at position 74 which is a characteristic feature for sublineage 1A. But there is no sublineage 1A specific amino acid substitution at position 72. This differentiates the isolates from the sublineage 1A specific strains. The strain JU-SHI-14 and JU-SOL-173 bears an additional substitution at position 91 and 268, respectively where amino acid T is substituted by A and I is replaced by V. These mutations support the fact that the Himachal isolates are diverse from the strains belonging to the five sublineages within lineage 1 and belong to a distinct sublineage.

Table 4.7: Nucleotide and Amino acid similarity between Himachal isolates and lineage 1 strains

	JU-SHI-14		JU-SOL-5		JU-SOL-58		JU-SOL-77		JU-SOL-173	
	N* (%)	AA# (%)	N (%)	AA (%)	N (%)	AA (%)	N (%)	AA (%)	N (%)	AA (%)
RVA/Human-wt/BGD/Dhaka8-02/2004/G1P8	96.1	97.6	96.5	98.2	96.3	97.6	96.5	98.2	96.3	97.6
RVA/Human-wt/IND/ISO-4/2002/G1PX	96.3	98.2	96.7	98.8	96.5	98.2	96.7	98.8	96.5	98.2
RVA/Human-wt/THA/Thai-804/2006/G1PX	95.9	97.6	96.3	98.2	96.1	97.6	96.3	98.2	96.1	97.6
RVA/Human-wt/VNM/VN-281/2006/G1PX	96.1	98.2	96.5	98.8	96.3	98.2	96.5	98.8	96.3	98.2
RVA/Human-wt/CHN/97S6/2000/G1PX	96.9	97.6	97.4	98.2	97.2	97.6	97.4	98.2	97.2	97.6
RVA/Human-wt/URY/Mvd9816/2002/G1PX	97.4	98.2	97.8	98.8	97.6	98.2	97.8	98.8	97.6	98.2
RVA/Human-wt/JPN/J-4689/2006/G1PX	96.3	98.2	96.7	98.2	96.5	97.6	96.7	98.2	96.5	97.6
RVA/Human-wt/JPN/6916/JP/2006/G1PX	96.1	98.2	96.5	98.2	96.3	97.6	96.5	98.2	96.3	97.6
RVA/Human-wt/TWN/CH631/1999/G1P8	95.9	96.4	96.3	97.0	96.1	96.4	96.3	97.0	96.1	96.4

*Nucleotide; #Amino acid

Table 4.8: Lineage and sublineage specific amino acid substitutions in VP7 protein

Sublineages under Lineage 1	Strain	Amino Acid positions and substitutions																			
		16	29	34	35	37	42	43	50	55	57	65	68	72	74	75	91	211	212	213	268
		I	I	I	Y	F	V	A	A	L	L	T	A	Q	G	I	T	N	V	D	I
1A	Dhaka 8-02	•	•	•	•	•	•	•	•	•	I	•	S	*R*	*E*	V	•	•	•	•	•
1B	Thai-804	*Q*	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	•
1C	Mvd9816	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	*V*
1D	6916	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	*N*	•	•	•	•
1E	CH631	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	•
	JU-SHI-14	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	*A*	•	•	•	-
	JU-SOL-5	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	-
	JU-SOL-58	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	-	-	-	-
	JU-SOL-77	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	-
	JU-SOL-173	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	*V*

Amino acids in *italics* represent sublineage specific mutations

4.9 Comparison of VP7 sequences of isolated G1 strains with the strains from other parts of India

The strains isolated in this study were compared with the other strains from different parts of India (Kolkata, Manipur, Pune and Haryana) on the basis of amino acid sequence of VP7 gene. Most of the amino acid variations lie in the antigenic regions (Preferably, A and C) (Figure 4.8). In the region A, a Himachal isolate, JU-SHI-14 showed the amino acid transition of T91A. Whereas, all the isolates from this study showed the substitution of amino acid E instead of D at position 100. Region C had three positions where Himachal isolates differ from the strains from other parts of the country. Strain JU-SOL-5 showed maximum amino acid differences at three positions (T210K, T217I and A219D). Two other strains, JU-SOL-77 and JU-SOL-173 were also having T217I transition. The isolates did not have any amino acid variations in the antigenic region B. Outside the antigenic regions, Himachal strains showed variations at two positions, 41 and 179 where F is replaced by S and G is replaced by E, respectively.

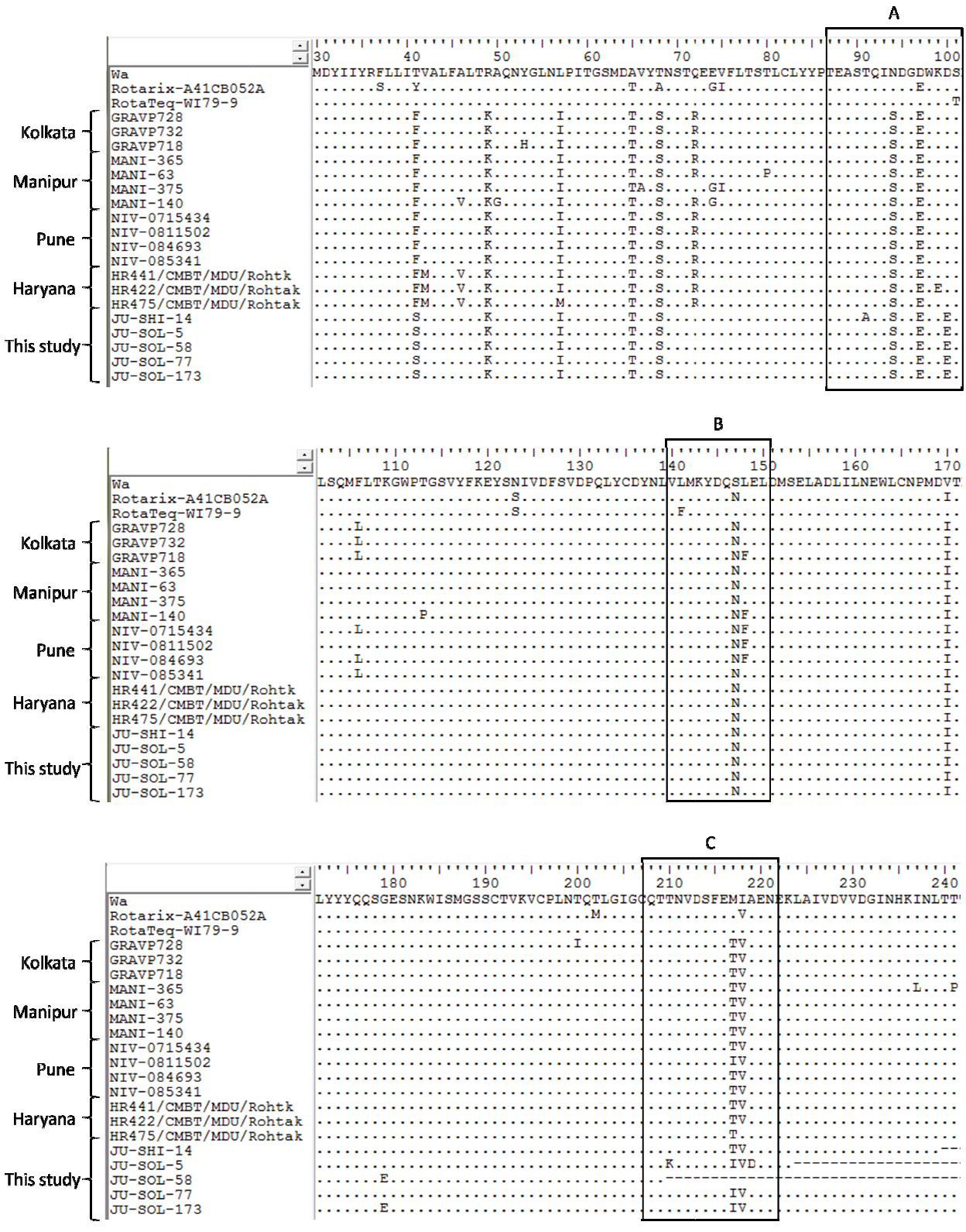

Figure 4.8: Alignment of amino acid sequences of VP7 protein of G1-Himachal isolates and strains from other parts of the country. Dots represent the conserved amino acid residues. The antigenic regions are indicated by black boxes.

4.10 Comparison of VP7 sequences of isolated G1 strains and vaccine strains

The comparison of isolated strains with Rotarix vaccine strain showed 92.8% - 93.3% and 93.9% - 94.5% similarity at nucleotide and amino acid level, respectively. Similarly, the isolated strains revealed 87% - 87.5% nucleotide and 91.9% - 92.6% amino acid similarity with the RotaTeq strain. These results demonstrate that the isolated strains are closer to the Rotarix strain on the nucleotide and amino acid levels than RotaTeq vaccine strains.

4.11 Comparison of VP7 epitopes of isolated G1 strains and vaccine strains

The amino acid differences in the antigenic epitope region between circulating strains and vaccine strains could have a substantial effect on the vaccine efficacy [344]. Therefore, the antigenic regions of the isolated G1 strains and vaccine strains were compared to ascertain the variability between these. The isolated strains showed five amino acid differences in the epitope region as compared with Rotarix strain, whereas RotaTeq showed a difference of seven amino acids (Table 4.9). A majority of the amino acid changes were found in epitope region 7-1a and it had amino acid changes at five positions. In this region, there was a transition between T→A, N→S, D (RotaTeq) →E, D→E and S→N at positions 91, 94, 97, 100 and 123, respectively. The region 7-2 has S (RotaTeq) →N and M→T/I amino acid change at positions 147 and 217, respectively. The significance of these mutations lies in the fact that they are the neutralization escape mutation sites except amino acid at position 123 [34]. As far as region 7-1b is concerned, it was found to be fully conserved between circulating strains and vaccine strains. Mapping of the amino acid variations in the antigenic regions of the isolates and vaccines on the trimeric structure of VP7 protein reveals that the differences were located inconsistently on the exposed surfaces of the protein in the epitope region (Figure 4.9).

Table 4.9: Alignment of antigenic amino acids in VP7 protein of Himachal isolates and vaccine strains

Rotavirus Strain	Epitope 7-1a														Epitope 7-1b						Epitope 7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
Rotarix-G1	**T**	**T**	**N**	**G**	**E**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**N**	**L**	**S**	**M**	**N**	**G**
RotaTeq-G1	**T**	**T**	**N**	**G**	**D**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**S**	**L**	**S**	**M**	**N**	**G**
JU-SHI-14	T	A	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	-	K	D	Q	N	L	S	T	N	-
JU-SOL-5	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	-	-	K	D	Q	N	L	S	I	N	-
JU-SOL-58	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	-	-	-	-	-	K	D	Q	N	L	S	-	-	-
JU-SOL-77	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	-
JU-SOL-173	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	-

Yellow color represents amino acid differences between Rotarix and RotaTeq. Magenta represents residues that differ from both Rotarix and RotaTeq. Cyan represents amino acid residues that differ from RotaTeq.

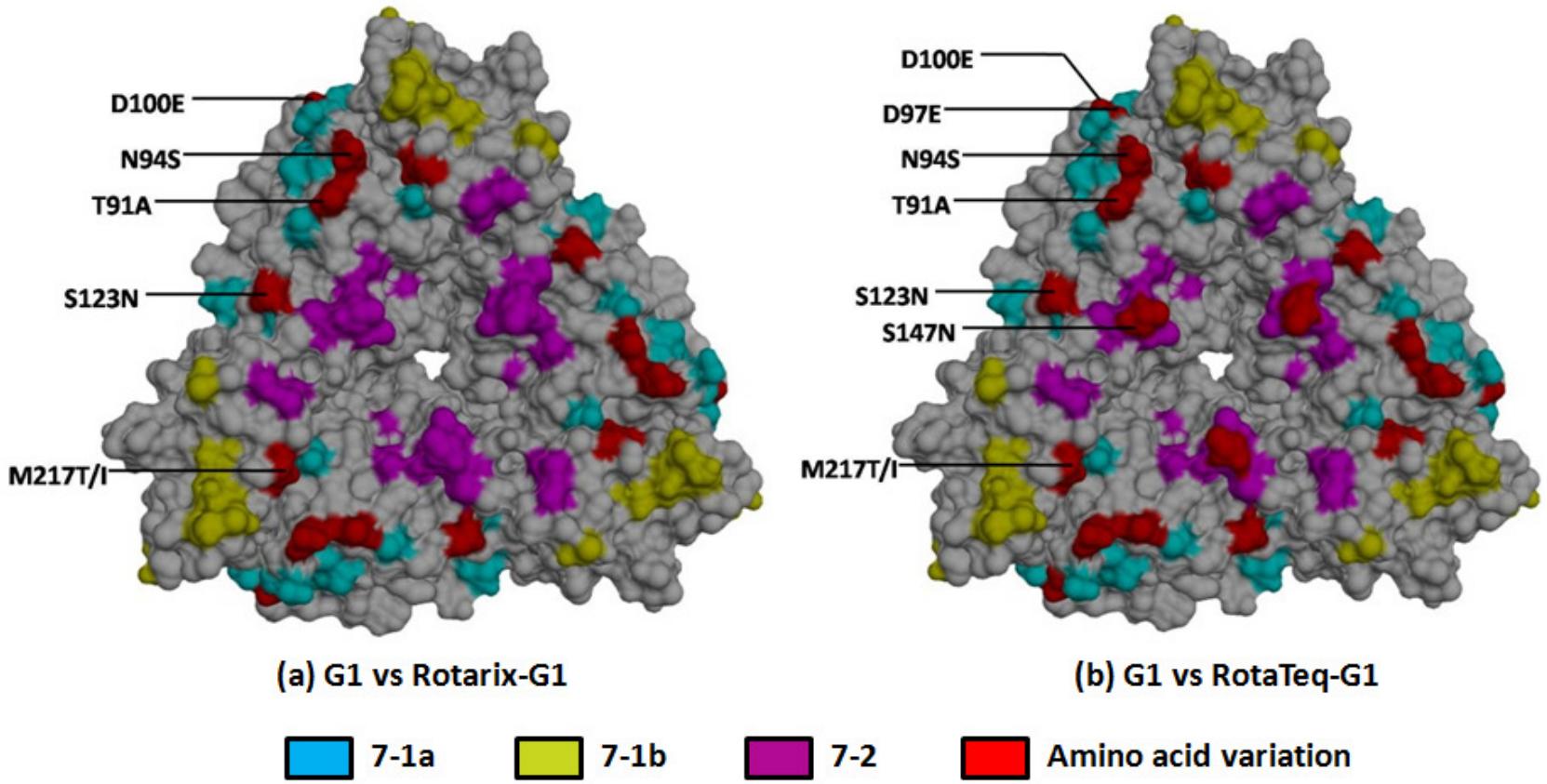

Figure 4.9: Structural representation of VP7 trimer proteins. Different regions of antigenic epitopes are shown in cyan (7-1a), yellow (7-1b) and magenta (7-2) colours. The amino acid differences between Himachal strains and vaccine strains are indicated with red colour.

4.12 Phylogenetic and sequence analysis of the VP4 sequence of P[6] rotavirus

The P[6] strains isolated from Himachal Pradesh during this study were 98%-100% and 99%-100% identical with each other on the nucleotide and amino acid level, respectively. Phylogenetic analysis of P[6] strains revealed that all the strains belong to sublineage Ia within lineage I (Figure 4.10).

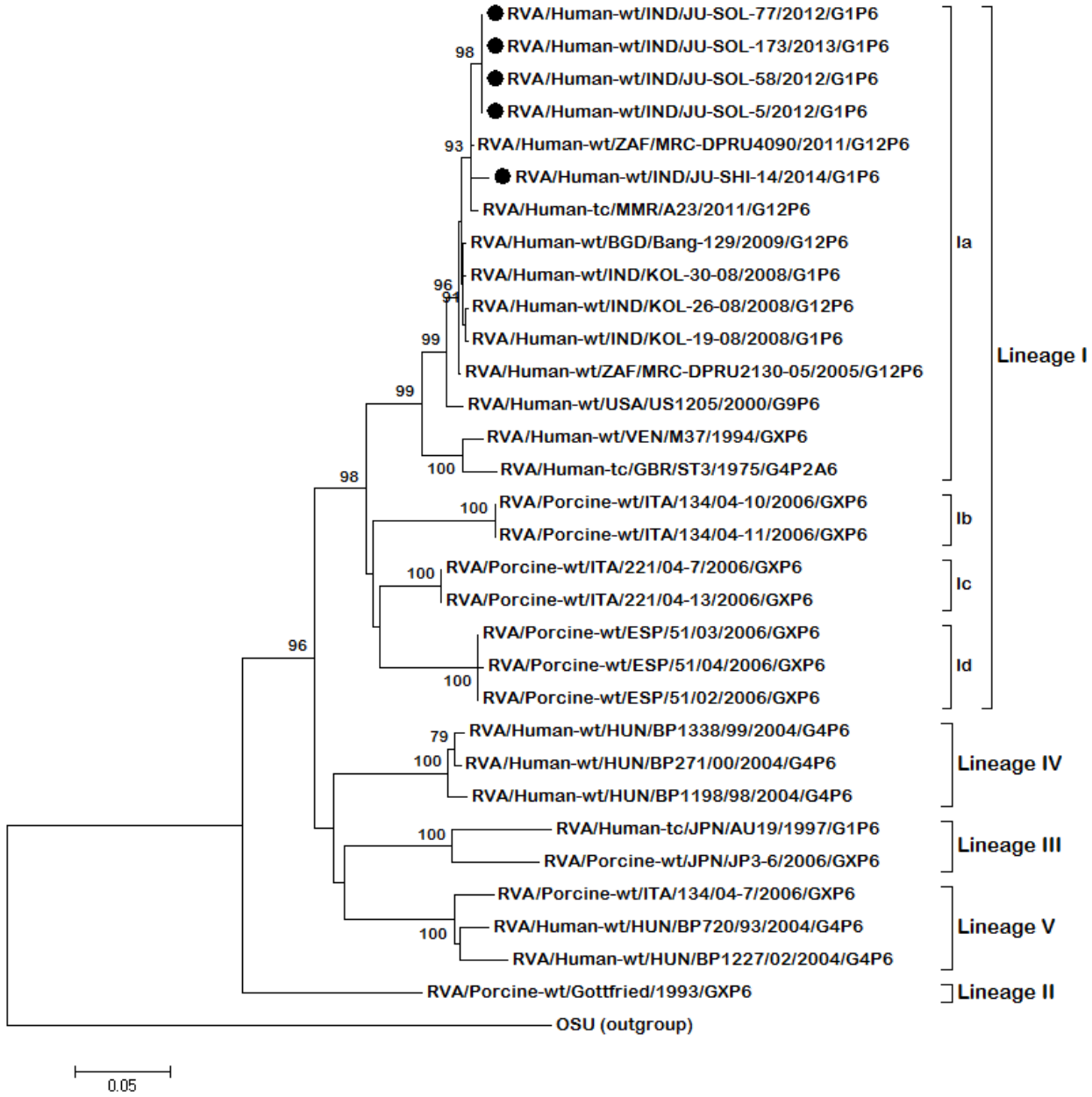

Figure 4.10: Phylogenetic tree on the basis of partial VP4 nucleotide sequence. The strains from the present study are indicated with black dots. The tree was rooted using OSU strain as an outgroup. The values at branch node are the bootstrap values and are expressed as percentages.

The Himachal strains showed 93% to 98% and 94% to 98% identity on nucleotide and amino acid level, respectively with the reference strains M37, US1205 and ST3. The Himachal strains showed 98% to 99% nucleotide and 99% amino acid similarity with Indian strains from Kolkata (KOL-30-08, KOL-26-08, KOL-19-08). Interestingly, Himachal strains also clustered with foreign strains MRC-DPRU5090 and MRC-DPRU2130-05 (South Africa), A23 (Myanmar) and Bang-129 (Bangladesh). All the strains showed very high (99% nucleotide and 99%-100% amino acid) identity with these foreign strains.

4.13 Protein modeling and comparative analysis

The 3D structures of vaccine and rotavirus strains were predicted using Rosetta Backrub Server. The structure of 3FMG was made to undergo mutations at the specific position of Rotarix-G1, RotaTeq-G1, Rotavac-G9, JU-SHI-14, JU-SOL-5 and JU-SOL-58. The changes in JU-SOL-77 and JU-SOL-173 were similar to JU-SOL-5 so its results could be implemented on JU-SOL-77 and JU-SOL-173, respectively. The mutations were carried out using multiple point mutation feature of Rosetta Backrub Server. It predicted 10 structures with different weighted score. The structure with minimum balanced score was carried forward for trimerization as it had maximum stability. The minimum scores for the isolates and vaccines are depicted in the Table 4.10.

Table 4.10: Minimum balanced score obtained for each model

Structure	Balanced Score
3FMG	1561.680
Rotarix-G1	1220.780
RotaTeq-G1	1215.980
Rotavac-G9	1197.120
JU-SHI-14	1193.412
JU-SOL-5	1213.045
JU-SOL-58	1211.697

For trimerization, the pdb files of monomer units were subjected to cluspro server which yielded 15 structures with different weighted scores. The structure with the least weighted score was selected for each protein and carried forward for comparative and docking

analysis. All the trimers showed good stereochemical qualities and have 98.3%-99.0% residues in the allowed and favoured regions of the Ramachandran plot (Table 4.11; Figure 4.11).

Table 4.11: Analysis of Ramachandran plots

Model	Residues in favoured region (%)	Residues in allowed region (%)	Residues in outlier region (%)
Rotarix	619 (88.6)	68 (9.7)	12 (1.7)
RotaTeq	617 (88.3)	72 (10.3)	10 (1.4)
Rotavac	636 (91.0)	55 (7.9)	8 (1.1)
JU-SHI-14	626 (89.6)	66 (9.4)	7 (1.0)
JU-SOL-5	625 (89.4)	65 (9.3)	9 (1.3)
JU-SOL-58	627 (89.7)	65 (9.3)	7 (1.0)

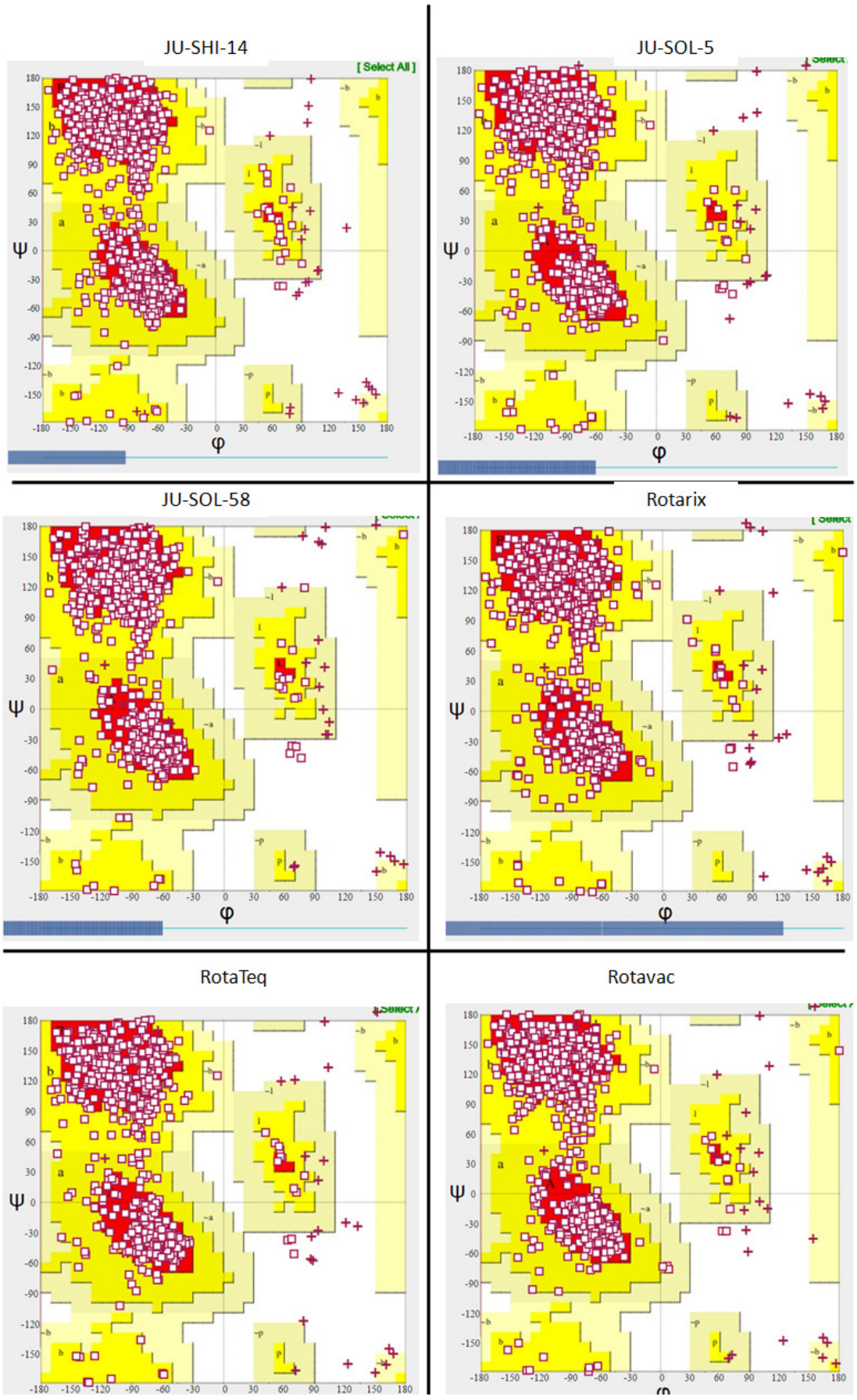

Figure 4.11: Ramachandran plots of the proteins modeled in the study.

To study the effect of amino acid variation on the structure of the proteins, the isolates were compared to the vaccine strains by superimposing them. The RMSD is the deviation of the position of atoms between the superimposed structures. The RMSD value of ≤ 2 Å signifies no significant differences between the superimposed structures. In this study, on comparing the isolates individually with Rotarix, RotaTeq and Rotavac, no significant difference in the protein structure was observed (Figure 4.12).

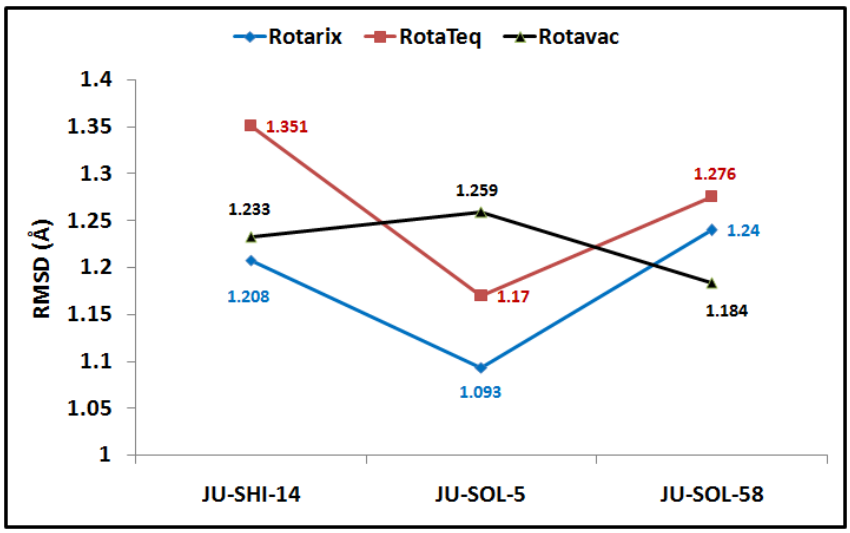

Figure 4.12: Comparison of vaccine strains with rotavirus strains using RMSD.

4.14 Antigen-antibody docking analysis

The protein structures of the isolates and vaccines were subjected to antibody docking, where the effect of amino acid variations on antibody binding was studied. The weighted scores obtained for the antigen antibody complex of the vaccines and isolates showed no considerable differences (Figure 4.13).

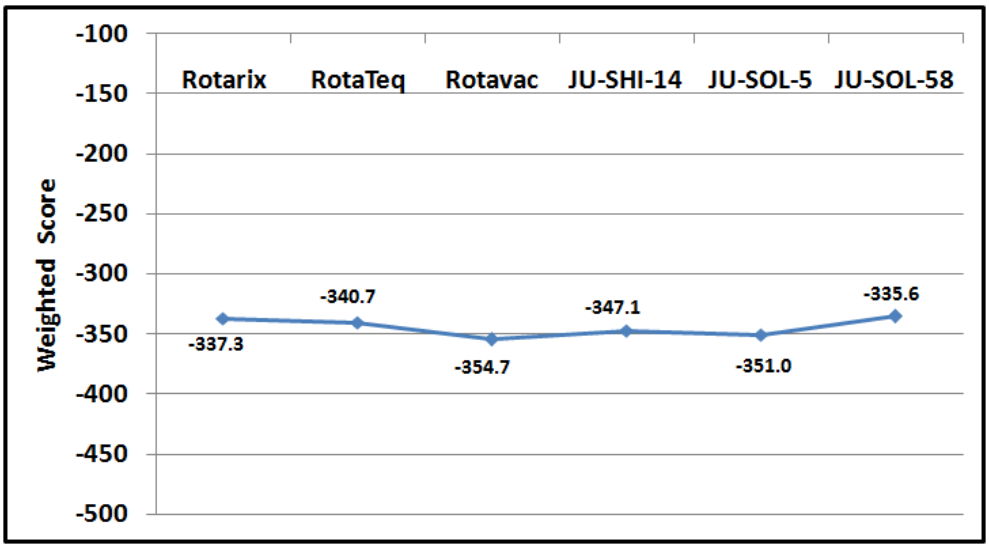

Figure 4.13: Scoring function in the form of weighted score obtained after antibody docking analysis.

Chapter 5

Discussion

DISCUSSION

In the words of Rose George, Journalist & Author of The Big Necessity, '*Diarrhea is the world's most effective weapon of mass destruction'*. Diarrheal disease poses a severe threat to mankind and is the second leading killer of children around the world. According to recent estimates by the World Health Organization, more than one million children die because of diarrhea each year [1]. The diarrhea associated mortality is highest in the under developed and developing countries of the world [1]. Infectious diarrhea is caused by a spectrum of bacterial, viral and parasitic pathogens. To reduce diarrheal mortality and morbidity, it is essential to study the burden of different diarrheagenic pathogens in a particular region of the country or the world. In the first phase of the present study, we have studied the prevalence of viral pathogens (rotavirus, norovirus and enterovirus) that cause diarrhea to ascertain the relative disease burden of these viruses in the state of Himachal Pradesh. The first phase of the study revealed the fact that among the viruses under investigation, rotaviruses emerged as the single most important pathogen responsible for diarrhea in the region, therefore a more extensive study over a longer period of time and including additional samples was performed to specifically study the rotavirus associated disease burden.

5.1 Prevalence of Rotavirus, Enterovirus and Norovirus

Rotavirus is the most common cause of childhood diarrhea. It is responsible for approximately 40% cases of diarrhea in children [8]. In India, rotavirus is the reason behind the death of around 79,000 children each year [9]. In the present study, rotavirus emerged as the single most important virus and was detected in 49% of children below the age of 5 years. According to different studies, rotavirus prevalence in the low income countries of Asia (India, Bangladesh, Pakistan, Vietnam, China) and Africa is very high, where 30%-40% of hospitalized children were positive for rotavirus infections [217, 345-350]. The rotavirus detection rate found in this study is much higher than these studies. As far as India is concerned, there are a large number of studies which have studied the epidemiological and clinical aspect of rotavirus in different parts of the country. According to recent studies, the rotavirus positivity in Indian children was found to be varying generally from 26%-40% [351-353]. In a multicentre study conducted by Kang et al. in 2013, overall rotavirus positivity in India was found to be 40% [351]. This study included 10 representative hospitals from different parts of the country. The rotavirus

positivity was highest in the southern region (44%) followed by the eastern (42%) and northern regions (40%). The lowest rate of rotavirus detection was observed in the western parts of India where 35% samples were positive for rotavirus. A recent study which included 12 medical centers across India reported the rotavirus detection rate of 26.4% during the 16 months of surveillance period [353]. Keeping in view the national and international figures of rotavirus prevalence, the high rotavirus positivity of 49% in the present study is a crucial finding from this part of country. However, there are studies from some parts of the country which reported the rotavirus prevalence of as high as 71% in Calicut [354], 53% in Kolkata [355] and 50% in Trichy [352]. Rotavirus infection in adults is not widely reported and the role of rotavirus in adult gastroenteritis is still not completely understood. But the outcome of this study suggests the significant role of rotaviruses in adult gastroenteritis where around 14% of diarrheic adults have rotaviruses in their stool samples. This detection rate is marginally higher than the studies from other parts of the country [356, 357] but is in accordance with study from Pune where this virus was detected in 17.2% of adults [356]. Although there are some reports from other parts of the world where this virus was responsible for as high as 63% incidences in adults [285]. These results signify that the rotavirus associated morbidity in adults cannot be ignored and warrants the need to extensively and compulsorily monitor rotavirus infection in adults.

The role of enterovirus as a diarrheagenic pathogen is not very well recognized. However, their diarrheagenic potential is gaining more recognition after the emergence of reports claiming it as a pathogen identified in a number of diarrhea surveillance studies [17, 20, 358-362]. In the present study, enterovirus emerged as the second most common virus identified in diarrheic patients with a detection rate of around 5% (though it was much less than rotavirus) among all age groups. In a recent study conducted by Patil et al. in the western part of India, the rate of enterovirus detection in the gastroenteritis patients (14%) were significantly higher than the non-gastroenteritis control group (5%) [18]. This shows an association of enteroviruses with acute gastroenteritis. It is interesting to note that, in our study, out of 16 enterovirus positive cases, 10 were adults. This shows that enteroviruses may cause diarrhea in adults too. Although the proportion of enterovirus isolated in the study is very less in comparison with rotavirus which was detected in as high as 49% of children and 14% of adults.

Norovirus is a well recognized viral pathogen which causes epidemic and sporadic diarrhea in both adults and children. Some studies have even reported it as the leading cause of diarrhea, especially in children where rotavirus is believed to be the foremost viral pathogen [5]. Among various norovirus genogroups, the outcome of majority of the studies shows that GII is the foremost cause of diarrhea in humans [307]. In this study also, norovirus GII was detected in 1.0 % cases in contrast to the 0.3 % prevalence of norovirus GI. However, it is very interesting to note that the prevalence of norovirus in the region is exceptionally low unlike majority of studies from India and other counties which reports norovirus as a prominent diarrheagenic pathogen in both epidemic and sporadic cases [363-365]. The outcomes of this study are indicative of the low circulation of norovirus in the region and it can be considered that norovirus is not an important etiological agent of diarrhea at least in this part of the country.

5.2 Age wise distribution of viruses

Altogether, among the three viruses, rotavirus was the only pathogen which was detected in all the age groups. The most vulnerable of all the age groups were children below the age of five years and it has been observed that children <5 years of age have an increased rotavirus infection rate in comparison with adults ($P<0.0001$). Studies have reported that the most rotavirus infections in children occurs before the age of 2 years [257, 366, 367] and in this study also the children <2 years of age demonstrated the highest rate (51 %) of rotavirus infections among all the patients belonging to different stages of life. Enterovirus infection was evenly distributed between the children (<5 years old) and >5 years old patient groups. This points out the diarrheagenic potential of enteroviruses in all stages of life and propels us to consider it as one of the major viruses involved in both infant and adult diarrhea. All the norovirus infections in the study emerged from the infants and thus strengthen the belief of considering norovirus as important diarrheagenic pathogens in childhood diarrhea. To summarize, the affect of the diarrheagenic viruses on the subjects of different age groups, it can be observed that the most susceptible population are the infants (<2 years) who suffer the major disease burden of the viruses included in the study. This could be because of the incompetence of the developing immune system of the children to set off the sufficient immune response which can combat the infecting pathogens. Studies have shown that the children between the age of 6-12 months show a diminished level of maternal antibodies and are least protected against rotavirus infections [368, 369]. Also children of this age group are in the process

of crawling and walking stages where they could pick up the unhygienic materials in their mouth thus making them more exposed to the pathogens [370]. The relatively low infection rate of viral pathogens in older children may be due to the immunity developed because of previous exposures during the early stages of life [371].

5.3 ELISA vs RT-PCR

In this study, rotavirus detection was carried out by two different techniques: ELISA and PCR. ELISA is the widely used method for the detection of rotavirus infections. Many surveillance studies rely on this method for studying the prevalence of the virus [351-353, 372, 373]. The ELISA based methods are economical, time saving and easy to perform. These qualities make them the preferred method of choice. But the efficiency of ELISA kits remains in question. The other commonly used technique for the detection of rotavirus is PCR. PCR, either conventional or real time, has greater sensitivity and specificity and can be employed to detect different genotypes [374]. In this study, we have compared the ELISA based method with the more efficient and sensitive method of RT-PCR. The sensitivity and specificity of the ELISA came out to be 84.48% and 98.61%, respectively confirming the high efficiency of RT-PCR.

5.4 Clinical association of rotavirus infection

Rotavirus infection exhibits a spectrum of symptoms, primarily being vomiting and dehydration. In this study, the association of clinical features (vomiting, fever and dehydration) with rotavirus infection was studied. It was interesting to note that the children with rotavirus infection have higher rates of vomiting, fever and dehydration in comparison with their rotavirus negative counterparts. This shows that rotavirus infection has a substantial association with the accompanying symptoms (along with diarrhea) and hence severity of illness is higher. During the study, it was found that vomiting was significantly associated with rotavirus infection ($P<0.05$) where 35.5% of the children with rotavirus diarrhea experienced vomiting in contrast to 23% children having non-rotavirus diarrhea. This high trend of vomiting in rotavirus infected patients is supported by the reports which state that vomiting is the common symptom associated with rotavirus infection and precede diarrhea in almost half of the rotavirus infection cases [375].

Rotavirus infection is also found to cause rigorous diarrhea resulting in severe dehydration [376]. In fact there are studies which reported that the children having severe diarrhea have two times more chances of having rotavirus diarrhea [377]. The clinical investigation of the diarrheal children in this study also showed the significant association of dehydration with rotavirus infection ($P<0.05$). Around 26% of children suffering from rotavirus gastroenteritis were found to be suffering from fever which is 5% higher than the rate of fever in non-rotavirus gastroenteritis cases. However this association of fever with rotavirus infection showed no statistical significance in Chi-square test. As a whole, these results are in concordance with the other studies which reported that rotavirus infected children suffer from more severe illness than children having non-rotavirus gastroenteritis [345, 353, 370, 378-380]. On the contrary, in the adult subjects, the rates of vomiting, fever and dehydration was considerably lower in rotavirus positive cases. This leads to the conviction that rotavirus infection results in more severe illness in children than adults and this might be an explanation for the higher rotavirus associated mortality in children than adults.

5.5 Seasonal distribution of rotavirus infection

The seasonal distribution of rotaviruses in this study revealed some interesting findings. Seasonal fluctuations in rotavirus infections are well established and extensively reported [381-383]. Seasonality of rotavirus infection varies widely around the globe. According to a common belief, rotavirus infections show a marked seasonality in the temperate regions of the globe, where rotavirus infections are at peak in the winter season with very rare infections in the months of summer. Whereas in the tropical parts of the world, rotavirus infections occur year round with no particular seasonal peaks [384]. A number of attempts have been made to correlate the seasonal patterns with the climatic conditions such as rainfall, humidity and temperature but no robust link could be established. Although, the outcomes of different studies concludes that risk of rotavirus infections increase with the low temperature, low relative humidity and low levels of rainfall [385-391]. In this study, rotavirus infections were observed in all the seasons of the year. The rotavirus positivity of the samples was highest in the months of winter where 38.5% samples were positive for rotavirus. These results are in accordance with the other studies from northern India which shows maximum rotavirus infections and hospitalizations in the winter season [345, 351, 353, 383, 392]. But unlike those studies, rotavirus infection rate in this study was comparatively higher in the other seasons also. In fact, summer

season (May-June) witnessed the second highest rotavirus detection rate with positivity in almost 29% samples. Equal figures were obtained in the spring season. However, as expected, the months between August to October (autumn) showed the least prevalence of rotavirus in the region but still having considerable rotavirus associated hospitalizations. This weird and different seasonality of rotavirus in Himachal Pradesh may be due to the geographical and climatic conditions of the region. Here the summers are not very hot because of the high altitude and hilly terrain. Also the region experiences low relative humidity which is favorable for spread of rotavirus. The year round prevalence of rotavirus in the region warrants the implementation of continuous surveillance and preventive measures against the virus throughout the year and not only in particular seasons.

5.6 Molecular epidemiology of rotavirus

The effectiveness of any vaccine in a particular region depends on its ability to protect against the circulating strains at that time [35, 393]. Therefore it becomes imperative to study the molecular epidemiology of the rotavirus which can be helpful in designing of the preventive strategies against the virus. Among 32 G-types reported till date, G1, G2, G3, G4, G9 and G12 are the most common genotypes isolated from humans [28]. Rotavirus G1 (VP7 type) is the most common genotype found in humans globally as well as from different parts of India [28, 161, 352, 353, 357, 378]. In our study, the frequency of G1 genotype is in accordance with the global and national trends. Here, G1 rotavirus is identified as the foremost G-type with frequency of 66.03%. The significance of high circulation of G1 lies in the fact that former studies have shown total absence of this genotype but predominance of G2 strain in the region [38, 39]. On the contrary, G2 was isolated only in one case during this study. Other globally common G-types (G3 and G4) were totally absent in the region. This pattern of circulating genotypes is different from the other parts of the country. G9 and G12 are believed to be the emerging genotypes and studies from various parts of the world have reported them as the predominant genotypes [355, 394-396]. G9 was first detected in humans in 1987 and eventually it has established itself as the important strain responsible for gastroenteritis. G12, for the first time was reported in India in 2003 [397] and since then numerous reports from different parts of the country have detected it in significant fractions [345, 351, 398, 399]. In our study, G9 and G12 strains were the second and third leading cause of gastroenteritis, respectively. This reassures the increasing importance of G9 and G12 strains in human gastroenteritis

and warrants the need to include them in the future vaccines. G10 is commonly associated with cattle but it has been found to infect humans also [187, 217, 400]. The molecular analyses of the G10 strains from human have shown the involvement of interspecies transmission and genetic reassortment events [400, 401]. G10 alone and in mixed infection with G9 was detected in two cases (both children) in the current study. Further analysis and characterization of these isolated G10 strains is required to study their origin and evolution.

In the present study, P[6] emerged as the predominant genotype with 66.7% incidence. Globally, P[4] and P[8] are the most common VP4 types found in humans. P[6] genotype is a rare genotype in industrialized countries but is prevalent in Asian and African parts of the world [175, 199, 402]. The latest Indian Rotavirus Surveillance Network (IRSN) study reported that 33% and 21% of rotavirus infections in India are attributed to P[8] and P[4] strains whereas P[6] strain was isolated in merely 10% cases [351]. This proportion of P[6] strain nationally is drastically lower than the one witnessed in this study. The predominance of P[6] strains in the present study is an interesting finding from this part of country. However, this high frequency of P[6] strains in Himachal Pradesh is not new to this region. Two previous reports on rotavirus diversity are available from this region and both the reports have documented significantly high rate (71.4% and 97.3%) of P[6] circulation in the region [38, 39]. This suggests the continuous elevated prevalence of P[6] in the region from last two decades. The second most common VP4 genotype in this study was P[8] with 13.36% positivity. The other globally and nationally common P-type, P[4] was not detected in any of the cases. This altered diversity of P-types is very crucial in designing and implementing the preventive strategies in the region.

As far as, G-P combinations are concerned G1P[8] is the most common genotype reported in the previous studies from the other parts of India and the world. The association of G1 with P[6] or any other genotype is considered unusual. But in this study, G1P[6] combination was the leading genotype responsible for 62.8% incidences. According to our knowledge, this is the first time in India when we are witnessing such high infection rate attributed to G1P[6] rotavirus strains. The detection of G9P[6], G12P[6] and G2P[6] in this study suggest that P[6] genotype is circulating in association with all the G-types identified. G9 and G12 in this study were found to be in exclusive association with P[6] strains in contrast to the previous studies from other parts of the country where both the genotypes were found in major association with P[8]. Similarly

G2 is commonly found to be associated with P[4], but in this study, the only G2 strain detected was having P[6] with it. This high prevalence of unusual combinations in the region is very fascinating and compels us to report such high prevalence of rotaviruses with unusual genotype combination. The relatively low frequency of globally predominant strain, G1P[8] in the region is also a notable finding of the study. Interestingly, there is no significant variation of circulating genotypes observed in the children and adult samples. This suggests the unbiased circulation of diverse rotavirus strain in the different age groups.

Currently, two rotavirus vaccines, RotaTeq and Rotarix are licensed in India and indigenously developed Rotavac emerged as a vaccine candidate for inclusion in vaccination program. The G1 genotype which emerged as predominant type in this study is a component of both RotaTeq and Rotarix whereas G9, the second leading genotype is the part of Rotavac vaccine. Studies have shown that the vaccines show a broad heterotypic protection with similar protection against homotypic and heterotypic strains [403]. It would be interesting to see that how efficiently the Rotavac vaccine having G9P[11] genotype would work against nationally predominant G1 strains.

5.7 Molecular characterization of VP7 and VP4 viral protein

The molecular characterization of the VP7 protein was performed in the present study. VP7 is the most abundant outer most protein of the virus and an important target for the immune response generated against the virus [50, 55, 61]. In the present study, we have found that G1 was the most common VP7 genotype identified. Therefore, we have analyzed the VP7 gene of G1 strains prevalent in Himachal Pradesh and compared them with the G1 components of the Rotarix and RotaTeq vaccines to investigate the genetic and antigenic variations. The phylogenetic analysis in our study reveals that the isolated Himachal strains belong to the subgenotypic lineage 1 whereas the Rotarix and RotaTeq strains clustered in the lineage 2 and 3, respectively. In such a case, will there be any effect on vaccine efficacy? There is no robust data or evidences to prove that. But there are studies which show that intragenotypic nucleotide differences have a correlation with the antigenic differences. A study by Matthijnssens et al. reported that a rotavirus vaccinated child got infected by rotavirus G1 strain belonging to different lineage than the vaccine strain [404]. In another study, Hoshino et al., proposed G9-lineage 1 rotavirus strains as more suitable vaccine candidate than strains belonging to lineage 2 or 3 [405].

They have constructed VP7 substitution mutants bearing VP7 gene from different G9 specific lineages to study the immunogenic potential of all the three G9 subgenotypic lineages by raising the antisera against them. The outcome of the study showed that the antibodies against lineage 1 strains were able to neutralize the lineage 2 and 3 strains very efficiently with high titer values whereas the lineage 2 and 3 specific antibodies were not as efficient in neutralizing the lineage 1 strains. It might be possible that mutations in the lineage and sublineage specific amino acids belonging to the antigenic region rendering antibody escaping to the strains.

In the present study, a comparative analysis between the G1 strains and vaccine (Rotarix & RotaTeq) strains revealed significant number of amino acid changes in the antigenic epitope regions 7-1 and 7-2. These amino acid differences could influence antigenicity of the strains and might give rise to neutralization antibody escape mutants [65, 344]. 1n 1996, Jin et al investigated a vaccine failure episode in USA where children vaccinated with the D strain of rotavirus suffered G1 rotavirus infection post immunization [406]. The interesting part of the study was that the break through strains which evaded the immunization elicited neutralizing antibodies belonged to the same serotype as the vaccine strain D and its identical Wa strains. On investigating the antigenic region of VP7 amino acid sequence, the authors found that the break through strains differ from the vaccine strains at positions 97 and 147. Both these positions are very critical components of neutralization epitopes and lies in the epitope region 7-1a and 7-2, respectively. This study supports the fact that difference in antigenic region of circulating strains and vaccine strains could lead to vaccine failure or at least reduce the vaccine efficacy.

In the epitope region, Himachal G1 strains and vaccine strains exhibited variations in the amino acid composition at crucial neutralization antibody binding sites (region 7-1a and 7-2). But these strains were found to be closer to Rotarix strain (having five amino acid substitutions) in contrast to the RotaTeq strain, where seven amino acid differences were present. As far as the antigenic epitopes of Rotarix and RotaTeq strains are concerned, they both differ at two amino acids at positions 97 and 147. In Rotarix, there is a Glutamic acid and Aspargine whereas RotaTeq has Aspartic acid and Serine at positions 97 and 147, respectively. All of the G1 strains under investigation were found to possess Glutamic acid and Aspargine at these positions. These results are in accordance with other studies which showed similar results with G1 strains from other part of India [37, 407-409]. If the amino acid variations between circulating strains and vaccine strains are

effective enough to reduce the vaccine efficacy then Rotarix emerges as the favorite vaccine candidate to be administered in the region. But it is yet to be established. Also, these amino acid substitutions in the neutralization antibody escape sites are not exclusive in this study. A number of other studies from different parts of the world have documented these mutations [410-415] and they seem to have no apparent adverse effect on vaccine effectiveness in their region [269, 270]. These results show that the immunization efficiency of the vaccine does not solely depend on the epitope region of VP7 protein but there may be some other regions or other proteins which are also responsible for immunogenic potential of the virus.

P[6] rotaviruses were first identified to be associated with asymptomatic infections in neonates [416]. Although, with time, P[6] emerged as an important rotavirus genotype to be associated with gastroenteritis in humans, especially in African countries [161]. The molecular analysis of human P[6] strains from different geographical locations reveals the predominance of lineage 1a across the globe [417-419]. Although a number of highly divergent lineages are found in humans in Hungary, Italy, China, Vietnam, Brazil, Argentina, India and Ecuador [417]. The molecular analysis of VP4 genes of P[6] strains isolated in this study also reveals that all the isolates cluster within lineage 1a. The high sequence similarity of the isolates with the African and other international strains from nearby countries is a crucial finding and impels us to carry out molecular characterization of other important genes (including VP7 and NSP4) so that the evolutionary characteristics of the P[6] strains can be studied.

5.8 *In silico* analysis

To ascertain the effect of amino acid variations on the structure of the VP7 protein of the isolates and then subsequent binding with the anti-VP7 antibody, we have carried out the *in silico* analysis. In this analysis, the VP7 protein structures of the vaccines and isolates were predicted/ modeled using Rosettabackrub and cluspro servers. These predicted structures of the isolates were compared with each of the vaccine strains to know if the amino acid variations in the epitope region have resulted in the alteration in the protein organization. The RMSD analysis revealed no significant variation in the structures of the isolates and vaccine strains. This is interesting to find that even after having five (Rotarix), seven (RotaTeq) and fifteen (Rotavac) amino acid differences, the VP7 protein of Himachal isolates did not vary from the vaccines on the structural level. The possible

explanation for this can be the chemical characteristics of the substituting amino acids. On observing the chemical characteristics of the amino acids under study, we can see that all the amino acids which are varying between the isolates and vaccines are having similar chemical characteristics except in isolate JU-SHI-14. For example, on comparing the isolates with Rotarix, it can be seen that at position 94, N is replaced by S where both are polar amino acids. Similarly RotaTeq and isolates vary at position 97 where D is replaced by E. Here also both the amino acids are having similar chemical characteristic and have acidic R group. The other amino acids undergoing substitution also show similar trend except one isolate (JU-SHI-14). It has been observed that if an amino acid is replaced by its counterpart having different chemical characteristic of polarity or charge, than it may alter the structure of the overall protein. But this has not been observed at least in this study where out of fifteen amino acid variations, half of them bear a substitution between amino acids having converse chemical characteristics. Further structural analysis and interaction studies are required to study this behavior of protein.

The objective of antigen antibody docking analysis was to study the antibody binding characteristics of the isolates and compare them with the vaccine strains. It has been reported that the change in amino acid in the epitope region can result in reduced or altered binding of the neutralizing antibody and thus may lead to weakened immune response. In the study, it was found that the binding energy (referred to as weighted score) of the antibody with the VP7 protein of isolates is almost similar to the binding energy of the vaccines under study. This reflects that in spite of amino acid differences, the isolates likely behave in a similar fashion to that of the vaccine, as far as their interaction with the antibody is concerned. Thus, it can be concluded on the basis of this basic *in silico* study that the antibody binding capability of the isolates is retained in the similar manner to that of the vaccines and thus it can be hypothesized that on the administration of these vaccines in the children of Himachal Pradesh there will be no negative effect on vaccine efficacy. Although this is merely an assumption which can be made, but actual inference can only be obtained after the availability of post vaccination data. However, before the availability of post vaccination data, serological analysis by wet lab experiments can generate more reliable information on this aspect. This study is limited by the fact that the only anti-VP7 antibody structure available in PDB were raised against rotavirus G3 strain and used for the present *in silico* docking study. The more

reliable results can only be obtained once the anti-G1 antibody structure is available and then used for docking studies against G1 strains.

Chapter 6

Conclusion

CONCLUSION & FUTURE PROSPECTS

Keeping in view the disease and economic burden associated with the diarrheal diseases, it becomes imperative to study the epidemiological and etiological features of this disease in the different regions of the country. This study indicates the predominance of rotavirus in the region and a significant disease burden can be attributed to this virus among all age groups. Enterovirus is also detected in considerable fraction and thus imposing a need to consider it as a potential pathogen accountable for human diarrhea. However, a very important diarrheagenic virus, norovirus was detected in a very low frequency. This study not only throws light on the relative prevalence of the important diarrheagenic viruses but also furnish preliminary data which would be helpful in formulating the preventive strategies against them. In particular, the high infection rate of rotavirus in children and its year round prevalence warrants immediate attention in order to reduce the morbidity and suspected mortality due to this pathogen.

G1P[6] was observed as the predominant cause of rotavirus diarrhea in the region. However, G9P[6] and G12P[6] were also detected in significant proportion. As a whole, this study provides crucial data on rotavirus epidemiology and strain diversity in children and adult population of the region. We anticipate that this data will be helpful in implementing the preventive strategies accordingly which would be helpful in reducing rotavirus associated disease burden in the otherwise unexplored state of Himachal Pradesh. Further molecular analysis of G10 isolates is required to study their evolutionary characteristics which could probably ascertain interspecies transmission mechanism.

The phylogenetic and molecular analysis of VP7 gene reveals the belonging of isolates to a novel sublineage within lineage 1. The isolates under investigation have considerable amino acid differences as compared to the vaccine strains in the epitope region and thus might have an adverse effect on the vaccine efficacy. Although, *in silico* analysis revealed no adverse effect on the binding of antibody to the antigen. But this cannot be ascertained without further serological analysis and extensive genetic and molecular studies including larger proportion of circulating strains at different time intervals (pre- and post-vaccination). However, this molecular analysis gives preliminary information on the genetic diversity of the circulating strains and can navigate the further approaches to study the diversity of G1 rotaviruses circulating in Himachal Pradesh, India.

In a nutshell, this study paves way for more extensive molecular and serological studies in future involving larger spectrum of viral and bacterial pathogens.

Chapter 7

References

REFERENCES

[1] World Health Organization. http://www.who.int/gho/mortality_burden_disease/causes_death/en/. 2015

[2] A. J. Eckardt and D. C. Baumgart, "Viral gastroenteritis in adults," *Recent Pat Antiinfect Drug Discov,* vol. 6, pp. 54-63, 2011.

[3] I. Wilhelmi, E. Roman, and A. Sanchez-Fauquier, "Viruses causing gastroenteritis," *Clin Microbiol Infect,* vol. 9, pp. 247-262, 2003.

[4] F. Rovida, G. Campanini, A. Piralla, K. M. Adzasehoun, A. Sarasini, and F. Baldanti, "Molecular detection of gastrointestinal viral infections in hospitalized patients," *Diagn Microbiol Infect Dis,* vol. 77, pp. 231-235, 2013.

[5] A. Thongprachum, S. Takanashi, A. F. Kalesaran, S. Okitsu, M. Mizuguchi, S. Hayakawa*, et al.*, "Four-year study of viruses that cause diarrhea in Japanese pediatric outpatients," *J Med Virol,* vol. 87, pp. 1141-1148, 2015.

[6] S. M. Banajeh and B. A. Abu-Asba, "The epidemiology of all-cause and rotavirus acute gastroenteritis and the characteristics of rotavirus circulating strains before and after rotavirus vaccine introduction in Yemen: analysis of hospital-based surveillance data," *BMC Infect Dis,* vol. 15, pp. 418-427, 2015.

[7] J. E. Tate, A. H. Burton, C. Boschi-Pinto, U. D. Parashar, M. Agocs, F. Serhan*, et al.*, "Global, regional, and national estimates of rotavirus mortality in children< 5 years of age, 2000–2013," *Clin Infect Dis,* vol. 62, pp. S96-S105, 2016.

[8] World Health Organization. http://www.wpro.who.int/mediacentre/factsheets/fs_20120229/en/. 2012

[9] J. John, R. Sarkar, J. Muliyil, N. Bhandari, M. K. Bhan, and G. Kang, "Rotavirus gastroenteritis in India, 2011-2013: revised estimates of disease burden and potential impact of vaccines," *Vaccine,* vol. 11, pp. 4, 2014.

[10] E. J. Anderson and S. G. Weber, "Rotavirus infection in adults," *Lancet Infect Dis,* vol. 4, pp. 91-99, 2004.

[11] H. Nakajima, T. Nakagomi, T. Kamisawa, N. Sakaki, K. Muramoto, T. Mikami*, et al.*, "Winter seasonality and rotavirus diarrhoea in adults," *Lancet,* vol. 357, pp. 1950, 2001.

[12] C. F. Lanata, C. L. Fischer-Walker, A. C. Olascoaga, C. X. Torres, M. J. Aryee, and R. E. Black, "Global causes of diarrheal disease mortality in children <5 years of age: a systematic review," *PLoS One,* vol. 8, pp. e72788, 2013.

[13] M. M. Patel, M.-A. Widdowson, R. I. Glass, K. Akazawa, J. Vinjé, and U. D. Parashar, "Systematic literature review of role of noroviruses in sporadic gastroenteritis," *Emerg Infect Dis,* vol. 14, pp. 1224-1231, 2008.

[14] A. Kroneman, J. Harris, H. Vennema, E. Duizer, Y. van Duynhoven, J. Gray*, et al.*, "Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses," *J Public Health,* vol. 30, pp. 82-90, 2008.

[15] D. P. Zheng, M. A. Widdowson, R. I. Glass, and J. Vinje, "Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006," *J Clin Microbiol,* vol. 48, pp. 168-77, 2010.

[16] M. Pons-Salort, E. P. Parker, and N. C. Grassly, "The epidemiology of non-polio enteroviruses: recent advances and outstanding questions," *Curr Opin Infect Dis,* vol. 28, pp. 479-487, 2015.

[17] S. Harada, M. Okada, S. Yahiro, K. Nishimura, S. Matsuo, J. Miyasaka*, et al.*, "Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto Prefecture, Japan," *J Med Virol,* vol. 81, pp. 1117-1127, 2009.

[18] P. R. Patil, S. D. Chitambar, and V. Gopalkrishna, "Molecular surveillance of non-polio enterovirus infections in patients with acute gastroenteritis in Western India: 2004-2009," *J Med Virol,* vol. 87, pp. 154-161, 2015.

[19] D. C. Rao, M. A. Babu, A. Raghavendra, D. Dhananjaya, S. Kumar, and P. Maiya, "Non-polio enteroviruses and their association with acute diarrhea in children in India," *Infect Genet Evol,* vol. 17, pp. 153-161, 2013.

[20] C. Scarcella, S. Carasi, F. Cadoria, L. Macchi, A. Pavan, M. Salamana*, et al.*, "An outbreak of viral gastroenteritis linked to municipal water supply, Lombardy, Italy, June 2009," *Euro Surveill,* vol. 14, pp. 506-513, 2009.

[21] J. Matthijnssens, P. H. Otto, M. Ciarlet, U. Desselberger, M. Van Ranst, and R. Johne, "VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation," *Arch Virol,* vol. 157, pp. 1177-1182, 2012.

[22] J. Matthijnssens, M. Ciarlet, S. M. McDonald, H. Attoui, K. Banyai, J. R. Brister*, et al.*, "Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG)," *Arch Virol,* vol. 156, pp. 1397-1413, 2011.

[23] Rotavirus Classification Working Group (RCWG). Newly assigned genotypes. https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg. 2016

[24] L. da Silva Soares, S. de Fatima Dos Santos Guerra, A. do Socorro Lima de Oliveira, F. da Silva Dos Santos, E. M. de Fatima Costa de Menezes, J. Mascarenhas*, et al.*, "Diversity of rotavirus strains circulating in Northern Brazil after introduction of a rotavirus vaccine: high prevalence of G3P[6] genotype," *J Med Virol,* vol. 86, pp. 1065-1072, 2014.

[25] M. D. Esona, G. E. Armah, and A. D. Steele, "Rotavirus VP4 and VP7 genotypes circulating in Cameroon: identification of unusual types," *J Infect Dis,* vol. 202, pp. S205-S211, 2010.

[26] R. Q. Gurgel, A. D. J. Alvarez, A. Rodrigues, R. R. Ribeiro, S. S. Dolabella, N. L. Da Mota*, et al.*, "Incidence of rotavirus and circulating genotypes in Northeast Brazil during 7 years of national rotavirus vaccination," *PloS One,* vol. 9, pp. e110217, 2014.

[27] A. Piekarska, A. Kacerka, E. Majda-Stanisławska, B. Jóźwiak, and M. Sidorkiewicz, "Predominance of genotype P [9] G3 in rotavirus gastroenteritis in Polish children," *Arch Med Sci,* vol. 11, pp. 577-583, 2015.

[28] World Health Organization. Global Rotavirus Surveillance and Information Bulletin, vol. 10, pp. 1–4, 2015.

[29] S. Jain, J. Vashistt, and H. Changotra, "Rotaviruses: Is their surveillance needed?," *Vaccine,* vol. 32, pp. 3367-3378, 2014.

[30] Q. D. Trinh, T. A. Nguyen, T. G. Phan, P. Khamrin, H. Yan, P. L. Hoang*, et al.*, "Sequence analysis of the VP7 gene of human rotavirus G1 isolated in Japan, China, Thailand, and Vietnam in the context of changing distribution of rotavirus G-types," *J Med Virol,* vol. 79, pp. 1009-16, 2007.

[31] M. L. Dyall-Smith, I. Lazdins, G. W. Tregear, and I. H. Holmes, "Location of the major antigenic sites involved in rotavirus serotype-specific neutralization," *Proc Natl Acad Sci U S A,* vol. 83, pp. 3465-3468, 1986.

[32] N. Kobayashi, K. Taniguchi, T. Urasawa, and S. Urasawa, "Analysis of the neutralization epitopes on human rotavirus VP7 recognized by monotype-specific monoclonal antibodies," *J Gen Virol,* vol. 72, pp. 1855-1861, 1991.

[33] K. Nishikawa, Y. Hoshino, K. Taniguchi, K. Y. Green, H. B. Greenberg, A. Z. Kapikian*, et al.*, "Rotavirus VP7 neutralization epitopes of serotype 3 strains," *Virology,* vol. 171, pp. 503-515, 1989.

[34] S. T. Aoki, E. C. Settembre, S. D. Trask, H. B. Greenberg, S. C. Harrison, and P. R. Dormitzer, "Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab," *Science,* vol. 324, pp. 1444-1447, 2009.

[35] C. M. Wang, S. C. Chen, and K. T. Chen, "Current status of rotavirus vaccines," *World J Pediatr,* vol. 11, pp. 300-308, 2015.

[36] M. Ciarlet and F. Schodel, "Development of a rotavirus vaccine: clinical safety, immunogenicity, and efficacy of the pentavalent rotavirus vaccine, RotaTeq," *Vaccine,* vol. 30, pp. 107, 2009.

[37] R. Kulkarni, R. Arora, and S. D. Chitambar, "Sequence analysis of VP7 and VP4 genes of G1P[8] rotaviruses circulating among diarrhoeic children in Pune, India: a comparison with Rotarix and RotaTeq vaccine strains," *Vaccine,* vol. 11, pp. 080, 2014.

[38] M. Ramachandran, B. K. Das, A. Vij, R. Kumar, S. S. Bhambal, N. Kesari*, et al.*, "Unusual diversity of human rotavirus G and P genotypes in India," *J Clin Microbiol,* vol. 34, pp. 436-439, 1996.

[39] V. Jain, B. K. Das, M. K. Bhan, R. I. Glass, and J. R. Gentsch, "Great diversity of group A rotavirus strains and high prevalence of mixed rotavirus infections in India," *J Clin Microbiol,* vol. 39, pp. 3524-3529, 2001.

[40] R. Bishop, G. Davidson, I. Holmes, and B. Ruck, "Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis," *Lancet,* vol. 302, pp. 1281-1283, 1973.

[41] A. Z. Kapikian, Y. Hoshino, R. M. Chanock, and I. Perez-Schael, "Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children," *J Infect Dis,* vol. 174, pp. S65-S72, 1996.

[42] D. I. Bernstein, "Rotavirus overview," *Pediatr Infect Dis J,* vol. 28, pp. S50-S53, 2009.

[43] K. L. Kotloff, J. P. Nataro, W. C. Blackwelder, D. Nasrin, T. H. Farag, S. Panchalingam*, et al.*, "Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study," *Lancet,* vol. 382, pp. 209-222, 2013.

[44] L. Liu, H. L. Johnson, S. Cousens, J. Perin, S. Scott, J. E. Lawn*, et al.*, "Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000," *Lancet,* vol. 379, pp. 2151-2161, 2012.

[45] E. C. Settembre, J. Z. Chen, P. R. Dormitzer, N. Grigorieff, and S. C. Harrison, "Atomic model of an infectious rotavirus particle," *EMBO J,* vol. 30, pp. 408-416, 2011.

[46] J. Z. Chen, E. C. Settembre, S. T. Aoki, X. Zhang, A. R. Bellamy, P. R. Dormitzer*, et al.*, "Molecular interactions in rotavirus assembly and uncoating seen by high-resolution cryo-EM," *Proc Natl Acad Sci,* vol. 106, pp. 10644-10648, 2009.

[47] B. McClain, E. Settembre, B. R. Temple, A. R. Bellamy, and S. C. Harrison, "X-ray crystal structure of the rotavirus inner capsid particle at 3.8 Å resolution," *J Mol Biol,* vol. 397, pp. 587-599, 2010.

[48] X. Zhang, E. Settembre, C. Xu, P. R. Dormitzer, R. Bellamy, S. C. Harrison*, et al.*, "Near-atomic resolution using electron cryomicroscopy and single-particle reconstruction," *Proc Natl Acad Sci,* vol. 105, pp. 1867-1872, 2008.

[49] S. M. McDonald and J. T. Patton, "Rotavirus VP2 core shell regions critical for viral polymerase activation," *J Virol,* vol. 85, pp. 3095-3105, 2011.

[50] B. V. Prasad, G. Wang, J. P. Clerx, and W. Chiu, "Three-dimensional structure of rotavirus," *J Mol Biol,* vol. 199, pp. 269-275, 1988.

[51] M. K. Estes H.B. Greenberg, "Rotaviruses," *Fields virology*, Kluwer/Lippincott, Williams and Wilkins, pp. 1348-1401, 2013.

[52] M. Mathieu, I. Petitpas, J. Navaza, J. Lepault, E. Kohli, P. Pothier*, et al.*, "Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion," *EMBO J,* vol. 20, pp. 1485-1497, 2001.

[53] B. V. Prasad and M. K. Estes, "Electron cryomicroscopy and computer image processing techniques," *Methods Mol Med,* vol. 34, pp. 9-31, 2000.

[54] M. Tihova, K. A. Dryden, A. R. Bellamy, H. B. Greenberg, and M. Yeager, "Localization of membrane permeabilization and receptor binding sites on the VP4 hemagglutinin of rotavirus: implications for cell entry," *J Mol Biol,* vol. 314, pp. 985-992, 2001.

[55] M. Yeager, K. A. Dryden, N. H. Olson, H. B. Greenberg, and T. S. Baker, "Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction," *J Cell Biol,* vol. 110, pp. 2133-2144, 1990.

[56] A. Shaw, R. Rothnagel, D. Chen, R. Ramig, W. Chiu, and B. V. Prasad, "Three-dimensional visualization of the rotavirus hemagglutinin structure," *Cell,* vol. 74, pp. 693-701, 1993.

[57] M. Yeager, J. Berriman, T. Baker, and A. Bellamy, "Three-dimensional structure of the rotavirus haemagglutinin VP4 by cryo-electron microscopy and difference map analysis," *EMBO J,* vol. 13, pp. 1011-1018, 1994.

[58] A. K. Banerjee and A. Shatkin, "Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase," *J Virol,* vol. 6, pp. 1-11, 1970.

[59] N. M. Bartlett, S. C. Gillies, S. Bullivant, and A. Bellamy, "Electron microscopy study of reovirus reaction cores," *J Virol,* vol. 14, pp. 315-326, 1974.

[60] R. E. Smith and Y. Furuichi, "The double-stranded RNA genome segments of cytoplasmic polyhedrosis virus are independently transcribed," *J Virol,* vol. 41, pp. 326-329, 1982.

[61] M. K. Estes and J. Cohen, "Rotavirus gene structure and function," *Microbiol Rev,* vol. 53, pp. 410-449, 1989.

[62] P. Gouet, J. M. Diprose, J. M. Grimes, R. Malby, J. N. Burroughs, S. Zientara*, et al.*, "The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography," *Cell,* vol. 97, pp. 481-490, 1999.

[63] J. B. Pesavento, M. K. Estes, and B. V. Prasad, "Structural organization of the genome in rotavirus," *Cold Spring Harb Perspect Med,* vol. 9, pp. 115-127, 2003.

[64] J. B. Pesavento, S. E. Crawford, M. K. Estes, and B. V. Prasad, "Rotavirus proteins: structure and assembly," *Curr Top Microbiol Immunol,* vol. 309, pp. 189-219, 2006.

[65] K. Y. Green and A. Z. Kapikian, "Identification of VP7 epitopes associated with protection against human rotavirus illness or shedding in volunteers," *J Virol,* vol. 66, pp. 548-53, 1992.

[66] J. E. Ludert, N. Feng, J. H. Yu, R. L. Broome, Y. Hoshino, and H. B. Greenberg, "Genetic mapping indicates that VP4 is the rotavirus cell attachment protein in vitro and in vivo," *J Virol,* vol. 70, pp. 487-93, 1996.

[67] J. E. Ludert, B. B. Mason, J. Angel, B. Tang, Y. Hoshino, N. Feng*, et al.*, "Identification of mutations in the rotavirus protein VP4 that alter sialic-acid-dependent infection," *J Gen Virol,* vol. 79, pp. 725-9, 1998.

[68] S. E. Crawford, S. K. Mukherjee, M. K. Estes, J. A. Lawton, A. L. Shaw, R. F. Ramig*, et al.*, "Trypsin cleavage stabilizes the rotavirus VP4 spike," *J Virol,* vol. 75, pp. 6052-6061, 2001.

[69] C. F. Arias, P. Romero, V. Alvarez, and S. Lopez, "Trypsin activation pathway of rotavirus infectivity," *J Virol,* vol. 70, pp. 5832-5839, 1996.

[70] M. Estes, D. Graham, and B. Mason, "Proteolytic enhancement of rotavirus infectivity: molecular mechanisms," *J Virol,* vol. 39, pp. 879-888, 1981.

[71] L. Fiore, H. B. Greenberg, and E. R. Mackow, "The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin," *Virology,* vol. 181, pp. 553-563, 1991.

[72] S. Lopez and C. Arias, "Attachment and post-attachment receptors for rotavirus," *Viral gastroenteritis*. Elsevier Science BV*,* pp. 143-163, 2003.

[73] M. Ciarlet, S. E. Crawford, and M. K. Estes, "Differential infection of polarized epithelial cell lines by sialic acid-dependent and sialic acid-independent rotavirus strains," *J Virol,* vol. 75, pp. 11834-11850, 2001.

[74] I. S. Kim, S. D. Trask, M. Babyonyshev, P. R. Dormitzer, and S. C. Harrison, "Effect of mutations in VP5* hydrophobic loops on rotavirus cell entry," *J Virol,* vol. 84, pp. 6200-6207, 2010.

[75] M. E. Chemello, O. C. Aristimuño, F. Michelangeli, and M.-C. Ruiz, "Requirement for vacuolar H+-ATPase activity and Ca2+ gradient during entry of rotavirus into MA104 cells," *J Virol,* vol. 76, pp. 13083-13087, 2002.

[76] J. Ludert, F. Michelangeli, F. Gil, F. Liprandi, and J. Esparza, "Penetration and uncoating of rotaviruses in cultured cells," *Intervirology,* vol. 27, pp. 95-101, 1987.

[77] J. D. Yoder, S. D. Trask, P. T. Vo, M. Binka, N. Feng, S. C. Harrison*, et al.*, "VP5* rearranges when rotavirus uncoats," *J Virol,* vol. 83, pp. 11372-11377, 2009.

[78] S. D. Trask, I. S. Kim, S. C. Harrison, and P. R. Dormitzer, "A rotavirus spike protein conformational intermediate binds lipid bilayers," *J Virol,* vol. 84, pp. 1764-1770, 2010.

[79] M. Gutiérrez, P. Isa, C. Sánchez-San Martin, J. Pérez-Vargas, R. Espinosa, C. F. Arias*, et al.*, "Different rotavirus strains enter MA104 cells through different endocytic pathways: the role of clathrin-mediated endocytosis," *J Virol,* vol. 84, pp. 9161-9169, 2010.

[80] J. A. Lawton, M. K. Estes, and B. V. Prasad, "Mechanism of genome transcription in segmented dsRNA viruses," *Adv Virus Res,* vol. 55, pp. 185-229, 2000.

[81] Y. Tao, D. L. Farsetta, M. L. Nibert, and S. C. Harrison, "RNA synthesis in a cage—structural studies of reovirus polymerase λ3," *Cell,* vol. 111, pp. 733-745, 2002.

[82] X. Lu, S. M. McDonald, M. A. Tortorici, Y. J. Tao, R. Vasquez-Del Carpio, M. L. Nibert*, et al.*, "Mechanism for coordinated RNA packaging and genome replication by rotavirus polymerase VP1," *Structure,* vol. 16, pp. 1678-1688, 2008.

[83] J. Lawton, M. Estes, and B. V. Prasad, "Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles," *Nat Struct Mol Biol,* vol. 4, pp. 118-121, 1997.

[84] D. Chen, C. L. Luongo, M. L. Nibert, and J. T. Patton, "Rotavirus open cores catalyze 5′-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase," *Virology,* vol. 265, pp. 120-130, 1999.

[85] S. Libersou, X. Siebert, M. Ouldali, L. F. Estrozi, J. Navaza, A. Charpilienne*, et al.*, "Geometric mismatches within the concentric layers of rotavirus particles: a potential regulatory switch of viral particle transcription activity," *J Virol,* vol. 82, pp. 2844-2852, 2008.

[86] E. Fabbretti, I. Afrikanova, F. Vascotto, and O. R. Burrone, "Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo," *J Gen Virol,* vol. 80, pp. 333-339, 1999.

[87] H. Jayaram, Z. Taraporewala, J. T. Patton, and B. V. Prasad, "Rotavirus protein involved in genome replication and packaging exhibits a HIT-like fold," *Nature,* vol. 417, pp. 311-315, 2002.

[88] X. Jiang, H. Jayaram, M. Kumar, S. J. Ludtke, M. K. Estes, and B. V. Prasad, "Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: implications for genome replication," *J Virol,* vol. 80, pp. 10829-10835, 2006.

[89] D. Martin, M. Ouldali, J. Ménétrey, and D. Poncet, "Structural organisation of the rotavirus nonstructural protein NSP5," *J Mol Biol,* vol. 413, pp. 209-221, 2011.

[90] J. Chnaiderman, M. Barro, and E. Spencer, "NSP5 phosphorylation regulates the fate of viral mRNA in rotavirus infected cells," *Arch Virol,* vol. 147, pp. 1899-1911, 2002.

[91] J. T. Patton, "Rotavirus VP1 alone specifically binds to the 3'end of viral mRNA, but the interaction is not sufficient to initiate minus-strand synthesis," *J Virol,* vol. 70, pp. 7940-7947, 1996.

[92] J. T. Patton, J. Chnaiderman, and E. Spencer, "Open reading frame in rotavirus mRNA specifically promotes synthesis of double-stranded RNA: template size also affects replication efficiency," *Virology,* vol. 264, pp. 167-180, 1999.

[93] M. J. Wentz, J. T. Patton, and R. F. Ramig, "The 3'-terminal consensus sequence of rotavirus mRNA is the minimal promoter of negative-strand RNA synthesis," *J Virol,* vol. 70, pp. 7833-7841, 1996.

[94] R. F. Ramig, "Genetics of the rotaviruses," *Annu Rev Microbiol,* vol. 51, pp. 225-255, 1997.

[95] D. Chen and J. T. Patton, "Rotavirus RNA replication requires a single-stranded 3′ end for efficient minus-strand synthesis," *J Virol,* vol. 72, pp. 7387-7396, 1998.

[96] M. A. Tortorici, T. J. Broering, M. L. Nibert, and J. T. Patton, "Template recognition and formation of initiation complexes by the replicase of a segmented double-stranded RNA virus," *J Biol Chem,* vol. 278, pp. 32673-32682, 2003.

[97] B. L. Petrie, H. B. Greenberg, D. Y. Graham, and M. K. Estes, "Ultrastructural localization of rotavirus antigens using colloidal gold," *Virus Res,* vol. 1, pp. 133-152, 1984.

[98] R. Gonzalez, R. Espinosa, P. Romero, S. Lopez, and C. Arias, "Relative localization of viroplasmic and endoplasmic reticulum-resident rotavirus proteins in infected cells," *Arch Virol,* vol. 145, pp. 1963-1973, 2000.

[99] C. Musalem and R. T. Espejo, "Release of progeny virus from cells infected with simian rotavirus SA11," *J Gen Virol,* vol. 66, pp. 2715-2724, 1985.

[100] N. Jourdan, M. Maurice, D. Delautier, A. M. Quero, A. L. Servin, and G. Trugnan, "Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus," *J Virol,* vol. 71, pp. 8268-8278, 1997.

[101] M. K. Estes, G. Kang, C. Q.-Y. Zeng, S. E. Crawford, and M. Ciarlet, "Pathogenesis of rotavirus gastroenteritis," *Novartis Foundation Symposium*, pp. 82-100, 2001.

[102] M. K. Estes, "The rotavirus NSP4 enterotoxin: Current status and challenges," *Perspectives in Medical Virology,* vol. 9, pp. 207-224, 2003.

[103] M. Estes and A. Morris, "A viral enterotoxin. A new mechanism of virus-induced pathogenesis," *Adv Exp Med Biol,* vol. 473, pp. 73, 1999.

[104] O. Lundgren and L. Svensson, "Pathogenesis of rotavirus diarrhea," *Microbes Infect,* vol. 3, pp. 1145-1156, 2001.

[105] O. Lundgren and L. Svensson, "The enteric nervous system and infectious diarrhea," *Perspectives in Medical Virology*, Elsevier, pp. 51-67, 2003.

[106] P. Tian, Y. Hu, W. P. Schilling, D. A. Lindsay, J. Eiden, and M. K. Estes, "The nonstructural glycoprotein of rotavirus affects intracellular calcium levels," *J Virol,* vol. 68, pp. 251-257, 1994.

[107] J. P. Brunet, J. Cotte-Laffitte, C. Linxe, A. M. Quero, M. Geniteau-Legendre, and A. Servin, "Rotavirus infection induces an increase in intracellular calcium concentration in human intestinal epithelial cells: role in microvillar actin alteration," *J Virol,* vol. 74, pp. 2323-2332, Mar 2000.

[108] J.-P. Brunet, N. Jourdan, J. Cotte-Laffitte, C. Linxe, M. Géniteau-Legendre, A. Servin*, et al.*, "Rotavirus infection induces cytoskeleton disorganization in human

intestinal epithelial cells: implication of an increase in intracellular calcium concentration," *J Virol,* vol. 74, pp. 10801-10806, 2000.

[109] K. G. Dickman, S. J. Hempson, J. Anderson, S. Lippe, L. Zhao, R. Burakoff*, et al.*, "Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells," *Am J Physiol Gastrointest Liver Physiol,* vol. 279, pp. G757-G766, 2000.

[110] F. Tafazoli, C. Q. Zeng, M. K. Estes, K. E. Magnusson, and L. Svensson, "NSP4 enterotoxin of rotavirus induces paracellular leakage in polarized epithelial cells," *J Virol,* vol. 75, pp. 1540-1546, 2001.

[111] F. Michelangeli and M. C. Ruiz, "I, 2. Physiology and pathophysiology of the gut in relation to viral diarrhea," *Perspect Med Virol,* vol. 9, pp. 23-50, 2003.

[112] J. Collins, W. G. Starkey, T. S. Wallis, G. J. Clarke, K. J. Worton, A. J. Spencer*, et al.*, "Intestinal enzyme profiles in normal and rotavirus-infected mice," *J Pediatr Gastroenterol Nutr,* vol. 7, pp. 264-272, 1988.

[113] A. P. Morris, J. K. Scott, J. M. Ball, C. Q.-Y. Zeng, W. K. O'Neal, and M. K. Estes, "NSP4 elicits age-dependent diarrhea and Ca2+ mediated I– influx into intestinal crypts of CF mice," *Am J Physiol Gastrointest Liver Physiol,* vol. 277, pp. G431-G444, 1999.

[114] M. Osborne, S. Haddon, A. Spencer, J. Collins, W. Starkey, T. Wallis*, et al.*, "An electron microscopic investigation of time-related changes in the intestine of neonatal infected with murine rotavirus," *J Pediatr Gastroenterol Nutr,* vol. 7, pp. 236-248, 1988.

[115] W. Starkey, J. Collins, T. Wallis, G. Clarke, A. Spencer, S. Haddon*, et al.*, "Kinetics, tissue specificity and pathological changes in murine rotavirus infection of mice," *J Gen Virol,* vol. 67, pp. 2625-2634, 1986.

[116] R. F. Ramig, "Pathogenesis of intestinal and systemic rotavirus infection," *J Virol,* vol. 78, pp. 10213-10220, 2004.

[117] S. Pedley, J. Bridger, J. Brown, and M. McCrae, "Molecular characterization of rotaviruses with distinct group antigens," *J Gen Virol,* vol. 64, pp. 2093-2101, 1983.

[118] S. Pedley, J. Bridger, D. Chasey, and M. McCrae, "Definition of two new groups of atypical rotaviruses," *J Gen Virol,* vol. 67, pp. 131-137, 1986.

[119] T. Flewett and G. Woode, "The rotaviruses," *Arch Virol,* vol. 57, pp. 1-23, 1978.

[120] L. Maunula and C.-H. von Bonsdorff, "Frequent reassortments may explain the genetic heterogeneity of rotaviruses: analysis of Finnish rotavirus strains," *J Virol,* vol. 76, pp. 11793-11800, 2002.

[121] J. L. Gombold and R. F. Ramig, "Analysis of reassortment of genome segments in mice mixedly infected with rotaviruses SA11 and RRV," *J Virol,* vol. 57, pp. 110-116, 1986.

[122] H. Greenberg, V. McAuliffe, J. Valdesuso, R. Wyatt, J. Flores, A. Kalica*, et al.*, "Serological analysis of the subgroup protein of rotavirus, using monoclonal antibodies," *Infect Immun,* vol. 39, pp. 91-99, 1983.

[123] Y. Hoshino, M. Gorziglia, J. Valdesuso, J. Askaa, R. I. Glass, and A. Z. Kapikian, "An equine rotavirus (FI-14 strain) which bears both subgroup I and subgroup II specificities on its VP6," *Virology,* vol. 157, pp. 488-496, 1987.

[124] L. Svensson, L. Grahnquist, C. Pettersson, M. Grandien, G. Stintzing, and H. Greenberg, "Detection of human rotaviruses which do not react with subgroup I- and II-specific monoclonal antibodies," *J Clin Microbiol,* vol. 26, pp. 1238-1240, 1988.

[125] K. Taniguchi, T. Urasawa, S. Urasawa, and T. Yasuhara, "Production of subgroup-specific monoclonal antibodies against human rotaviruses and their application to an enzyme-linked immunosorbent assay for subgroup determination," *J Med Virol,* vol. 14, pp. 115-125, 1984.

[126] J. Matthijnssens, M. Ciarlet, E. Heiman, I. Arijs, T. Delbeke, S. M. McDonald*, et al.*, "Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains," *J Virol,* vol. 82, pp. 3204-3219, 2008.

[127] R. Bishop, G. Davidson, I. Holmes, and B. Ruck, "Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis," *Lancet,* vol. 303, pp. 149-151, 1974.

[128] T. Flewett, A. Bryden, and H. Davies, "Virus particles in gastroenteritis," *Lancet,* vol. 2, pp. 1497, 1973.

[129] A. Z. Kapikian, H. W. Kim, R. G. Wyatt, W. J. Rodriguez, S. Ross, W. L. Cline, *et al.*, "Reoviruslike agent in stools: association with infantile diarrhea and development of serologic tests," *Science,* vol. 185, pp. 1049-1053, 1974.

[130] C. Mebus, N. Underdahl, M. Rhodes, and M. Twiehaus, *Calf diarrhea (scours): reproduced with a virus from a field outbreak*, University of Nebraska USA, 1969.

[131] L. Svensson, M. Grandien, and C. A. Pettersson, "Comparison of solid-phase immune electron microscopy by use of protein A with direct electron microscopy and enzyme-linked immunosorbent assay for detection of rotavirus in stool," *J Clin Microbiol,* vol. 18, pp. 1244-1249, 1983.

[132] M. Thouless, A. Bryden, T. Flewett, G. Woode, J. Bridger, D. Snodgrass, *et al.*, "Serological relationships between rotaviruses from different species as studied by complement fixation and neutralization," *Arch Virol,* vol. 53, pp. 287-294, 1977.

[133] G. Zissis, J. Lambert, and D. De Kegel, "Routine diagnosis of human rotaviruses in stools," *J Clin Pathol,* vol. 31, pp. 175-178, 1978.

[134] P. Grauballe, J. Genner, A. Meyling, and A. Hornsleth, "Rapid diagnosis of rotavirus infections: Comparison of electron microscopy and immunoelectro-osmophoresis for the detection of rotavirus in human infantile gastroenteritis," *J Gen Virol,* vol. 35, pp. 203-218, 1977.

[135] P. Middleton, M. Petric, C. Hewitt, M. Szymanski, and J. Tam, "Counter-immunoelectro-osmophoresis for the detection of infantile gastroenteritis virus (orbi-group) antigen and antibody," *J Clin Pathol,* vol. 29, pp. 191-197, 1976.

[136] L. Spence, M. Fauvel, R. Petro, and S. Bloch, "Comparison of Counterimmuno-electrophoresis and electron microscopy for laboratory diagnosis of human

reovirus-like agent-associated infantile gastroenteritis," *J Clin Microbiol,* vol. 5, pp. 248, 1977.

[137] G. Hammond, G. Ahluwalia, B. Klisko, and P. Hazelton, "Human rotavirus detection by counterimmunoelectrophoresis versus enzyme immunoassay and electron microscopy after direct ultracentrifugation," *J Clin Microbiol,* vol. 19, pp. 439-441, 1984.

[138] M. Clementi, P. Pauri, P. Bagnarelli, G. Carloni, and L. Calegari, "Diagnosis of human rotavirus infections: comparison of an electrophoretic method, a modified complement fixation test and electron microscopy for rotavirus detection," *Arch Virol,* vol. 67, pp. 341-344, 1981.

[139] G. Cukor, M. K. Berry, and N. R. Blacklow, "Simplified radioimmunoassay for detection of human rotavirus in stools," *J Infect Dis,* vol. 138, pp. 906-910, 1978.

[140] A. R. Kalica, R. H. Purcell, M. M. Sereno, R. G. Wyatt, H. W. Kim, R. M. Chanock*, et al.*, "A microtiter solid phase radioimmunoassay for detection of the human reovirus-like agent in stools," *J Immunol,* vol. 118, pp. 1275-1279, 1977.

[141] P. Middleton, M. Holdaway, M. Petric, M. Szymanski, and J. Tam, "Solid-phase radioimmunoassay for the detection of rotavirus," *Infect Immun,* vol. 16, pp. 439-444, 1977.

[142] H. K. Sarkkinen, P. E. Halonen, and P. P. Arstila, "Comparison of four-layer radioimmunoassay and electron microscopy for detection of human rotavirus," J M*ed Virol,* vol. 4, pp. 255-260, 1979.

[143] R. Scherrer and S. Bernard, "Application of enzyme-linked immunosorbent assay (ELISA) to the detection of calf rotavirus and rotavirus antibodies," *Ann Microbiol*, 1976, pp. 499-510.

[144] R. Yolken, H. Kim, T. Clem, R. Wyatt, A. Kalica, R. Chanock*, et al.*, "Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis," *Lancet,* vol. 310, pp. 263-267, 1977.

[145] S. M. Matsui, E. R. Mackow, S. Matsuno, P. S. Paul, and H. B. Greenberg, "Sequence analysis of gene 11 equivalents from" short" and" super short" strains of rotavirus," *J Virol,* vol. 64, pp. 120-124, 1990.

[146] P. G. Miotti, J. Eiden, and R. H. Yolken, "Comparative efficiency of commercial immunoassays for the diagnosis of rotavirus gastroenteritis during the course of infection," *J Clin Microbiol,* vol. 22, pp. 693-698, 1985.

[147] R. Moosai, R. Alcock, T. Bell, F. Laidler, J. Peiris, A. Wyn-Jones*, et al.*, "Detection of rotavirus by a latex agglutination test, Rotalex: comparison with electron microscopy, immunofluorescence, polyacrylamide gel electrophoresis, and enzyme linked immunosorbent assay," *J Clin Pathol,* vol. 38, pp. 694-700, 1985.

[148] R. Othman, M. Jaliha, N. Rasool, and S. Lam, "Asian group for rapid viral diagnosis-evaluation of two commercial kits for the detection of rotavirus in faecal samples," *Virus Information Exch. Newsl,* vol. 3, pp. 5-6, 1986.

[149] M. Sambourg, A. Goudeau, C. Courant, G. Pinon, and F. Denis, "Direct appraisal of latex agglutination testing, a convenient alternative to enzyme immunoassay for the detection of rotavirus in childhood gastroenteritis, by comparison of two enzyme immunoassays and two latex tests," *J Clin Microbiol,* vol. 21, pp. 622-625, 1985.

[150] A. Hasegawa, S. Matsuno, S. Inouye, R. Kono, Y. Tsurukubo, A. Mukoyama*, et al.*, "Isolation of human rotaviruses in primary cultures of monkey kidney cells," *J Clin Microbiol,* vol. 16, pp. 387-390, 1982.

[151] K. Sato, Y. Inaba, T. Shinozaki, R. Fujii, and M. Matumoto, "Isolation of human rotavirus in cell cultures," *Arch Virol,* vol. 69, pp. 155-160, 1981.

[152] R. L. Ward, J. D. Clemens, D. A. Sack, D. R. Knowlton, M. M. McNeal, N. Huda*, et al.*, "Culture adaptation and characterization of group A rotaviruses causing diarrheal illnesses in Bangladesh from 1985 to 1986," *J Clin Microbiol,* vol. 29, pp. 1915-1923, 1991.

[153] F. Morinet, F. Ferchal, R. Colimon, and Y. Perol, "Comparison of six methods for detecting human rotavirus in stools," *European J Clin Microbiol,* vol. 3, pp. 136-140, 1984.

[154] V. Gouvea, R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester*, et al.*, "Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens," *J Clin Microbiol,* vol. 28, pp. 276-82, 1990.

[155] J. R. Gentsch, R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores*, et al.*, "Identification of group A rotavirus gene 4 types by polymerase chain reaction," *J Clin Microbiol,* vol. 30, pp. 1365-73, 1992.

[156] M. Iturriza-Gomara, G. Kang, and J. Gray, "Rotavirus genotyping: keeping up with an evolving population of human rotaviruses," *J Clin Virol,* vol. 31, pp. 259-65, 2004.

[157] X. L. Pang, B. Lee, N. Boroumand, B. Leblanc, J. K. Preiksaitis, and C. C. Yu Ip, "Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea," *J Med Virol,* vol. 72, pp. 496-501, 2004.

[158] S. Ramani, P. Sankaran, R. Arumugam, R. Sarkar, I. Banerjee, I. Mohanty*, et al.*, "Comparison of viral load and duration of virus shedding in symptomatic and asymptomatic neonatal rotavirus infections," *J Med Virol,* vol. 82, pp. 1803-1807, 2010.

[159] G. Kang, M. Iturriza-Gomara, J. G. Wheeler, P. Crystal, B. Monica, S. Ramani*, et al.*, "Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: Correlation with clinical severity in children in South India," *J Med Virol,* vol. 73, pp. 118-122, 2004.

[160] S. Das, G. C. Sahoo, P. Das, U. K. Singh, A. K. Jaiswal, P. Singh*, et al.*, "Evaluating the Impact of Breastfeeding on Rotavirus Antigenemia and Disease Severity in Indian Children," *PloS One,* vol. 11, pp. e0146243, 2016.

[161] K. Bányai, B. László, J. Duque, A. D. Steele, E. A. S. Nelson, J. R. Gentsch*, et al.*, "Systematic review of regional and temporal trends in global rotavirus strain

diversity in the pre rotavirus vaccine era: insights for understanding the impact of rotavirus vaccination programs," *Vaccine,* vol. 30, pp. A122-A130, 2012.

[162] World Health Organization. Global Rotavirus Surveillance and Information Bulletin, vol. 10, pp. 1–4, 2015.

[163] J. Blackhall, A. Fuentes, and G. Magnusson, "Genetic stability of a porcine rotavirus RNA segment during repeated plaque isolation," *Virology,* vol. 225, pp. 181-190, 1996.

[164] M. Iturriza-Gomara, D. Cubitt, D. Steele, J. Green, D. Brown, G. Kang*, et al.*, "Characterisation of rotavirus G9 strains isolated in the UK between 1995 and 1998," *J Med Virol,* vol. 61, pp. 510-517, 2000.

[165] J. Matthijnssens and M. Van Ranst, "Genotype constellation and evolution of group A rotaviruses infecting humans," *Curr Opin Virol,* vol. 2, pp. 426-433, 2012.

[166] T. Nakagomi, Y. H. Doan, W. Dove, B. Ngwira, M. Iturriza-Gomara, O. Nakagomi*, et al.*, "G8 rotaviruses with conserved genotype constellations detected in Malawi over 10 years (1997-2007) display frequent gene reassortment among strains co-circulating in humans," *J Gen Virol,* vol. 94, pp. 1273-1295, 2013.

[167] J. Matthijnssens, M. Rahman, M. Ciarlet, M. Zeller, E. Heylen, T. Nakagomi*, et al.*, "Reassortment of human rotavirus gene segments into G11 rotavirus strains," *Emerg Infect Dis,* vol. 16, pp. 625-630, 2010.

[168] S. E. Midgley, C. K. Hjulsager, L. E. Larsen, G. Falkenhorst, and B. Bottiger, "Suspected zoonotic transmission of rotavirus group A in Danish adults," *Epidemiol Infect,* vol. 140, pp. 1013-1017, 2012.

[169] R. P. Maestri, J. H. Kaiano, D. L. Neri, S. Soares Lda, F. Guerra Sde, S. Oliveira Dde*, et al.*, "Phylogenetic analysis of probable non-human genes of group A rotaviruses isolated from children with acute gastroenteritis in Belem, Brazil," *J Med Virol,* vol. 84, pp. 1993-2002, 2012.

[170] N. Schnepf, C. Deback, A. Dehee, E. Gault, N. Parez, and A. Garbarg-Chenon, "Rearrangements of rotavirus genomic segment 11 are generated during acute

infection of immunocompetent children and do not occur at random," *J Virol,* vol. 82, pp. 3689-3696, 2008.

[171] F. Hundley, M. McIntyre, B. Clark, G. Beards, D. Wood, I. Chrystie*, et al.*, "Heterogeneity of genome rearrangements in rotaviruses isolated from a chronically infected immunodeficient child," *J Virol,* vol. 61, pp. 3365-3372, 1987.

[172] S. D. Chitambar, R. Arora, and P. Chhabra, "Molecular characterization of a rare G1P[19] rotavirus strain from India: evidence of reassortment between human and porcine rotavirus strains," *J Med Microbiol,* vol. 58, pp. 1611-1615, 2009.

[173] N. M. Abdel-Haq, R. A. Thomas, B. I. Asmar, V. Zacharova, and W. D. Lyman, "Increased prevalence of G1P[4] genotype among children with rotavirus-associated gastroenteritis in metropolitan Detroit," *J Clin Microbiol,* vol. 41, pp. 2680-2, 2003.

[174] S. Ghosh, N. Urushibara, M. Chawla-Sarkar, T. Krishnan, and N. Kobayashi, "Whole genomic analyses of asymptomatic human G1P[6], G2P[6] and G3P[6] rotavirus strains reveal intergenogroup reassortment events and genome segments of artiodactyl origin," *Infect Genet Evol,* vol. 16, pp. 165-173, 2013.

[175] M. I. Adah, A. Wade, and K. Taniguchi, "Molecular epidemiology of rotaviruses in Nigeria: detection of unusual strains with G2P [6] and G8P [1] specificities," *J Clin Microbiol,* vol. 39, pp. 3969-3975, 2001.

[176] H. F. Clark, D. Lawley, D. DiStefano, M. Maliga, B. Kilby, G. Kulnis*, et al.*, "An unusual outbreak of rotavirus genotype G2P[6] during the 2005-2006 epidemic season in Philadelphia," *Diagn Microbiol Infect Dis,* vol. 70, pp. 218-222, 2011.

[177] T. K. Fischer, J. Eugen-Olsen, A. G. Pedersen, K. Molbak, B. Bottiger, K. Rostgaard*, et al.*, "Characterization of rotavirus strains in a Danish population: high frequency of mixed infections and diversity within the VP4 gene of P[8] strains," *J Clin Microbiol,* vol. 43, pp. 1099-1104, 2005.

[178] S. Ghosh, Z. Gatheru, J. Nyangao, N. Adachi, N. Urushibara, and N. Kobayashi, "Full genomic analysis of a simian SA11-like G3P[2] rotavirus strain isolated

from an asymptomatic infant: identification of novel VP1, VP6 and NSP4 genotypes," *Infect Genet Evol,* vol. 11, pp. 57-63, 2011.

[179] P. Khamrin, N. Maneekarn, S. Peerakome, F. Yagyu, S. Okitsu, and H. Ushijima, "Molecular characterization of a rare G3P[3] human rotavirus reassortant strain reveals evidence for multiple human-animal interspecies transmissions," *J Med Virol,* vol. 78, pp. 986-994, 2006.

[180] A. Luchs, A. Cilli, S. G. Morillo, C. Carmona Rde, and C. Timenetsky Mdo, "Rare G3P[3] rotavirus strain detected in Brazil: possible human-canine interspecies transmission," *J Clin Virol,* vol. 54, pp. 89-92, 2012.

[181] L. Grant, M. Esona, J. Gentsch, J. Watt, R. Reid, R. Weatherholtz*, et al.*, "Detection of G3P[3] and G3P[9] rotavirus strains in American Indian children with evidence of gene reassortment between human and animal rotaviruses," *J Med Virol,* vol. 83, pp. 1288-1299, 2011.

[182] P. Khamrin, N. Maneekarn, S. Peerakome, S. Tonusin, T. G. Phan, S. Okitsu*, et al.*, "Molecular characterization of rare G3P[9] rotavirus strains isolated from children hospitalized with acute gastroenteritis," *J Med Virol,* vol. 79, pp. 843-851, 2007.

[183] K. Khananurak, V. Vutithanachot, N. Simakachorn, A. Theamboonlers, V. Chongsrisawat, and Y. Poovorawan, "Prevalence and phylogenetic analysis of rotavirus genotypes in Thailand between 2007 and 2009," *Infect Genet Evol,* vol. 10, pp. 537-545, 2010.

[184] L. S. Ch'ng, W. S. Lee, and C. D. Kirkwood, "Rare rotavirus strains in children with severe diarrhea, Malaysia," *Emerg Infect Dis,* vol. 17, pp. 948-950, 2011.

[185] Y. H. Wang, B. B. Pang, X. Zhou, S. Ghosh, W. F. Tang, J. S. Peng*, et al.*, "Complex evolutionary patterns of two rare human G3P[9] rotavirus strains possessing a feline/canine-like H6 genotype on an AU-1-like genotype constellation," *Infect Genet Evol,* vol. 16, pp. 103-112, 2013.

[186] T. G. Phan, Q. D. Trinh, P. Khamrin, K. Kaneshi, Y. Ueda, S. Nakaya*, et al.*, "Emergence of new variant rotavirus G3 among infants and children with acute gastroenteritis in Japan during 2003-2004," *Clin Lab,* vol. 53, pp. 41-48, 2007.

[187] A. Mukherjee, S. Mullick, N. Kobayashi, and M. Chawla-Sarkar, "The first identification of rare human group A rotavirus strain G3P[10] with severe infantile diarrhea in eastern India," *Infect Genet Evol,* vol. 12, pp. 1933-1937, 2012.

[188] P. Khamrin, N. Maneekarn, S. Peerakome, R. Malasao, A. Thongprachum, W. Chan-It*, et al.*, "Molecular characterization of VP4, VP6, VP7, NSP4, and NSP5/6 genes identifies an unusual G3P[10] human rotavirus strain," *J Med Virol,* vol. 81, pp. 176-182, 2009.

[189] A. Theamboonlers, P. Bhattarakosol, V. Chongsrisawat, T. Sungkapalee, N. Wutthirattanakowit, and Y. Poovorawan, "Molecular characterization of group A human rotaviruses in Bangkok and Buriram, Thailand during 2004-2006 reveals the predominance of G1P[8], G9P[8] and a rare G3P[19] strain," *Virus Genes,* vol. 36, pp. 289-298, 2008.

[190] F. T. Wu, K. Banyai, J. C. Huang, H. S. Wu, F. Y. Chang, C. A. Hsiung*, et al.*, "Human infection with novel G3P[25] rotavirus strain in Taiwan," *Clin Microbiol Infect,* vol. 17, pp. 1570-1573, 2011.

[191] A. Mukherjee, S. Ghosh, P. Bagchi, D. Dutta, S. Chattopadhyay, N. Kobayashi*, et al.*, "Full genomic analyses of human rotavirus G4P[4], G4P[6], G9P[19] and G10P[6] strains from North-eastern India: evidence for interspecies transmission and complex reassortment events," *Clin Microbiol Infect,* vol. 17, pp. 1343-1346, 2011.

[192] V. Gouvea, L. de Castro, M. C. Timenetsky, H. Greenberg, and N. Santos, "Rotavirus serotype G5 associated with diarrhea in Brazilian children," *J Clin Microbiol,* vol. 32, pp. 1408-1409, 1994.

[193] J. P. Leite, A. A. Alfieri, P. A. Woods, R. I. Glass, and J. R. Gentsch, "Rotavirus G and P types circulating in Brazil: characterization by RT-PCR, probe hybridization, and sequence analysis," *Arch Virol,* vol. 141, pp. 2365-2374, 1996.

[194] K. Bok, N. Castagnaro, A. Borsa, S. Nates, C. Espul, O. Fay*, et al.*, "Surveillance for rotavirus in Argentina," *J Med Virol,* vol. 65, pp. 190-198, 2001.

[195] N. Coluchi, V. Munford, J. Manzur, C. Vazquez, M. Escobar, E. Weber, *et al.*, "Detection, subgroup specificity, and genotype diversity of rotavirus strains in children with acute diarrhea in Paraguay," *J Clin Microbiol,* vol. 40, pp. 1709-1714, 2002.

[196] M. D. Esona, G. E. Armah, A. Geyer, and A. D. Steele, "Detection of an unusual human rotavirus strain with G5P[8] specificity in a Cameroonian child with diarrhea," *J Clin Microbiol,* vol. 42, pp. 441-444, 2004.

[197] D. D. Li, Z. J. Duan, Q. Zhang, N. Liu, Z. P. Xie, B. Jiang, *et al.*, "Molecular characterization of unusual human G5P[6] rotaviruses identified in China," *J Clin Virol,* vol. 42, pp. 141-148, 2008.

[198] Z. Mladenova, H. Papp, G. Lengyel, P. Kisfali, A. Steyer, A. F. Steyer, *et al.*, "Detection of rare reassortant G5P[6] rotavirus, Bulgaria," *Infect Genet Evol,* vol. 12, pp. 1676-1684, 2012.

[199] J. Nordgren, L. W. Nitiema, S. Sharma, D. Ouermi, A. S. Traore, J. Simpore, *et al.*, "Emergence of unusual G6P [6] rotaviruses in children, Burkina Faso, 2009-2010," *Emerg Infect Dis,* vol. 18, 2012.

[200] S. Mullick, A. Mukherjee, S. Ghosh, G. P. Pazhani, D. Sur, B. Manna, *et al.*, "Genomic analysis of human rotavirus strains G6P[14] and G11P[25] isolated from Kolkata in 2009 reveals interspecies transmission and complex reassortment events," *Infect Genet Evol,* vol. 14, pp. 15-21, 2013.

[201] G. F. Browning, D. R. Snodgrass, O. Nakagomi, E. Kaga, A. Sarasini, and G. Gerna, "Human and bovine serotype G8 rotaviruses may be derived by reassortment," *Arch Virol,* vol. 125, pp. 121-128, 1992.

[202] S. Todd, N. A. Page, A. Duncan Steele, I. Peenze, and N. A. Cunliffe, "Rotavirus strain types circulating in Africa: Review of studies published during 1997-2006," *J Infect Dis,* vol. 202 Suppl, pp. S34-S42, 2010.

[203] M. Iturriza-Gomara, T. Dallman, K. Banyai, B. Bottiger, J. Buesa, S. Diedrich, *et al.*, "Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined by EuroRotaNet, a pan-European collaborative strain surveillance network," *Epidemiol Infect,* vol. 139, pp. 895-909, 2011.

[204] G. A. Weinberg, D. C. Payne, E. N. Teel, S. Mijatovic-Rustempasic, M. D. Bowen, M. Wikswo*, et al.*, "First reports of human rotavirus G8P[4] gastroenteritis in the United States," *J Clin Microbiol,* vol. 50, pp. 1118-1121, 2012.

[205] A. Mukherjee, S. Mullick, A. K. Deb, S. Panda, and M. Chawla-Sarkar, "First report of human rotavirus G8P[4] gastroenteritis in India: evidence of ruminants-to-human zoonotic transmission," *J Med Virol,* vol. 85, pp. 537-545, 2013.

[206] V. P. Le, J. Y. Kim, S. L. Cho, S. W. Nam, I. Lim, H. J. Lee*, et al.*, "Detection of unusual rotavirus genotypes G8P[8] and G12P[6] in South Korea," *J Med Virol,* vol. 80, pp. 175-182, 2008.

[207] H. F. Clark, Y. Hoshino, L. M. Bell, J. Groff, G. Hess, P. Bachman*, et al.*, "Rotavirus isolate WI61 representing a presumptive new human serotype," *J Clin Microbiol,* vol. 25, pp. 1757-1762, 1987.

[208] S. Arista, E. Vizzi, M. C. Migliore, E. Di Rosa, and A. Cascio, "High incidence of G9P181 rotavirus infections in Italian children during the winter season 1999-2000," *Eur J Epidemiol,* vol. 18, pp. 711-714, 2003.

[209] P. Khamrin, S. Peerakome, L. Wongsawasdi, S. Tonusin, P. Sornchai, V. Maneerat*, et al.*, "Emergence of human G9 rotavirus with an exceptionally high frequency in children admitted to hospital with diarrhea in Chiang Mai, Thailand," *J Med Virol,* vol. 78, pp. 273-280, 2006.

[210] L. Ribas Mde, S. Nagashima, A. Calzado, G. Acosta, Y. Tejero, Y. Cordero*, et al.*, "Emergence of G9 as a predominant genotype of human rotaviruses in Cuba," *J Med Virol,* vol. 83, pp. 738-744, 2011.

[211] L. E. Esteban, R. P. Rota, J. R. Gentsch, B. Jiang, M. Esona, R. I. Glass*, et al.*, "Molecular epidemiology of group A rotavirus in Buenos Aires, Argentina 2004-2007: reemergence of G2P[4] and emergence of G9P[8] strains," *J Med Virol,* vol. 82, pp. 1083-1093, 2010.

[212] V. Martella, V. Terio, G. Del Gaudio, M. Gentile, P. Fiorente, S. Barbuti*, et al.*, "Detection of the emerging rotavirus G9 serotype at high frequency in Italy," *J Clin Microbiol,* vol. 41, pp. 3960-3963, 2003.

[213] J. Diez-Domingo, J. M. Baldo, M. Patrzalek, P. Pazdiora, J. Forster, L. Cantarutti, *et al.*, "Primary care-based surveillance to estimate the burden of rotavirus gastroenteritis among children aged less than 5 years in six European countries," *Eur J Pediatr,* vol. 170, pp. 213-222, 2011.

[214] A. Mukherjee, D. Dutta, S. Ghosh, P. Bagchi, S. Chattopadhyay, S. Nagashima, *et al.*, "Full genomic analysis of a human group A rotavirus G9P[6] strain from Eastern India provides evidence for porcine-to-human interspecies transmission," *Arch Virol,* vol. 154, pp. 733-746, 2009.

[215] M. Zeller, E. Heylen, S. De Coster, M. Van Ranst, and J. Matthijnssens, "Full genome characterization of a porcine-like human G9P[6] rotavirus strain isolated from an infant in Belgium," *Infect Genet Evol,* vol. 12, pp. 1492-1500, 2012.

[216] S. Ghosh, N. Urushibara, K. Taniguchi, and N. Kobayashi, "Whole genomic analysis reveals the porcine origin of human G9P[19] rotavirus strains Mc323 and Mc345," *Infect Genet Evol,* vol. 12, pp. 471-477, 2012.

[217] G. E. Armah, Y. Hoshino, N. Santos, F. Binka, S. Damanka, R. Adjei, *et al.*, "The global spread of rotavirus G10 strains: detection in Ghanaian children hospitalized with diarrhea," *J Infect Dis,* vol. 202, pp. S231-S238, 2010.

[218] S. Ramani, M. Iturriza-Gomara, A. K. Jana, K. A. Kuruvilla, J. J. Gray, D. W. Brown, *et al.*, "Whole genome characterization of reassortant G10P[11] strain (N155) from a neonate with symptomatic rotavirus infection: identification of genes of human and animal rotavirus origin," *J Clin Virol,* vol. 45, pp. 237-244, 2009.

[219] M. D. Esona, K. Banyai, K. Foytich, M. Freeman, S. Mijatovic-Rustempasic, J. Hull, *et al.*, "Genomic characterization of human rotavirus G10 strains from the African Rotavirus Network: relationship to animal rotaviruses," *Infect Genet Evol,* vol. 11, pp. 237-241, 2011.

[220] A. Mukherjee, M. K. Nayak, T. Roy, S. Ghosh, T. N. Naik, N. Kobayashi, *et al.*, "Detection of human G10 rotavirus strains with similarity to bovine and bovine-like equine strains from untypable samples," *Infect Genet Evol,* vol. 12, pp. 467-470, 2012.

[221] Y. Matsushima, E. Nakajima, T. A. Nguyen, H. Shimizu, A. Kano, Y. Ishimaru*, et al.*, "Genome sequence of an unusual human G10P[8] rotavirus detected in Vietnam," *J Virol,* vol. 86, pp. 10236-10237, 2012.

[222] M. Rahman, J. Matthijnssens, S. Nahar, G. Podder, D. A. Sack, T. Azim*, et al.*, "Characterization of a novel P[25],G11 human group a rotavirus," *J Clin Microbiol,* vol. 43, pp. 3208-3212, 2005.

[223] J. O. Shim, I. H. Baek, V. P. Le, E. M. Ko, W. S. Seok, Y. Uh*, et al.*, "Molecular characterization of rotavirus diarrhea among children in South Korea: detection of an unusual G11 strain," *Arch Virol,* vol. 156, pp. 887-892, 2011.

[224] S. K. Hong, S. G. Lee, S. A. Lee, J. H. Kang, J. H. Lee, J. H. Kim*, et al.*, "Characterization of a G11,P[4] strain of human rotavirus isolated in South Korea," *J Clin Microbiol,* vol. 45, pp. 3759-3761, 2007.

[225] K. Taniguchi, T. Urasawa, N. Kobayashi, M. Gorziglia, and S. Urasawa, "Nucleotide sequence of VP4 and VP7 genes of human rotaviruses with subgroup I specificity and long RNA pattern: implication for new G serotype specificity," *J Virol,* vol. 64, pp. 5640-5664, 1990.

[226] M. Rahman, J. Matthijnssens, X. Yang, T. Delbeke, I. Arijs, K. Taniguchi*, et al.*, "Evolutionary history and global spread of the emerging g12 human rotaviruses," *J Virol,* vol. 81, pp. 2382-2390, 2007.

[227] P. A. Kokkinos, P. G. Ziros, M. Monini, P. Lampropoulou, A. Karampini, E. Papachatzi*, et al.*, "Rare types of rotaviruses isolated from children with acute gastroenteritis in Patras, Greece," *Intervirology,* vol. 56, pp. 237-241, 2013.

[228] A. A. Castello, T. Nakagomi, O. Nakagomi, B. Jiang, J. O. Kang, R. I. Glass*, et al.*, "Characterization of genotype P[9]G12 rotavirus strains from Argentina: high similarity with Japanese and Korean G12 strains," *J Med Virol,* vol. 81, pp. 371-381, 2009.

[229] Y. Pongsuwanna, R. Guntapong, M. Chiwakul, R. Tacharoenmuang, N. Onvimala, M. Wakuda*, et al.*, "Detection of a human rotavirus with G12 and P[9] specificity in Thailand," *J Clin Microbiol,* vol. 40, pp. 1390-1394, 2002.

[230] J. J. Hull, E. N. Teel, T. K. Kerin, M. M. Freeman, M. D. Esona, J. R. Gentsch*, et al.*, "United States rotavirus strain surveillance from 2005 to 2008: genotype prevalence before and after vaccine introduction," *Pediatr Infect Dis J,* vol. 30, pp. S42-S47, 2011.

[231] Australian Rotavirus Surveillance Program annual report, 2005/2006, 2006/2007, 2007/2008, 2008/2009, 2009/2010, 2010/2011. Communicable diseases intelligence quarterly report. 2006-2011.

[232] M. Zeller, M. Rahman, E. Heylen, S. De Coster, S. De Vos, I. Arijs*, et al.*, "Rotavirus incidence and genotype distribution before and after national rotavirus vaccine introduction in Belgium," *Vaccine,* vol. 28, pp. 7507-7513, 2010.

[233] J. R. Gentsch, J. J. Hull, E. N. Teel, T. K. Kerin, M. M. Freeman, M. D. Esona*, et al.*, "G and P types of circulating rotavirus strains in the United States during 1996-2005: nine years of prevaccine data," *J Infect Dis,* vol. 1, pp. S99-S105, 2009.

[234] J. P. Leite, F. A. Carvalho-Costa, and A. C. Linhares, "Group A rotavirus genotypes and the ongoing Brazilian experience: a review," *Mem Inst Oswaldo Cruz,* vol. 103, pp. 745-753, 2008.

[235] S. M. McDonald and J. T. Patton, "Assortment and packaging of the segmented rotavirus genome," *Trends Microbiol,* vol. 19, pp. 136-144, 2011.

[236] F. A. Carvalho-Costa, M. Volotao Ede, R. M. de Assis, A. M. Fialho, S. de Andrade Jda, L. N. Rocha*, et al.*, "Laboratory-based rotavirus surveillance during the introduction of a vaccination program, Brazil, 2005-2009," *Pediatr Infect Dis J,* vol. 30, 2011.

[237] G. A. Weinberg, E. N. Teel, S. Mijatovic-Rustempasic, D. C. Payne, S. Roy, K. Foytich*, et al.*, "Detection of novel rotavirus strain by vaccine postlicensure surveillance," *Emerg Infect Dis,* vol. 19, pp. 1321-1323, 2013.

[238] C. D. Kirkwood, K. Boniface, G. L. Barnes, and R. F. Bishop, "Distribution of rotavirus genotypes after introduction of rotavirus vaccines, Rotarix(R) and RotaTeq(R), into the National Immunization Program of Australia," *Pediatr Infect Dis J,* vol. 30, pp. S48-S53, 2011.

[239] R. Q. Gurgel, L. E. Cuevas, S. C. Vieira, V. C. Barros, P. B. Fontes, E. F. Salustino*, et al.*, "Predominance of rotavirus P[4]G2 in a vaccinated population, Brazil," *Emerg Infect Dis,* vol. 13, pp. 1571-1573, 2007.

[240] W. Atkinson, J. Hamborsky, and L. McIntyre "Epidemiology and Prevention of Vaccine-Preventable Diseases.," *Centers for Disease Control and Prevention*, Public Health Foundation, pp. 295-306, 2007.

[241] M. A. Staat, P. H. Azimi, T. Berke, N. Roberts, D. I. Bernstein, R. L. Ward*, et al.*, "Clinical presentations of rotavirus infection among hospitalized children," *Pediatr Infect Dis J,* vol. 21, pp. 221-227, 2002.

[242] W. J. Rodriguez, H. W. Kim, C. D. Brandt, R. H. Schwartz, M. K. Gardner, B. Jeffries*, et al.*, "Longitudinal study of rotavirus infection and gastroenteritis in families served by a pediatric medical practice: clinical and epidemiologic observations," *Pediatr Infect Dis J,* vol. 6, pp. 170-176, 1987.

[243] F. R. Velazquez, J. J. Calva, M. L. Guerrero, D. Mass, R. I. Glass, L. K. Pickering*, et al.*, "Cohort study of rotavirus serotype patterns in symptomatic and asymptomatic infections in Mexican children," *Pediatr Infect Dis J,* vol. 12, pp. 54-61, 1993.

[244] W. J. Rodriguez, H. W. Kim, J. O. Arrobio, C. D. Brandt, R. M. Chanock, A. Z. Kapikian*, et al.*, "Clinical features of acute gastroenteritis associated with human reovirus-like agent in infants and young children," *J Pediatr,* vol. 91, pp. 188-193, 1977.

[245] H.-G. H. Chang, R. I. Glass, P. F. Smith, H. G. Cicirello, R. C. Holman, and D. L. Morse, "Disease burden and risk factors for hospitalizations associated with rotavirus infection among children in New York State, 1989 through 2000," *Pediatr Infect Dis J,* vol. 22, pp. 808-814, 2003.

[246] E. S. Bass, D. A. Pappano, and S. G. Humiston, "Rotavirus," *Pediatr Rev,* vol. 28, p. 183, 2007.

[247] R. I. Glass, U. Parashar, M. Patel, J. Gentsch, and B. Jiang, "Rotavirus vaccines: successes and challenges," *J Infect,* vol. 68, pp. S9-S18, 2014.

[248] S. E. Blutt, C. D. Kirkwood, V. Parreño, K. L. Warfield, M. Ciarlet, M. K. Estes, *et al.*, "Rotavirus antigenaemia and viraemia: a common event?," *Lancet,* vol. 362, pp. 1445-1449, 2003.

[249] M. Patel, M. A. Rench, J. A. Boom, J. E. Tate, L. C. Sahni, J. A. Hull, *et al.*, "Detection of rotavirus antigenemia in routinely obtained serum specimens to augment surveillance and vaccine effectiveness evaluations," *Pediatr Infect Dis J,* vol. 29, pp. 836-839, 2010.

[250] L. Maureen, L. Brian, A. Parvin, J. Gentsch, C. Glaser, S. Gilliam, *et al.*, "Rotavirus and central nervous system symptoms: cause or contaminant? Case reports and review," *Clin Infect Dis,* vol. 33, pp. 932-938, 2001.

[251] I. Oishi, T. Kimura, T. Murakami, K. Haruki, K. Yamazaki, Y. Seto, *et al.*, "Serial observations of chronic rotavirus infection in an immunodeficient child," *Microbiol Immunol,* vol. 35, pp. 953-961, 1991.

[252] F. T. Saulsbury, J. A. Winkelstein, and R. H. Yolken, "Chronic rotavirus infection in immunodeficiency," *J Pediatr,* vol. 97, pp. 61-65, 1980.

[253] C. Williams, A. Lobanov, and R. Pebody, "Estimated mortality and hospital admission due to rotavirus infection in the WHO European region," *Epidemiol Infect,* vol. 137, pp. 607-616, 2009.

[254] S. Ramani, T. V. Sowmyanarayanan, B. P. Gladstone, K. Bhowmick, J. R. Asirvatham, A. K. Jana, *et al.*, "Rotavirus infection in the neonatal nurseries of a tertiary care hospital in India," *Pediatr Infect Dis J,* vol. 27, pp. 719-723, 2008.

[255] L. Bodhidatta, N. T. P. Lan, B. T. Hien, N. V. Lai, A. Srijan, O. Serichantalergs, *et al.*, "Rotavirus disease in young children from Hanoi, Vietnam," *Pediatr Infect Dis J,* vol. 26, pp. 325-328, 2007.

[256] J. Mrukowicz, H. Szajewska, and T. Vesikari, "Options for the prevention of rotavirus disease other than vaccination," *J Pediatr Gastroenterol Nutr,* vol. 46, pp. S32-S37, 2008.

[257] F. R. Velazquez, D. O. Matson, J. J. Calva, L. Guerrero, A. L. Morrow, S. Carter-Campbell, *et al.*, "Rotavirus infections in infants as protection against subsequent infections," *N Engl J Med,* vol. 335, pp. 1022-1028, 1996.

[258] J. Joensuu, E. Koskenniemi, X.-L. Pang, and T. Vesikari, "Randomised placebo-controlled trial of rhesus-human reassortant rotavirus vaccine for prevention of severe rotavirus gastroenteritis," *Lancet,* vol. 350, pp. 1205-1209, 1997.

[259] I. Perez-Schael, M. J. Guntiñas, M. Pérez, V. Pagone, A. M. Rojas, R. González, *et al.*, "Efficacy of the rhesus rotavirus–based quadrivalent vaccine in infants and young children in Venezuela," *N Engl J Med,* vol. 337, pp. 1181-1187, 1997.

[260] M. B. Rennels, R. I. Glass, P. H. Dennehy, D. I. Bernstein, M. E. Pichichero, E. T. Zito, *et al.*, "Safety and efficacy of high-dose rhesus-human reassortant rotavirus vaccines—report of the National Multicenter Trial," *Pediatrics,* vol. 97, pp. 7-13, 1996.

[261] M. Santosham, L. H. Moulton, R. Reid, J. Croll, R. Weatherholt, R. Ward, *et al.*, "Efficacy and safety of high-dose rhesus-human reassortant rotavirus vaccine in Native American populations," *J Pediatr,* vol. 131, pp. 632-638, 1997.

[262] G. Peter and M. G. Myers, "Intussusception, rotavirus, and oral vaccines: summary of a workshop," *Pediatrics,* vol. 110, pp. e67-e67, 2002.

[263] T. V. Murphy, P. M. Gargiullo, M. S. Massoudi, D. B. Nelson, A. O. Jumaan, C. A. Okoro, *et al.*, "Intussusception among infants given an oral rotavirus vaccine," *N Engl J Med* vol. 344, pp. 564-572, 2001.

[264] P. Kramarz, E. K. France, F. Destefano, S. B. Black, H. Shinefield, J. I. Ward, *et al.*, "Population-based study of rotavirus vaccination and intussusception," *Pediatr Infect Dis J,* vol. 20, pp. 410-416, 2001.

[265] T. Vesikari, D. O. Matson, P. Dennehy, P. Van Damme, M. Santosham, Z. Rodriguez, *et al.*, "Safety and efficacy of a pentavalent human–bovine (WC3) reassortant rotavirus vaccine," *N Engl J Med,* vol. 354, pp. 23-33, 2006.

[266] U. D. Parashar, J. P. Alexander, R. I. Glass, and A. C. o. I. Practices, "Prevention of rotavirus gastroenteritis among infants and children," *Recommendations of the*

Advisory Committee on Immunization Practices (ACIP) MMWR Recomm Rep, vol. 55, pp. 1-13, 2006.

[267] P. H. Dennehy, "Rotavirus vaccines: an overview," *Clin Microbiol Rev,* vol. 21, pp. 198-208, 2008.

[268] D. I. Bernstein, D. A. Sack, E. Rothstein, K. Reisinger, V. E. Smith, D. O'Sullivan*, et al.*, "Efficacy of live, attenuated, human rotavirus vaccine 89–12 in infants: a randomised placebo-controlled trial," *Lancet,* vol. 354, pp. 287-290, 1999.

[269] G. M. Ruiz-Palacios, I. Perez-Schael, F. R. Velazquez, H. Abate, T. Breuer, S. C. Clemens*, et al.*, "Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis," *N Engl J Med,* vol. 354, pp. 11-22, 2006.

[270] T. Vesikari, A. Karvonen, R. Prymula, V. Schuster, J. C. Tejedor, R. Cohen*, et al.*, "Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study," *Lancet,* vol. 370, pp. 1757-1763, 2007.

[271] N. Bhandari, T. Rongsen-Chandola, A. Bavdekar, J. John, K. Antony, S. Taneja*, et al.*, "Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial," *Lancet,* vol. 383, pp. 2136-2143, 2014.

[272] M. Bhan, J. F. Lew, S. Sazawal, B. K. Das, J. R. Gentsch, and R. I. Glass, "Protection conferred by neonatal rotavirus infection against subsequent rotavirus diarrhea," *J Infect Dis,* vol. 168, pp. 282-287, 1993.

[273] N. Bhandari, P. Sharma, R. I. Glass, P. Ray, H. Greenberg, S. Taneja*, et al.*, "Safety and immunogenicity of two live attenuated human rotavirus vaccine candidates, 116E and I321, in infants: results of a randomised controlled trial," *Vaccine,* vol. 24, pp. 5817-5823, 2006.

[274] N. Bhandari, P. Sharma, S. Taneja, T. Kumar, T. Rongsen-Chandola, M. B. Appaiahgari*, et al.*, "A dose-escalation safety and immunogenicity study of live attenuated oral rotavirus vaccine 116E in infants: a randomized, double-blind, placebo-controlled trial," *J Infect Dis,* vol. 200, pp. 421-429, 2009.

[275] A. Kapikian, R. Wyatt, M. Levine, R. Yolken, D. VanKirk, R. Dolin*, et al.*, "Oral administration of human rotavirus to volunteers: induction of illness and correlates of resistance," *J Infect Dis,* vol. 147, pp. 95-106, 1983.

[276] R. L. Ward, D. I. Bernstein, E. C. Young, J. R. Sherwood, D. R. Knowlton, and G. M. Schiff, "Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection," *J Infect Dis,* vol. 154, pp. 871-880, 1986.

[277] R. L. Ward, D. I. Bernstein, R. Shukla, M. M. McNeal, J. R. Sherwood, E. C. Young*, et al.*, "Protection of adults rechallenged with a human rotavirus," *J Infect Dis,* vol. 161, pp. 440-445, 1990.

[278] J. Jewkes, H. Larson, A. Price, P. Sanderson, and H. Davies, "Aetiology of acute diarrhoea in adults," *Gut,* vol. 22, pp. 388-392, 1981.

[279] J. Loosli, K. Gyr, H. Stalder, G. Stalder, W. Vischer, J. Voegtlin*, et al.*, "Etiology of acute infectious diarrhea in a highly industrialized area of Switzerland," *Gastroenterology,* vol. 88, pp. 75-79, 1985.

[280] B. Svenungsson, Å. Lagergren, E. Ekwall, B. Evengård, K. O. Hedlund, A. Kärnell*, et al.*, "Enteropathogens in adult patients with diarrhea and healthy control subjects: a 1-year prospective study in a Swedish clinic for infectious diseases," *Clin Infect Dis,* vol. 30, pp. 770-778, 2000.

[281] P. Echeverria, N. Blacklow, G. Cukor, S. Vibulbandhitkit, S. Changchawalit, and P. Boonthai, "Rotavirus as a cause of severe gastroenteritis in adults," *J Clin Microbiol,* vol. 18, pp. 663-667, 1983.

[282] J. S. Koopman and A. S. Monto, "The tecumseh study XV: Rotavirus infection and pathogenicity," *Am J Epidemiol,* vol. 130, pp. 750-759, 1989.

[283] W. Pryor, W. Bye, D. Curran, and G. Grohmann, "Acute diarrhoea in adults: a prospective study," *Med J Aust,* vol. 147, pp. 490-493, 1987.

[284] B. A. Oyofo, D. Subekti, P. Tjaniadi, N. Machpud, S. Komalarini, B. Setiawan*, et al.*, "Enteropathogens associated with acute diarrhea in community and hospital

patients in Jakarta, Indonesia," *FEMS Immunol Med Microbiol,* vol. 34, pp. 139-146, 2002.

[285] M. A. del Refugio González-Losa, G. G. Polanco-Marín, L. Manzano-Cabrera, and M. n. Puerto-Solís, "Acute gastroenteritis associated with rotavirus in adults," *Arch Med Res,* vol. 32, pp. 164-167, 2001.

[286] S. Foster, E. Palmer, G. Gary, M. Martin, K. Herrmann, P. Beasley*, et al.*, "Gastroenteritis due to rotavirus in an isolated Pacific island group: an epidemic of 3,439 cases," *J Infect Dis,* vol. 141, pp. 32-39, 1980.

[287] O. Meurman and M. Laine, "Rotavirus epidemic in adults," *N Engl J Med,* vol. 296, pp. 1298-1299, 1977.

[288] M. G. Friedman, A. Galil, B. Sarov, M. Margalith, G. Katzir, K. Midthun*, et al.*, "Tho Sequential Outbreaks of Rotavirus Gastroenteritis: Evidence for Symptomatic and Asymptomatic Reinfections," *J Infect Dis,* vol. 158, pp. 814-822, 1988.

[289] R. Bolivar, R. H. Conklin, J. J. Vollet, L. K. Pickering, H. L. DuPont, D. L. Walters*, et al.*, "Rotavirus in travelers' diarrhea: study of an adult student population in Mexico," *J Infect Dis,* vol. 137, pp. 324-327, 1978.

[290] J. J. Vollet, C. D. Ericsson, G. Gibson, L. K. Pickering, H. L. Dupont, S. Kohl*, et al.*, "Human rotavirus in an adult population with travelers' diarrhea and its relationship to the location of food consumption," *J Med Virol,* vol. 4, pp. 81-87, 1979.

[291] B. Keswick, N. Blacklow, G. Cukor, H. DuPont, and J. Vollet, "Norwalk Virus and Rotavirus in Travellers' diarrhoea in Mexico," *Lancet,* vol. 319, pp. 109-110, 1982.

[292] R. Ryder, C. Oquist, H. Greenberg, D. Taylor, F. Ørskov, I. Ørskov*, et al.*, "Travelers' diarrhea in Panamanian tourists in Mexico," *J Infect Dis,* vol. 144, pp. 442-448, 1981.

[293] J. F. Sheridan, L. Aurelian, G. Barbour, M. Santosham, R. B. Sack, and R. Ryder, "Traveler's diarrhea associated with rotavirus infection: analysis of virus-specific immunoglobulin classes," *Infect Immun,* vol. 31, pp. 419-429, 1981.

[294] A. Z. Kapikian, R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock, "Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis," *J Virol,* vol. 10, pp. 1075-1081, 1972.

[295] J. Zahorsky, "Hyperemesis hiemis or the winter vomiting disease," *Arch Pediatr,* vol. 46, pp. 391-395, 1929.

[296] R. Goodgame, "Norovirus gastroenteritis," *Curr Gastroenterol Rep,* vol. 8, pp. 401-408, 2006.

[297] M.-A. Widdowson, S. S. Monroe, and R. I. Glass, "Are noroviruses emerging," *Emerg Infect Dis,* vol. 11, pp. 735-737, 2005.

[298] S. M. Ahmed, A. J. Hall, A. E. Robinson, L. Verhoef, P. Premkumar, U. D. Parashar*, et al.*, "Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis," *Lancet Infect Dis,* vol. 14, pp. 725-730, 2014.

[299] X. Jiang, M. Wang, K. Wang, and M. K. Estes, "Sequence and genomic organization of Norwalk virus," *Virology,* vol. 195, pp. 51-61, 1993.

[300] L. G. Thorne and I. G. Goodfellow, "Norovirus gene expression and replication," *J Gen Virol,* vol. 95, pp. 278-291, 2014.

[301] G. Belliot, S. V. Sosnovtsev, T. Mitra, C. Hammer, M. Garfield, and K. Y. Green, "In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells," *J Virol,* vol. 77, pp. 10957-10974, 2003.

[302] S. Green, K. Dingle, P. Lambden, E. Caul, C. Ashley, and I. Clarke, "Human enteric Caliciviridae; a new prevalent SRSV group defined by RNA-dependent RNA polymerase and capsid diversity," *J. Gen. Virol,* vol. 75, pp. 1883-1888, 1994.

[303] K. Green, R. Chanock, and A. Kapikian, "Human caliciviruses," *Fields virology,* vol. 1, pp. 841-874, 2001.

[304] E. Robilotti, S. Deresinski, and B. A. Pinsky, "Norovirus," *Clin Microbiol Rev,* vol. 28, pp. 134-64, 2015.

[305] S. G. Morillo and M. d. C. S. T. Timenetsky, "Norovirus: an overview," *Rev Assoc Med Bras,* vol. 57, pp. 462-467, 2011.

[306] C. I. Gallimore, J. Green, D. Lewis, A. F. Richards, B. A. Lopman, A. D. Hale*, et al.*, "Diversity of noroviruses cocirculating in the north of England from 1998 to 2001," *J Clin Microbiol,* vol. 42, pp. 1396-1401, 2004.

[307] L. Verhoef, J. Hewitt, L. Barclay, S. M. Ahmed, R. Lake, A. J. Hall*, et al.*, "Norovirus genotype profiles associated with foodborne transmission, 1999-2012," *Emerg Infect Dis,* vol. 21, pp. 592-9, 2015.

[308] A. W. Mounts, T. Ando, M. Koopmans, J. S. Bresee, J. Noel, and R. I. Glass, "Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses," *J Infect Dis,* vol. 181, pp. S284-S287, 2000.

[309] B. Rockx, M. de Wit, H. Vennema, J. Vinjé, E. de Bruin, Y. van Duynhoven*, et al.*, "Natural history of human calicivirus infection: a prospective cohort study," *Clin Infect Dis,* vol. 35, pp. 246-253, 2002.

[310] B. A. Lopman, M. H. Reacher, I. B. Vipond, J. Sarangi, and D. W. Brown, "Clinical manifestation of norovirus gastroenteritis in health care settings," *Clin Infect Dis,* vol. 39, pp. 318-324, 2004.

[311] J. E. Kaplan, R. Feldman, D. S. Campbell, C. Lookabaugh, and G. W. Gary, "The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis," *Am J Public Health,* vol. 72, pp. 1329-1332, 1982.

[312] R. G. Wyatt, R. Dolin, N. R. Blacklow, H. L. DuPont, R. F. Buscho, T. S. Thornhill*, et al.*, "Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers," *J Infect Dis,* vol. 129, pp. 709-714, 1974.

[313] J. E. Kaplan, G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, *et al.*, "Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis," *Ann Intern Med,* vol. 96, pp. 756-761, 1982.

[314] Y. Sakai, S. Nakata, S. Honma, M. Tatsumi, K. Numata-Kinoshita, and S. Chiba, "Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan," *Pediatr Infect Dis J,* vol. 20, pp. 849-853, 2001.

[315] C. D. Kirkwood and R. F. Bishop, "Molecular detection of human calicivirus in young children hospitalized with acute gastroenteritis in Melbourne, Australia, during 1999," *J Clin Microbiol,* vol. 39, pp. 2722-2724, 2001.

[316] E. O. Caul, "Small round structured viruses: airborne transmission and hospital control," *Lancet,* vol. 343, pp. 1240-1242, 1994.

[317] H. Leclerc, L. Schwartzbrod, and E. Dei-Cas, "Microbial agents associated with waterborne diseases," *Crit Rev Microbiol,* vol. 28, pp. 371-409, 2002.

[318] R. L. Fankhauser, J. S. Noel, S. S. Monroe, T. Ando, and R. I. Glass, "Molecular epidemiology of “Norwalk-like viruses” in outbreaks of gastroenteritis in the United States," *J Infect Dis,* vol. 178, pp. 1571-1578, 1998.

[319] E. Haramoto, H. Katayama, and S. Ohgaki, "Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater," *J Appl Environ Microbiol,* vol. 70, pp. 2154-2160, 2004.

[320] T. Nishida, H. Kimura, M. Saitoh, M. Shinohara, M. Kato, S. Fukuda, *et al.*, "Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters," *J Appl Environ Microbiol,* vol. 69, pp. 5782-5786, 2003.

[321] P. F. Teunis, C. L. Moe, P. Liu, S. E Miller, L. Lindesmith, R. S. Baric, *et al.*, "Norwalk virus: how infectious is it?," *J Med Virol,* vol. 80, pp. 1468-1476, 2008.

[322] E. Duizer, P. Bijkerk, B. Rockx, A. De Groot, F. Twisk, and M. Koopmans, "Inactivation of caliciviruses," *J Appl Environ Microbiol,* vol. 70, pp. 4538-4543, 2004.

[323] S. Clay, S. Maherchandani, Y. S. Malik, and S. M. Goyal, "Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses," *Am J Infect Control ,* vol. 34, pp. 41-43, 2006.

[324] U. Parashar, E. S. Quiroz, A. W. Mounts, S. S. Monroe, R. L. Fankhauser, T. Ando*, et al.*, "" Norwalk-like viruses". Public health consequences and outbreak management," *MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports/Centers for Disease Control,* vol. 50, pp. 1-17, 2001.

[325] X. Jiang, M. Wang, D. Y. Graham, and M. K. Estes, "Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein," *J Virol,* vol. 66, pp. 6527-6532, 1992.

[326] R. A. Bull, E. T. Tu, C. J. McIver, W. D. Rawlinson, and P. A. White, "Emergence of a new norovirus genotype II. 4 variant associated with global outbreaks of gastroenteritis," *J Clin Microbiol,* vol. 44, pp. 327-333, 2006.

[327] S. Castriciano, K. Luinstra, A. Petrich, M. Smieja, C. Lee, D. Jang*, et al.*, "Comparison of the RIDASCREEN® norovirus enzyme immunoassay to IDEIA NLV GI/GII by testing stools also assayed by RT-PCR and electron microscopy," *J Virol Methods,* vol. 141, pp. 216-219, 2007.

[328] R. L. Atmar and M. K. Estes, "Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses," *Clin Microbiol Rev,* vol. 14, pp. 15-37, 2001.

[329] A. A. Trujillo, K. A. McCaustland, D.-P. Zheng, L. A. Hadley, G. Vaughn, S. M. Adams*, et al.*, "Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus," *J Clin Microbiol,* vol. 44, pp. 1405-1412, 2006.

[330] C. D. Rao, "Non-Polio Enteroviruses, the Neglected and Emerging Human Pathogens: Are we Waiting for the Sizzling Enterovirus Volcano to Erupt?," *Proc Indian Natl Sci Acad*, pp. 447-462, 2015.

[331] M. Pallansch and R. Roos, "Enteroviruses: Polioviruses, Coxsackieviruses, echoviruses, and newer enteroviruses," *Fields virology*, Kluwer/Lippincott, Williams and Wilkins, pp. 839-893, 2013.

[332] W. Huang, G. Wang, J. Zhuge, S. M. Nolan, N. Dimitrova, and J. T. Fallon, "Whole-Genome Sequence Analysis Reveals the Enterovirus D68 Isolates during the United States 2014 Outbreak Mainly Belong to a Novel Clade," *Sci Rep,* vol. 5, 2015.

[333] D. C. Rao, H. Reddy, K. Sudheendra, A. Raghavendra, V. Varadharaj, S. Edula*, et al.*, "Non-polio enterovirus association with persistent diarrhea in children as revealed by a follow-up study of an Indian cohort during the first two years of life," *J Clin Virol,* vol. 61, pp. 125-131, 2014.

[334] A. Mondal, K. Sharma, Y. S. Malik, and S. N. Joardar, "Detection of group a rotavirus in faeces of diarrhoeic bovine porcine and human population from eastern India by reverse transcriptase–polymerase chain reaction," *Adv Anim Vet Sci,* vol. 1(1S), pp. 18-19, 2013.

[335] G. J. Zoll, W. J. Melchers, H. Kopecka, G. Jambroes, H. J. van der Poel, and J. M. Galama, "General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections," *J Clin Microbiol,* vol. 30, pp. 160-165, 1992.

[336] G. P. Richards, M. A. Watson, R. L. Fankhauser, and S. S. Monroe, "Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers," *Appl Environ Microbiol,* vol. 70, pp. 7179-7184, 2004.

[337] S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller*, et al.*, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Res,* vol. 25, pp. 3389-3402, 1997.

[338] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0," *Mol Biol Evol,* vol. 30, pp. 2725-2729, 2013.

[339] F. Lauck, C. A. Smith, G. F. Friedland, E. L. Humphris, and T. Kortemme, "RosettaBackrub-a web server for flexible backbone protein structure modeling and design," *Nucleic Acids Res,* vol. 38, pp. 12, 2010.

[340] S. R. Comeau, D. W. Gatchell, S. Vajda, and C. J. Camacho, "ClusPro: a fully automated algorithm for protein-protein docking," *Nucleic Acids Res,* vol. 32, pp. W96-9, 2004.

[341] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng*, et al.*, "UCSF Chimera-a visualization system for exploratory research and analysis," *J Comput Chem,* vol. 25, pp. 1605-1612, 2004.

[342] R. Brenke, D. R. Hall, G. Y. Chuang, S. R. Comeau, T. Bohnuud, D. Beglov*, et al.*, "Application of asymmetric statistical potentials to antibody-protein docking," *Bioinformatics,* vol. 28, pp. 2608-2614, 2012.

[343] T. G. Phan, P. Khamrin, T. D. Quang, S. K. Dey, S. Takanashi, S. Okitsu*, et al.*, "Detection and genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan," *J Virol,* vol. 81, pp. 4645-4653, 2007.

[344] M. L. O'Ryan, D. O. Matson, M. K. Estes, and L. K. Pickering, "Anti-rotavirus G type-specific and isotype-specific antibodies in children with natural rotavirus infections," *J Infect Dis,* vol. 169, pp. 504-511, 1994.

[345] G. Kang, R. Arora, S. D. Chitambar, J. Deshpande, M. D. Gupte, M. Kulkarni*, et al.*, "Multicenter, hospital-based surveillance of rotavirus disease and strains among indian children aged <5 years," *J Infect Dis,* vol. 1, pp. S147-S153, 2009.

[346] K. Zaman, M. Yunus, A. Faruque, S. El Arifeen, I. Hossain, T. Azim*, et al.*, "Surveillance of rotavirus in a rural diarrhoea treatment centre in Bangladesh, 2000–2006," *Vaccine,* vol. 27, pp. F31-F34, 2009.

[347] R. Qazi, S. Sultana, S. Sundar, H. Warraich, A. Rais, and A. K. Zaidi, "Population-based surveillance for severe rotavirus gastroenteritis in children in Karachi, Pakistan," *Vaccine,* vol. 27, pp. F25-F30, 2009.

[348] D. D. Anh, V. D. Thiem, T. K. Fischer, T. T. Minh, N. Van Man, P. Kilgore*, et al.*, "The burden of rotavirus diarrhea in Khanh Hoa Province, Vietnam: baseline assessment for a rotavirus vaccine trial," *Pediatr Infect Dis J,* vol. 25, pp. 37-40, 2006.

[349] J.-T. Lou, X.-J. Xu, Y.-D. Wu, R. Tao, and M.-Q. Tong, "Epidemiology and burden of rotavirus infection among children in Hangzhou, China," *J Clin Virol ,* vol. 50, pp. 84-87, 2011.

[350] K. Kawai, M. A. O'Brien, M. G. Goveia, T. C. Mast, and A. C. El Khoury, "Burden of rotavirus gastroenteritis and distribution of rotavirus strains in Asia: a systematic review," *Vaccine,* vol. 30, pp. 1244-1254, 2012.

[351] G. Kang, R. Desai, R. Arora, S. Chitamabar, T. N. Naik, T. Krishnan*, et al.*, "Diversity of circulating rotavirus strains in children hospitalized with diarrhea in India, 2005-2009," *Vaccine,* vol. 31, pp. 2879-2883, 2013.

[352] S. Babji, R. Arumugam, A. Sarvanabhavan, P. D. Moses, A. Simon, I. Aggarwal*, et al.*, "Multi-center surveillance of rotavirus diarrhea in hospitalized children <5 years of age in India, 2009-2012," *Vaccine,* vol. 11, pp. 1, 2014.

[353] T. Saluja, S. D. Sharma, M. Gupta, R. Kundu, S. Kar, A. Dutta*, et al.*, "A multicenter prospective hospital-based surveillance to estimate the burden of rotavirus gastroenteritis in children less than five years of age in India," *Vaccine,* vol. 11, pp. 30, 2014.

[354] S. Broor, D. Ghosh, and P. Mathur, "Molecular epidemiology of rotaviruses in India," *Indian J Med Res,* vol. 118, pp. 59, 2003.

[355] S. Mullick, P. Mandal, M. K. Nayak, S. Ghosh, P. De, K. Rajendran*, et al.*, "Hospital based surveillance and genetic characterization of rotavirus strains in children (<5 years) with acute gastroenteritis in Kolkata, India, revealed resurgence of G9 and G2 genotypes during 2011-2013," *Vaccine,* vol. 11, pp. 18, 2014.

[356] V. S. Tatte, J. R. Gentsch, and S. D. Chitambar, "Characterization of group A rotavirus infections in adolescents and adults from Pune, India: 1993-1996 and 2004-2007," *J Med Virol,* vol. 82, pp. 519-527, 2010.

[357] S. Anandan, R. Peter, R. Aramugam, N. Ismail, B. Veeraraghavan, and G. Kang, "Group A rotavirus gastroenteritis in older children and adults at a hospital in Southern India," *Vaccine,* vol. 32, pp. A33-A35, 2014.

[358] J. Nyangao, P. Kingori, and F. Okoth, "Detection and identification of echovirus 7 from a child with gastro-enteritis," *East Afr Med J ,* vol. 83, pp. 666-669, 2006.

[359] J. Patel, J. Daniel, and V. Mathan, "An epidemic of acute diarrhoea in rural southern India associated with echovirus type 11 infection," *J Hyg,* vol. 95, pp. 483-492, 1985.

[360] T. G. Phan, T. A. Nguyen, H. Shimizu, F. Yagyu, S. Okitsu, W. E. Müller*, et al.*, "Identification of enteroviral infection among infants and children admitted to hospital with acute gastroentritis in Ho Chi Minh City, Vietnam," *J Med Virol,* vol. 77, pp. 257-264, 2005.

[361] P. A. Silva, K. Stark, F. P. Mockenhaupt, K. Reither, T. Weitzel, R. Ignatius*, et al.*, "Molecular characterization of enteric viral agents from children in northern region of Ghana," *J Med Virol,* vol. 80, pp. 1790-1798, 2008.

[362] S. B. Rai, H. W. Mansor, T. Vasantha, I. Norizah, and K. Chua, "An outbreak of echovirus 11 amongst neonates in a confinement home in Penang, Malaysia," *Med J Malaysia,* vol. 62, pp. 223-226, 2007.

[363] S. B. Ballard, E. J. Reaves, C. G. Luna, M. E. Silva, C. Rocha, K. Heitzinger*, et al.*, "Epidemiology and genetic characterization of noroviruses among adults in an endemic setting, Peruvian Amazon Basin, 2004–2011," *PloS One,* vol. 10, pp. e0131646, 2015.

[364] V. K. Menon, S. George, S. Ramani, J. Illiayaraja, R. Sarkar, A. K. Jana*, et al.*, "Genogroup IIb norovirus infections and association with enteric symptoms in a neonatal nursery in southern India," *J Clin Microbiol,* vol. 48, pp. 3212-3215, 2010.

[365] X. Wu, J. Han, L. Chen, D. Xu, Y. Shen, Y. Zha*, et al.*, "Prevalence and genetic diversity of noroviruses in adults with acute gastroenteritis in Huzhou, China, 2013-2014," *Arch Virol,* vol. 160, pp. 1705-1713, 2015.

[366] F. Albano, E. Bruzzese, A. Bella, A. Cascio, L. Titone, S. Arista*, et al.*, "Rotavirus and not age determines gastroenteritis severity in children: a hospital-based study," *Eur J Pediatr,* vol. 166, pp. 241-247, 2007.

[367] B. Salinas, G. Gonzalez, R. Gonzalez, M. Escalona, M. Materan, and I. P. Schael, "Epidemiologic and clinical characteristics of rotavirus disease during five years of surveillance in Venezuela," *Pediatr Infect Dis J,* vol. 23, pp. S161-S167, 2004.

[368] B. Zheng, G. Ma, J. Tam, S. Lo, M. Ng, B. Lam*, et al.*, "The effects of maternal antibodies on neonatal rotavirus infection," *Pediatr Infect Dis J,* vol. 10, pp. 865-868, 1991.

[369] L. Mata, A. Simhon, J. Urrutia, R. A. Kronmal, R. Fernandez, and B. Garcia, "Epidemiology of rotaviruses in a cohort of 45 Guatamalan Mayan Indian children observed from birth to the age of three years," *J Infect Dis,* vol. 148, pp. 452-461, 1983.

[370] A. Paul, B. P. Gladstone, I. Mukhopadhya, and G. Kang, "Rotavirus infections in a community based cohort in Vellore, India," *Vaccine,* vol. 32, pp. A49-A54, 2014.

[371] S. J. Moyo, N. Gro, V. Kirsti, M. I. Matee, J. Kitundu, S. Y. Maselle*, et al.*, "Prevalence of enteropathogenic viruses and molecular characterization of group A rotavirus among children with diarrhea in Dar es Salaam Tanzania," *BMC Public Health,* vol. 7, pp. 1, 2007.

[372] H. Nirwati, T. Wibawa, A. T. Aman, A. Wahab, and Y. Soenarto, "Detection of group A rotavirus strains circulating among children with acute diarrhea in Indonesia," *SpringerPlus,* vol. 5, pp. 97, 2016.

[373] M. A. Neves, H. H. Pinheiro, R. S. Silva, A. C. Linhares, L. D. Silva, Y. B. Gabbay*, et al.*, "High prevalence of G12P[8] rotavirus strains in Rio Branco, Acre, Western Amazon, in the post-rotavirus vaccine introduction period," *J Med Virol,* vol. 15, pp. 24404, 2015.

[374] M. I. Gómara, J. Green, and J. Gray, "Methods of rotavirus detection, sero-and genotyping, sequencing, and phylogenetic analysis," *Rotaviruses: Methods and Protocols,* pp. 189-216, 2000.

[375] I. Haffejee, "The pathophysiology, clinical features and management of rotavirus diarrhoea," *QJ Med,* vol. 79, pp. 289-299, 1991.

[376] H. B. Greenberg, H. F. Clark, and P. A. Offit, "Rotavirus pathology and pathophysiology," *Curr Top Microbiol Immunol,* vol. 185, pp. 255-283, 1994.

[377] F. N. Binka, F. K. Anto, A. R. Oduro, E. A. Awini, A. K. Nazzar, G. E. Armah*, et al.*, "Incidence and risk factors of paediatric rotavirus diarrhoea in northern Ghana," *TM & IH,* vol. 8, pp. 840-846, 2003.

[378] S. D. Chitambar, S. S. Ranshing, G. N. Pradhan, V. R. Kalrao, R. K. Dhongde, and A. R. Bavdekar, "Changing trends in circulating rotavirus strains in Pune, western India in 2009-2012: Emergence of a rare G9P [4] rotavirus strain," *Vaccine,* vol. 32, pp. A29-A32, 2014.

[379] G. S. Namjoshi, M. Mitra, S. K. Lalwani, A. Sachdeva, S. Balasubramanian, S. Babji*, et al.*, "Rotavirus gastroenteritis among children less than 5 years of age in private outpatient setting in urban India," *Vaccine,* vol. 11, pp. 70, 2014.

[380] B. R. Alkali, A. Daneji, A. Magaji, and L. Bilbis, "Clinical symptoms of human rotavirus infection observed in children in Sokoto, Nigeria," *Adv Virol,* Article ID:890957, 2015.

[381] S. Cook, R. Glass, C. LeBaron, and M. S. Ho, "Global seasonality of rotavirus infections," *Bull World Health Organ,* vol. 68, pp. 171, 1990.

[382] K. Levy, A. E. Hubbard, and J. N. Eisenberg, "Seasonality of rotavirus disease in the tropics: a systematic review and meta-analysis," *Int J Epidemiol,* vol. 38, pp. 1487-1496, 2009.

[383] J. S. Jagai, R. Sarkar, D. Castronovo, D. Kattula, J. McEntee, H. Ward*, et al.*, "Seasonality of rotavirus in South Asia: a meta-analysis approach assessing associations with temperature, precipitation, and vegetation index," *PloS One,* vol. 7, pp. e38168, 2012.

[384] M. M. Patel, V. Pitzer, W. J. Alonso, D. Vera, B. Lopman, J. Tate*, et al.*, "Global seasonality of rotavirus disease," *Pediatr Infect Dis J,* vol. 32, pp. e134, 2013.

[385] C. Brandt, H. Kim, W. Rodriguez, J. Arrobio, B. Jeffries, and R. Parrott, "Rotavirus gastroenteritis and weather," *J Clin Microbiol,* vol. 16, pp. 478-482, 1982.

[386] M. Paul and E. Erinle, "Influence of humidity on rotavirus prevalence among Nigerian infants and young children with gastroenteritis," *J Clin Microbiol,* vol. 15, pp. 212-215, 1982.

[387] S. Ram, S. Khurana, S. Khurana, S. Sharma, D. Vadehra, and S. Broor, "Bioecological factors & rotavirus diarrhoea," *Indian J Med Res,* vol. 91, pp. 167-170, 1990.

[388] N. Gomwalk, U. Umoh, L. Gosham, and A. Ahmad, "Influence of climatic factors on rotavirus infection among children with acute gastroenteritis in Zaria, northern Nigeria," *J Trop Pediatr,* vol. 39, pp. 293-297, 1993.

[389] G. Armah, J. Mingle, A. Dodoo, A. Anyanful, R. Antwi, J. Commey*, et al.*, "Seasonality of rotavirus infection in Ghana," *Ann Trop Paediatr,* vol. 14, pp. 223-229, 1993.

[390] M. Hashizume, B. Armstrong, Y. Wagatsuma, A. Faruque, T. Hayashi, and D. A. Sack, "Rotavirus infections and climate variability in Dhaka, Bangladesh: a time-series analysis," *Epidemiol Infect,* vol. 136, pp. 1281-1289, 2008.

[391] R. D'souza, G. Hall, and N. Becker, "Climatic factors associated with hospitalizations for rotavirus diarrhoea in children under 5 years of age," *Epidemiol Infect,* vol. 136, pp. 56-64, 2008.

[392] A. Mathew, P. S. Rao, T. V. Sowmyanarayanan, and G. Kang, "Severity of rotavirus gastroenteritis in an Indian population: report from a 3 year surveillance study," *Vaccine,* vol. 32, pp. A45-A48, 2014.

[393] T. Cherian, S. Wang, and C. Mantel, "Rotavirus vaccines in developing countries: the potential impact, implementation challenges, and remaining questions," *Vaccine,* vol. 30, pp. A3-A6, 2012.

[394] A. Sánchez-Fauquier, V. González-Galán, S. Arroyo, A. Cabornero, A. Ruiz-Burruecos, and I. Wilhelmi-De Cal, "Monitoring of children with acute gastroenteritis in Madrid, Spain, during 2010–2011: rotavirus genotype distribution after the vaccines introduction," *Enferm Infecc Microbiol Clin,* vol. 32, pp. 280-284, 2014.

[395] N. Abdel-Haq, M. Amjad, E. McGrath, P. Chearskul, A. Amer, H. Salimnia*, et al.*, "Emergence of human rotavirus genotype G9 in metropolitan Detroit between 2007 and 2009," *J Med Microbiol,* vol. 60, pp. 761-767, 2011.

[396] J. A. Stupka, J. I. Degiuseppe, and G. I. Parra, "Increased frequency of rotavirus G3P[8] and G12P[8] in Argentina during 2008-2009: whole-genome characterization of emerging G12P[8] strains," *J Clin Virol,* vol. 54, pp. 162-167, 2012.

[397] S. Das, V. Varghese, S. Chaudhury, P. Barman, S. Mahapatra, K. Kojima*, et al.*, "Emergence of novel human group A rotavirus G12 strains in India," *J Clin Microbiol,* vol. 41, pp. 2760-2762, 2003.

[398] S. Sharma, P. Ray, J. R. Gentsch, R. I. Glass, V. Kalra, and M. K. Bhan, "Emergence of G12 rotavirus strains in Delhi, India, in 2000 to 2007," *J Clin Microbiol,* vol. 46, pp. 1343-1348, 2008.

[399] S. Samajdar, V. Varghese, P. Barman, S. Ghosh, U. Mitra, P. Dutta*, et al.*, "Changing pattern of human group A rotaviruses: emergence of G12 as an important pathogen among children in eastern India," *J Clin Virol,* vol. 36, pp. 183-188, 2006.

[400] P. Rajendran and G. Kang, "Molecular epidemiology of rotavirus in children and animals and characterization of an unusual G10P [15] strain associated with bovine diarrhea in south India," *Vaccine,* vol. 32, pp. A89-A94, 2014.

[401] G. Ianiro, R. Delogu, L. Fiore, and F. M. Ruggeri, "Genomic characterization and molecular investigation of VP7 epitopes of uncommon G10P [8] group A rotavirus strains detected in Italy in 2009," *J Gen Virol,* vol. 96, pp. 1801-1810, 2015.

[402] M. G. Miles, K. D. Lewis, G. Kang, U. D. Parashar, and A. D. Steele, "A systematic review of rotavirus strain diversity in India, Bangladesh, and Pakistan," *Vaccine,* vol. 30, pp. A131-A139, 2012.

[403] A. D. Steele, K. M. Neuzil, N. A. Cunliffe, S. A. Madhi, P. Bos, B. Ngwira*, et al.*, "Human rotavirus vaccine Rotarix™ provides protection against diverse

circulating rotavirus strains in African infants: a randomized controlled trial," *BMC Infect Dis,* vol. 12, pp. 1, 2012.

[404] J. Matthijnssens, J. Bilcke, M. Ciarlet, V. Martella, K. Banyai, M. Rahman*, et al.*, "Rotavirus disease and vaccination: impact on genotype diversity," *Future Microbiol,* vol. 4, pp. 1303-1316, 2009.

[405] Y. Hoshino, R. W. Jones, J. Ross, S. Honma, N. Santos, J. R. Gentsch*, et al.*, "Rotavirus serotype G9 strains belonging to VP7 gene phylogenetic sequence lineage 1 may be more suitable for serotype G9 vaccine candidates than those belonging to lineage 2 or 3," *J Virol,* vol. 78, pp. 7795-7802, 2004.

[406] Q. Jin, R. L. Ward, D. R. Knowlton, Y. B. Gabbay, A. C. Linhares, R. Rappaport*, et al.*, "Divergence of VP7 genes of G1 rotaviruses isolated from infants vaccinated with reassortant rhesus rotaviruses," *Arch Virol,* vol. 141, pp. 2057-2076, 1996.

[407] R. Arora, P. Chhabra, and S. D. Chitambar, "Genetic diversity of genotype G1 rotaviruses co-circulating in western India," *Virus Res*, vol. 146, pp. 36-40, 2009.

[408] R. Arora and S. D. Chitambar, "Full genomic analysis of Indian G1P[8] rotavirus strains," *Infect Genet Evol,* vol. 11, pp. 504-511, 2011.

[409] V. S. Tatte and S. D. Chitambar, "Diversity in the VP7 encoding genes of rotavirus strains isolated from adolescent and adult cases of acute gastroenteritis," *J Med Virol,* vol. 84, pp. 1481-1488, 2012.

[410] W. Chan-it, A. Thongprachum, S. K. Dey, T. G. Phan, P. Khamrin, S. Okitsu*, et al.*, "Detection and genetic characterization of rotavirus infections in non-hospitalized children with acute gastroenteritis in Japan, 2007-2009," *Infect Genet Evol,* vol. 11, pp. 415-422, 2011.

[411] B. Laszlo, J. Konya, E. Dandar, J. Deak, A. Farkas, J. Gray*, et al.*, "Surveillance of human rotaviruses in 2007-2011, Hungary: exploring the genetic relatedness between vaccine and field strains," *J Clin Virol,* vol. 55, pp. 140-146, 2012.

[412] A. G. Maranhao, J. L. Vianez-Junior, F. J. Benati, P. M. Bisch, and N. Santos, "Polymorphism of rotavirus genotype G1 in Brazil: in silico analysis of variant

strains circulating in Rio de Janeiro from 1996 to 2004," *Infect Genet Evol,* vol. 12, pp. 1397-1404, 2012.

[413] S. M. McDonald, A. O. McKell, C. M. Rippinger, J. K. McAllen, A. Akopov, E. F. Kirkness*, et al.*, "Diversity and relationships of cocirculating modern human rotaviruses revealed using large-scale comparative genomics," *J Virol,* vol. 86, pp. 9148-9162, 2012.

[414] Y. Nagaoka, M. Tatsumi, T. Tsugawa, Y. Yoto, and H. Tsutsumi, "Phylogenetic and computational structural analysis of VP7 gene of group a human rotavirus G1P [8] strains obtained in Sapporo, Japan from 1987 to 2000," *J Med Virol,* vol. 84, pp. 832-838, 2012.

[415] T. Nakagomi, O. Nakagomi, W. Dove, Y. H. Doan, D. Witte, B. Ngwira*, et al.*, "Molecular characterization of rotavirus strains detected during a clinical trial of a human rotavirus vaccine in Blantyre, Malawi," *Vaccine,* vol. 30, pp. A140-A151, 2012.

[416] J. R. Gentsch, A. R. Laird, B. Bielfelt, D. D. Griffin, K. Banyai, M. Ramachandran*, et al.*, "Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs," *J Infect Dis,* vol. 1, pp. S146-S159, 2005.

[417] K. P. Hwang, F. T. Wu, K. Banyai, H. S. Wu, D. C. Yang, Y. C. Huang*, et al.*, "Identification of porcine rotavirus-like genotype P[6] strains in Taiwanese children," *J Med Microbiol,* vol. 61, pp. 990-997, 2012.

[418] V. P. Le, J. B. Kim, D. H. Shon, I. S. Chung, Y. Yoon, K. Kim*, et al.*, "Molecular characterization of rare G12P[6] rotavirus isolates closely related to G12 strains from the United States, CAU 195 and CAU 214," *Arch Virol,* vol. 156, pp. 511-516, 2011.

[419] K. C. Jere, L. Mlera, H. G. O'Neill, A. C. Potgieter, N. A. Page, M. L. Seheri*, et al.*, "Whole genome analyses of African G2, G8, G9, and G12 rotavirus strains using sequence-independent amplification and 454(R) pyrosequencing," *J Med Virol,* vol. 83, pp. 2018-2042, 2011.

Chapter 8

Appendices

Reagents and Buffers

All the reagents and buffers for RNA work were prepared in nuclease free Milli Q grade water and sterilized by autoclaving for 15 min at 15-psi pressure unless otherwise indicated.

Phosphate Buffered Saline (PBS), per litre

KH_2PO_4	0.34 g
K_2HPO_4	1.21 g
NaCl	8.0 g
pH	7.3

TAE Buffer (50X)

Tris Base	242 g
Glacial Acetic Acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
Final Volume	1000 mL

6X loading dye for agarose gel elctrophoresis

Bromophenol Blue	0.25 %
Xylene cyanol	0.25 %
Glycerol	30 %

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Dr. R. S. Chauhan
Professor & Head

Dated: Nov. 23, 2011

Ethics Committee Approval

"Identification, characterization of diarrheagenic pathogens and estimation of diarrhea burden in Himachal Pradesh".

The Ethics committee meeting held on date 09/11/11 discussed the above mentioned project. The project was cleared form the ethical angle. While granting ethical clearance, the investigators were advised to obtain written informed consent from patients on consent forms and to submit six monthly progress reports to the ethics committee.

(R.S. Chauhan)
Member Secretary- Ethics committee

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Dr. R. S. Chauhan
Professor & Head

Dated: Oct 18, 2011

The Chief Medical Officer,
District Hospital,
Solan, H.P
INDIA

Sub: Request for approval of collection of stool specimens from diarrheal patients

Dear Sir,

Our department is planning to initiate research work on diarrhea (Dr. Jitendraa Vashistt and Dr. Harish Changotra). In the first phase of this project, we will look for the incidence of causative agents associated with this disease; bacterial as well as viral agents. In this research project, we need your help regarding clinical samples from the patients of District Hospital, Solan, which is the biggest organization in this region.
The outcome of the project will be beneficial in the better diagnosis (molecular) as well as in the treatment of the patients.
We will be highly obliged if you please give your kind approval for the collection of clinical samples from your hospital. We are positively looking for collaboration with your hospital for inter-disciplinary studies in the area of medical biotechnology. Your assistance will be duly acknowledged.
With best regards.

Sincerely Yours

(Prof. R.S. Chauhan)

Endst No : HFW-SLN-IDSP-General /2012 1706 Date 2-2-12 the Solan

Copy forwarded to:
(1) Dr. R.K. Baria, Medical Superintendent, RH Solan (H.P.) for information and kindly cooperate in the above said project.
(2) Sh. Prem Dass , Computer, CMO Office Solan for information and also provide the data related to Diarrhea.
(3) Dr. R.S. Chauhan (Prof. & Head), Jaypee University of Information Tech. Waknaghat, P.O.-Dumehar Banj., Kandaghat, Solan -173215 for information and Kindly contact to the above mentioned Officers/Official.

Medical Officer of Health
Solan District/Solan

Allowed

LIST OF PUBLICATIONS

Journal Publications

- **Jain S,** Thakur N, Vashistt J, Grover N, Krishnan T, Changotra H. Predominance of unusual rotavirus G1P[6] strain in North India: an evidence from hospitalized children and adult diarrheal patients. *Infection, Genetics and Evolution* 2016;46:65-70. (Impact factor: 2.8)
- **Jain S,** Vashistt J, Gupta K, Kumar A, Changotra H. Molecular analysis of VP7 gene of rotavirus G1 strains isolated from Himachal Pradesh, North India. *Current Microbiology* 2016;73(6):781-9. (Impact factor: 1.5)
- **Jain S,** Thakur N, Grover N, Vashistt J, Changotra H. Prevalence of rotavirus, norovirus and enterovirus in diarrheal diseases in Himachal Pradesh, India. *VirusDisease* 2016;27(1):77-83. (Impact factor: 0.4)
- **Jain S,** Vashistt J, Changotra H. Rotaviruses: Is their surveillance needed?. *Vaccine* 2014;32:3367-78. (Impact factor: 3.4)
- Nutan, **Jain S**, Shilpa, Tomar A, Changotra H, Vashistt J. Computational Tools; Indispensable Armamentarium of Medical Biotechnology. *Indian Journal of Science and Technology* 2016;9(32):1-5.

Conference Publications

- **Jain S,** Vashistt J, Changotra H (2015). Predominance of Rotavirus G1P[6] strain in Himachal Pradesh, India. *Proceedings of the 5th Asian Vaccine Conference.* Held at Hanoi, Vietnam on 12-14 June 2015. (Travel grant of Rs. 71,600/- from ICMR, India)
- **Jain S,** Singh TR, Vashistt J, Changotra H (2015). Molecular analysis of G1 rotavirus strains isolated from Himachal Pradesh. *Proceedings of the National Conference on Emerging Trends in Host-Microbe Interactions.* Held at Jalandhar, India on 17-18 April 2015.
- **Jain S**, Kumar N, Thakur N, Vashistt J, Changotra H (2014). Phylogenetic analysis to study the evolution of Rotavirus G1P[6] strain isolated from Himachal Pradesh. *Proceedings of the International Conference on Stem Cell Research, Cancer Biology, Biomedical Sciences, Bioinformatics and Applied Biotechnology.* Held at New Delhi, India on 1 -2 November 2014.
- Nutan, **Jain S**, Changotra H, Shrivastava R, Vashistt J (2015). Metagenomic Studies in Relation to Diarrheal Diseases. *Proceedings of the International Congress on*

Friedreichs Ataxia and DNA Structure in Health & Disease. Held at New Delhi, India on 11-13 April 2015.

- Nutan, **Jain S,** Grover N, Kumar Y, Changotra H, Vashistt J (2014). Identification, characterization of prevalent diarrheagenic bacterial pathogens from regions of Himachal Pradesh. *Proceedings of the International Conference on Life science Biotechnology & Bioinformatics* Held at Noida, India on 29-30 August 2014.