Chapter 2

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 up to 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.
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]).](image)

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])

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

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 αβ3, α4β1, α2β1 integrins. 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 assembly 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 trafficking 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 phosphoprotein 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$^{2+}$ concentration. The increase in Ca$^{2+}$ concentration 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, SGIII, 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 (Glycoprotein) and P stands for VP4 gene (Protease 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]**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Protein</th>
<th>Genotype</th>
<th>Number of genotypes identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP7</td>
<td>G</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>VP4</td>
<td>P</td>
<td>47</td>
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<tr>
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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.](image-url)
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]

<table>
<thead>
<tr>
<th>G-Type</th>
<th>Commonly associated P-Type</th>
<th>Unusual P-type association</th>
</tr>
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</table>

Table 2.4: Unusual G genotypes of rotavirus [29]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Associated P-Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>G5</td>
<td>P[6], P[8]</td>
</tr>
<tr>
<td>G6</td>
<td>P[6], P[14], P[9]</td>
</tr>
<tr>
<td>G8</td>
<td>P[1], P[4], P[6], P[14], P[8]</td>
</tr>
<tr>
<td>G10</td>
<td>P[8], P[6], P[11], P[4]</td>
</tr>
<tr>
<td>G11</td>
<td>P[8], P[6], P[4], P[25]</td>
</tr>
<tr>
<td>G12</td>
<td>P[4], P[9], P[6], P[8]</td>
</tr>
</tbody>
</table>
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
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].

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 deafness, 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])](image)

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].