Human Rotavirus was first recognized as a major etiological agent of severe gastroenteritis in infants and young children by Bishop and coworkers (1973). Before this, rotaviruses had already been described in diarrheic mice, monkeys and cattle (Adams and Kraft, 1963; Malherbe and Harwin, 1963; Mebus et al., 1969). Since that time, rotaviruses have been detected in various species such as bats (Estes and Greenberg, 2013) and birds (Kindler et al., 2013; Otto et al., 2012). Virus particles with similar morphology were observed in biopsies of duodenal mucosa from nine children suffering with acute nonbacterial diarrhea in thin sections by electron microscopy. Primarily, the viruses were identified as member of Orbivirus group. But after a year, scientists observed that the viruses could also be detected in negatively stained specimens of the stool samples of diarrheic patient (Bishop et al., 1974). It was found that viruses causing diarrhea in children and newborn of animals were indistinguishable in morphology (Flewett et al., 1974). The convalescent phase sera from humans and new borne calves agglutinated human and animal virus capsids under immunoelectron microscopy.

2.1 Structure of Rotavirus

Very soon after the detection of rotavirus in human infants, the scientific community was eager to elucidate the virus structure. Very detailed electron microscopic analyses were performed, but most of the observations were later shown to be incorrect. Under electron microscope, the virus particles appeared as ‘wheel shaped’ (Figure 2.1) and from its shape the name Rotavirus was derived as in Latin, rota means wheel (Flewett et al., 1974). The fully infectious rotavirus virion is composed of three capsid layers and due to this; it is also known as triple layered particle (TLP). The structure of icosahedral symmetry has been elucidated using cryo-electron microscopy and image reconstruction data (Jayaram et al., 2004). A total of 120 molecules of VP2 (viral protein 2) forms the single layered particle/ core of virion. These VP2 particles are arranged in a T=1 symmetry as 60 dimers. A decamer is formed after fusion of five of the dimers around the fivefold symmetry axis. Core protein layer is formed by rest of 12 decamers which is almost uniform except the points having pores along the 5 fold axis (McClain et al., 2010). The N terminus of VP2 was thought to contribute to the fivefold hub projecting in the interior core, but now it has been found that it is VP1, which is projected as a hub in the core instead of VP2 (Estrozi et al., 2013). Replication
complexes (RC), formed by VP1 and VP3 are located on the interior surface of VP2 layer along the fivefold axis and these complexes are intense contact with one specific dsRNA segment via VP1 protein (Periz et al., 2013). The VP2 capsid layer (core shell) encapsulates the 11 segments of dsRNA (as genome), RNA dependent RNA polymerase (RdRp), the capping enzyme (VP3) and VP1.

Jayaram and coworkers (2004) proposed that ds RNA genomic segments form conical cylinders around RC (replication complex) but a lot more work is to be done to explore the ds RNA structure within the core. In a double layered particle which is actively transcribing, it has been found that the proportion of VP6 layer decreases and that of core content increase (Kam et al., 2014). A similar kind of differential expression of dsRNA segments was also observed in case of Bluetongue virus (Gouet et al., 1999). The completely conserved 5’ and 3’ terminal sequences are very short in the 11 dsRNA segments. The UTR (untranslated region) of the dsRNA genome is small. The UTR sequences present at 3’end serves as recognition signals for viral RNA dependent RNA polymerase (Tortorici et al., 2003) and interact with nonstructural protein 3 also. These UTR sequences have been found to be involved in activation of the translation process.

The resolution of RNA dependent RNA polymerase has been achieved up to 2.9 Å (Lu et al., 2008). Polymerization of VP1 leads to formation of large cage like structure with four tunnels (Figure 2.2). The functions of these tunnels are proposed as follows:

1. First tunnel mediates the entry of free nucleoside triphosphates
2. Second tunnel mediates the entry of template ssRNA
3. Third tunnel mediates the release of (+) ssRNA
4. Fourth tunnel mediates the release of (-) ssRNA or dsRNA

The location of tunnel 3 is towards the class I channel (present in VP2 capsid layer) and it mediates the release of (+) ssRNA in the cytoplasm (Settembre et al., 2011; Estrozi et al., 2013). The newly replicated dsRNA segments are directed toward the core anterior through tunnel 4. Rotavirus RNA dependent RNA polymerase is highly similar to the corresponding enzyme of orthoreovirus in many aspects (Tao et al., 2002).

The core of rotavirus is enclosed by VP6 protein (260 trimers), which constitutes the middle layer of rotavirus capsid. Viral protein constitutes more than
51% of viral capsid protein and is known as major capsid protein. VP6 in combination with core constitute a double layered particle (DLP) which is a transcriptionally active state. The structure of VP6 has been elucidated (Mathieu et al., 2001) and it has been found that it interact with both; the core (VP2 protein) as well as outermost capsid proteins (VP4 & VP7) (Charpilienne et al., 2002). In other words, VP6 act as sandwich between the two important biological function i.e. virus entry in the cell and transcription and genome replication. The DLPs are further encapsulated by 60 spikes of VP4 trimers (total 180 molecules) and 260 trimers of VP7 (total 780 molecules) to form the triple layered particles (TLPs). The infectious virus particles have 132 channels along the fivefold axes (Class I channel number: 12), threefold and twofold axes respectively (Jayaram et al., 2004).

The rotavirus three dimensional structure has been determined using electron cryomicroscopy and single particle tomography and the resolution was found to be 4.3 Å (Settembre et al., 2011). From these experiments, it was found that VP4 interact intensely with both VP6 & VP7 protein. Previously, it has been demonstrated that VP4 trimers can only be observed in presence of tyepsin (Crawfold et al., 2001). Because the stoichiometry of VP4 in rotavirus, particles grown in the presence or absence of trypsin appeared different, it was proposed that VP4 spikes in absence of trypsin are icosahedrally disordered. By using cryo electron microscopy (Cryo-EM) and cryo-ET (Cryo electron tomography), it has been demonstrated recently that VP4 protein structure in rotavirus particles grown in the presence or absence of trypsin remains indistinguishable (Rodriguez et al., 2014). The previous deficiency in the observation was mainly due to superimposition of icosahedral structure requirement on previous cryo-electron microscopy data.

2.2 Rotavirus classification

Rotavirus is member of the genus Rotavirus which is one of the genera of family Reoviridae. It shares following common characteristics of the family:

- Non enveloped
- Isocahedral symmetry
- Virion particle of 65-75 nm in diameter
- Protease facilitate the growth in cell culture
- Isolated genomic RNA is not infectious

The family *Reoviridae* is further subdivided into the sub-families *Sedoreovirinae* and *Spinareovirinae*. *Sedoreovirinae* include six genera: Cardoreovirus, Minoreovirus, Orbivirus, Rotavirus, Phytoreovirus and Seadornavirus whereas sub-family *Spinareovirinae* include nine genera: Aquareovirus, Cypovirus, Coltivirus, Fijivirus, Dinovernavirus, Idnoreovirus, Mycoreovirus, Orthoreovirus and Oryzavirus. On the basis of serological epitopes and genetic diversity of VP6, eight groups (Rotavirus group A to H) have been described (Matthijnssens *et al.*, 2012). On the basis of glycoprotein VP7 (G-type) and Protease sensitive protein (P-type) genotyping of group A rotavirus has been done. Molecular analysis has identified 27 G-types and 37 P-types infecting both human and animals (Matthijnssens *et al.*, 2011; Trojnar *et al.*, 2013; Rotavirus classification working group, 2013). Advances in high throughput sequencing based genotyping have complemented serotyping methods and is commonly used. The Rotavirus classification working group has proposed RVA classification on the basis of all of its segments and are referred to as the Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx genotypes, respectively on the basis of particular cut off point of nucleotide sequence identity (Matthijnssens *et al.*, 2008a, 2008b, 2011a). Serotypes on the basis of VP7 protein are synonymous with genotypes whereas number of P- genotypes are much more than serotypes available i.e. in P2A[4], serotype is P2A while genotype is P[4] (Estes and Greenberg, 2013).

### 2.3 Rotavirus genome and its characteristics

Because rotaviruses exhibited morphological similarity with the *orbi-* and *reoviruses* as visualized by EM, it was easy to predict that they would also have a similar genome (segmented dsRNA). Neonatal calf diarrhea virus was reported to have 11 dsRNA segments (Newman *et al.*, 1975; Rodger *et al.*, 1975) similar to human rotavirus (Schnagl and Holmes, 1976). Native polyacrylamide gel electrophoresis was used to determine the molecular weight of RNA segments (Loening *et al.*, 1969). Detectable variations were reported in the molecular weights of specific genome segments of different RVA isolates (Schnagl and Holmes, 1976). Rotavirus genome is approximately 18,555 bp in size and consists of 11 segments of dsRNA. The molecular weight of these segments lies in the range of $2 \times 10^5$ to $2.2 \times 10^6$ Dalton with a size range of 667 to 3302 base pairs. Separation of dsRNA with PAGE was soon established

10
as one of the most commonly used methods for molecular epidemiology of rotavirus. The RNA segments migrate and cluster into four size classes as evident by PAGE of RNA (Kalica et al., 1978; Mattion et al., 1994). The numbering of the RNA segments is done in order of migration during PAGE.

In case of RVA, there are four large segments, two medium sized segments, three small sized segments. A characteristic 4:2:3:2 distribution is observed in PAGE for RVA. Avian rotaviruses are exceptions to the above pattern where segments 4 is close to segment 5 and segment 10 and 11 co-migrate and are difficult to resolve. In some of HRVA strain, the last segment (11th) RNA segment moves more slowly than usual resulting in short pattern. This short pattern is unique characteristic of almost all antigenic subgroup 1 (i.e G2 type) human rotaviruses. Long pattern is also reported dependent on relative migration last two segments, this being characteristic of all subgroup 2 (i.e. G1, G3, G4, G9 type) human rotaviruses. Super short pattern in some of the cases has been reported from human and bovine. Some rotavirus strains exhibit migration pattern different from RVA, known as non-group A rotaviruses and these include group B, C, D, E, F, G & H. Variations between rotaviruses of different host as well as rotaviruses of a single host can be predicted by comparing the genome with bovine, human, avian and simian rotavirus isolates (Rodger and Holmes, 1979). On the basis of hybridization experiments, it has been reported that animal and human rotavirus genome segments showed partial homology under less stringent conditions (Schroeder et al., 1982), whereas slight differences were evident even between rotavirus isolates of a single species under conditions of high stringency.

Molecular cloning and expression techniques have enabled the nucleotide sequence determination of the RNA segments and characterization of recombinant proteins after expression of the inserted plasmids in E. coli. Most of the information on gene coding assignment has been obtained by using SA-11 strain because this strain was the first rotavirus to be adapted to cell culture (Malherbe et al., 1963). The coding assignment of different genes has been established mostly by in vitro transcription technique and resort analysis (Mason et al., 1980; Offit et al., 1986; Urasawa et al., 1986). The genome of the SA-11 strain is AU-rich and only 6.3% of it is noncoding. Analysis of terminal sequences of RNA segments has shown that these sequences are paired end to end and contain the same terminal structures with a cap structure in the plus strand (5’ terminus). A stretch of eight conserved nucleotide 5’-AUGUGACC-3’ at
the 3'-terminus of the plus-strand was observed in human, bovine and porcine rotaviruses (McCrae and McCorquodale, 1983). The secondary structure of rotaviral mRNA is crucial in genome replication, translation, assembly and maturation. These structures for SA-11 gene 8 and OSU gene 9 were deduced using the mfold program (Patton et al., 1993; Chen and Patton, 1998).

Table 2.1: Number of RVA genotypes

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of rotaviral protein (VP)</th>
<th>Acronym</th>
<th>Number of genotypes till date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP1</td>
<td>RNA dependent RNA polymerase (RdRp)</td>
<td>9R</td>
</tr>
<tr>
<td>2</td>
<td>VP2</td>
<td>Core protein</td>
<td>9C</td>
</tr>
<tr>
<td>3</td>
<td>VP3</td>
<td>Methyltransferase</td>
<td>8M</td>
</tr>
<tr>
<td>4</td>
<td>VP4</td>
<td>Protease sensitive protein</td>
<td>37P</td>
</tr>
<tr>
<td>5</td>
<td>VP6</td>
<td>Major inner capsid protein</td>
<td>18I</td>
</tr>
<tr>
<td>6</td>
<td>VP7</td>
<td>Glycoprotein</td>
<td>27G</td>
</tr>
<tr>
<td>1</td>
<td>NSP1</td>
<td>Interferon antagonist</td>
<td>19A</td>
</tr>
<tr>
<td>2</td>
<td>NSP2</td>
<td>NTPase</td>
<td>10N</td>
</tr>
<tr>
<td>3</td>
<td>NSP3</td>
<td>Enhancer of translation</td>
<td>12T</td>
</tr>
<tr>
<td>4</td>
<td>NSP4</td>
<td>Enterotoxin (ET)</td>
<td>15E</td>
</tr>
<tr>
<td>5</td>
<td>NSP5</td>
<td>Phosphoprotein</td>
<td>11H</td>
</tr>
</tbody>
</table>

(Matthijnssens et al., 2008, 2008)

2.4 Rotavirus polypeptides and their characteristics

Characterization of RVA polypeptides became the area of researcher’s attention during the late 1970s and early 1980s. Five proteins (two major and three minor) were detected for the first time by Newman and coworkers (1975) with PAGE. Similar findings were observed by Cohen and coworkers (1979). However, most of the
researchers felt that RV particles contained eight structural proteins when purified virus proteins were resolved in PAGE (Rodger et al., 1975; Rodger et al., 1977; Thouless, 1979; Kalica and Theodore, 1979; Matsuno and Mukoyama, 1979). In infected MA-104 cells, three nonstructural proteins were frequently detected (Matsuno and Mukoyama, 1979; Thouless, 1979). The 85-90 kDa polypeptide was supposed to be an inner capsid protein until Espejo and coworkers (1981) demonstrated that it was being cleaved by trypsin into two smaller products and was proposed to be an outer capsid protein.

Then the polypeptide was named as VP3 for many years, since it often comigrated with VP3 in PAGE until it was shown to be the fourth largest polypeptide VP4 (Dyall-Smith and Holmes, 1981). Radiolabelling studies of rotavirus grown in cell culture and iodination reaffirmed the earlier reports and established that VP2 & VP6 are the major proteins in the inner capsid, comprising about 20% and 80% of the viral proteins, respectively (Novo and Esparza, 1981). A total of five structural proteins (125, 94, 88, 41 and 38 kDa), three nonstructural proteins (53, 35 and 34 kDa) and two proteins (26 and 20 kDa) were detected by Ericson and coworkers (1982).

Glycosylation of the major outer capsid protein (p34K) with the periodic acid-Schiff test was revealed by Rodger and coworkers (1977), and the findings were confirmed by concanavalin A (Cohen et al., 1979). It has been found that the rotavirus dsRNA genome encodes six structural and six non-structural polypeptides (Figure 2.3). However, Rotavirus C does not encode for NSP6. The structural proteins were designated as VP while non-structural proteins as NSP followed by the number according to the molecular weight of the protein (Arias et al., 1982; Mason et al., 1983).

Important Rotavirus Protein characterized in this study

In the present study, viral proteins which are most important in inducing immune response, virus replication and maturation were chosen for analysis. VP4, the outer spike proteins plays important role in virus entry in the enterocyte. The glycosylated protein, VP7 plays important role in inducing neutralizing antibodies. Most of rotavirus detection assays are based on VP6 protein. Moreover, VP6 act as sandwich between outer capsid layer and core (Figure 2.4). NSP4 protein acts as
Table 2.2: Protein coding assignments, protein function and position of proteins in Rotavirus A

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Genome segment</th>
<th>Molecular size (bp)</th>
<th>Protein encoded</th>
<th>Size of protein (kDa)</th>
<th>Functions</th>
<th>Number of molecules per virion</th>
<th>Position in virion structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>3302</td>
<td>VP-1</td>
<td>125</td>
<td>RNA dependent RNA polymerase</td>
<td>12</td>
<td>Core</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2687</td>
<td>VP-2</td>
<td>94</td>
<td>Core shell protein</td>
<td>120</td>
<td>Core</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2592</td>
<td>VP-3</td>
<td>88</td>
<td>Guanylyl transferase, methyl transferase</td>
<td>12</td>
<td>Core</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2362</td>
<td>VP-4</td>
<td>86</td>
<td>Attachment, protease sensitive</td>
<td>180</td>
<td>Outer capsid</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>1581</td>
<td>NSP-1</td>
<td>58</td>
<td>Antagonist to interferon</td>
<td>780</td>
<td>NS (Non structural)</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1356</td>
<td>VP-6</td>
<td>43.5</td>
<td>Group and subgroup specific antigens, intracellular protection</td>
<td>780</td>
<td>Middle layer protein</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>1062</td>
<td>VP-7</td>
<td>37</td>
<td>Major neutralization protein, glycoprotein</td>
<td>Few</td>
<td>Outer capsid</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>1059</td>
<td>NSP-2</td>
<td>36</td>
<td>NTPase, NDP kinase, helix destabilization</td>
<td>Few</td>
<td>NS</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>1074</td>
<td>NSP-3</td>
<td>34</td>
<td>Inhibitor of host protein translation</td>
<td>Few</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>751</td>
<td>NSP-4</td>
<td>20</td>
<td>Viroporin</td>
<td>Few</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>666</td>
<td>NSP-5</td>
<td>21</td>
<td>Essential for viroplasm formation</td>
<td>Few</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td></td>
<td>NSP-6</td>
<td>12</td>
<td>Viroplasm formation</td>
<td>Few</td>
<td>NS</td>
</tr>
</tbody>
</table>
enterotoxin and was shown to exhibit cross reactivity with antirotavirus polyclonal serum.

### 2.4.1 VP7 protein

It is a major outer capsid protein, constitutes about 30% of the rotavirus capsid protein (Mattion et al., 1994). It is the major neutralization antigen identified by immune system. Matsuno and Mukoyama (1979) reported that this protein in glycosylated at various sites. On the basis of composition of antigenic determinants, it is utilized for G- genotype classification of virus (Estes and Kapikian, 2007). This protein is encoded by one of three dsRNA gene segment 7 (rhesus rotavirus), 8 (UK bovine virus) & 9 (SA-11), on the basis of strain (Mattion et al., 1990). Till date, 27 G-genotypes have been identified on the basis of variation in nucleotide sequence at an important antigenic region (Trojnar et al., 2013). Amino acid composition of VP7 has been deduced from the nucleotide sequence and comparison of amino acid sequences from different genotypes indicated that nine regions of sequences are highly divergent (Matthijnssens et al., 2012).

Initially single glycosylation site was found to be present at aa 69-71 of simian SA-11 rotavirus VP7 (Both et al., 1983a). Another important site of glycosylation was found at position 238-240 (Aoki et al., 2009) which could alter the immune response. Alignment of amino acid sequences from various genotype indicated two hyrophobic regions, H1 (aa 6-23) and H2 (aa 32-48), towards N-terminal (Both et al., 1983b). Mutants having deletion in H2 region of VP7 were not retained in endoplasmic reticulum and secreted despite whether glycosylated or not (Poruchynsky et al., 1985). Intensive research has been done to reveal the amino acid patch required for endoplasmic reticulum retention. Two regions (51-61 & 62-111) were detected to be necessary for endoplasmic reticulum retention, however neither of two were alone sufficient (Poruchynsky and Atkinson, 1988). Intriguingly, amino acid substitution mutation at sites 59 (Isoleucine), 60 (Threonine) & 61 (Glycine). Although, the portion of VP7 associated with endoplasmic reticulum is not fully characterized. Because signal peptide is quickly cleaved, it cannot act as an anchor (Desselberger, 2014)

Sedimentation analyses on sucrose gradient have indicated the occurrence of oligomerization in VP7 (dimers) and NSP4 (tetramers) in MA-104 grown SA-11
rotavirus (Maass and Atkinson, 1990). An association of three viral proteins (VP4, VP7 & NSP4) were detected which indicated the sites on the endoplasmic reticulum participating in the bud formation of single layered rotavirus particle. rVP7 experiments have revealed that free Ca\(^{2+}\) is important for maintaining the stability of VP7 protein in absence of other rotaviral proteins (Dormitzer and Greenberg, 1992). The concentration of Ca\(^{2+}\) required for stabilization vary between different strains (Estes and Greenberg, 2013).

2.4.1.1 Genetic variability and phylogeny based evolutionary analysis of VP7 gene

Phylogenetic analysis indicated clustering of G9P[6] and G9P[8] UK rotavirus strains in to three lineages (I to III) and into two sub lineage within lineage I. Correlations were established between VP7 sequence clustering, geographical area of G9 strains origin and P type (Iturriza et al., 2000). Khetawat and coworkers (2001) observed that the local circulating WD33 human rotavirus strain exhibited similarity with G1 serotypes after alignment of nucleotide and amino acid sequence. However, the dendrogram constructed revealed that clustering occurs with sublineage of lineage IV and not with the reference strains Wa. Rotavirus strain isolation from a 13 month old HIV negative baby of an HIV positive woman from Belgium was carried out (Rahman et al., 2003) and its VP4 & VP7 genes were sequenced. Based on the comparison of its nucleotide and deduced amino acid sequence with 15 other known genotyped strains revealed that VP7 of this strain was most closely associated with American (Se584) and an Italian (PA151) human G6 strain. The close association of VP7, VP4 and NSP4 of a human strain B4106 from Belgium with those of lapine strain of rotavirus was reported by Matthijnssens et al. (2006) and this unusual strain was proposed to be either natural reassortant between animal and human or a lapine origin strain. Further analysis of this strain was done by nucleotide sequencing of VP1-3, VP6, NSP1-3 and NSP5-6 gene.

Iturriza-Gomara and coworkers (2001) reported large scale re-emergence of Taiwan local circulating G2 strains. It was due to evasion of the immune response as a result of altered antigenic domains, conferred by point mutation (amino acid substitution) at loci 96 in the immunogenic 7-1a (Zao et al., 1999). Post-vaccination reports from Brazil and Belgium has indicated a substantial rise in number of local circulating G2P[4] strains which didn’t have any surface capsid protein common with
vaccine strain (Nakagomi et al., 2008; Zeller et al., 2010). Kulkarni and coworkers (2014) reported that Pune G1 strains have maximum similarity with each other and cluster in lineage 1 within G1 genotype and showed more similarity with Rotarix (92.8-95.2% at nucleotide and 92.9-95.4% at amino acid level) as compared to RotaTeq (89.9-92% at nucleotide and 92-94.4% at amino acid level). Belgian VP7 of G1 rotavirus lineage 1 & 2 exhibited more distance (93.3-93.6% & 94.5-96% similarity, respectively) to G1 VP7 of lineage3 strain of RotaTeq 179-9 (Zeller et al., 2012).

Amino acid variations at the important antigenic positions on the outer capsid protein VP7 can alter the ability of antibodies to interact and neutralize the rotavirus (Estes and Kapikian, 2007). It may be the probable explanation for lower efficacy of existing rotavirus vaccine in developing countries (Madhi et al., 2010). The position of antigenic domains was defined by mapping escape mutants for neutralization and determination of surface exposed amino acids that exhibited intergenotypic variations among common G genotypes (Aoki et al., 2009; Dormitzer et al., 2004; Dormitzer et al., 2002b; McDonald et al., 2002). The glycoprotein VP7 contains two structurally defined domains - 7-1 & 7-2. The 7-1 is further subdivided in to 7-1a & 7-1b (Aoki et al., 2009).

Mutants at site 238-240 has previously been shown to reduce neutralization of animal group A rotavirus by monoclonal antibodies and polyclonal hyperimmune sera (Ciarlet et al., 1994; 1997). Several studies have demonstrated that change in glycosylation status of viral proteins of Human Respiratory Syncitial virus, Influenza virus and Human Immunodeficiency virus resulted in dramatically altered immunogenicity (Palombo et al., 2000; Skehel et al., 1984, Utachee et al., 2010). Aoki and coworkers (2009) reported that the variations at position 94 and 217 could be significant as these positions are part of recognition site of antibody. Only three sites (98, 104, 201) were found conserved among all VP7 of Belgian strains and vaccine strains (Zeller et al., 2012). Fredj and coworkers (2013) found only two sites (104 & 201) completely conserved among all the Tunisian strains and vaccine strains. Kulkarni et al. (2014) found four positions conserved in VP7 epitopes of all the Pune, India strains.
2.4.2 VP4 protein

VP4 is located on the outer surface of the virion particle in the form of protruding spikes. Number of spike protein in a rotavirus particle is 60 and the length of each spike protein is about 10-12 nm (Mattion et al., 1994). This protein constitutes 1.5% of whole virus protein (Espejo et al., 1981). It is a multifunctional protein having antibody neutralization, antigenic, hemeagglutinating and fusion properties. The 88 kDa VP4 is sensitive to cleavage by protease especially by trypsin in the small intestine and yield two subunits: VP5* (60 kDa) & VP8* (28kDa) (Clark et al., 1981; Estes et al., 1981). This cleavage augments the infectivity of rotavirus and this characteristic was found to be related to 4th segment of genomic RNA. The exact site of cleavage in VP4 protein was mapped to be located in the amino acid region 241 to 247 (Lopez et al., 1985; Arias et al., 1987). The pathway of cleavage of VP4 increasing the infectivity was studied and it was found that attachment of rotavirus to sialic acid receptor is independent of protease treatment (Clark et al., 1981), however it was proposed that trypsin treatment was required for selective entry of Rotavirus (Fukuhara et al., 1988). Replication of the virus has been found to increase in the presence of trypsin (Babiuk et al., 1977; Theil et al., 1977). It has also been proposed that VP4 has a role in disease severity as it result in brush border changes and it induced action remodeling that occur after enteroctyes infection by rotavirus (Gardet et al., 2006).

2.4.2.1 Genetic variability and phylogeny based evolutionary analysis of VP4 gene

Nucleotide sequencing of VP4 genes for a number of rotaviruses has been done and alignment of nucleotide and amino acid sequence has allowed the general characteristics of VP4 to be deduced. Since the creation of first rDNA molecule in 1972, the tremendous development in molecular biology and genetic engineering have had a greater effect on functional characterization of nucleotide sequence using reverse genetics. Comparison of nucleotide and deduced amino acid sequences of local circulating strains with representative rotavirus strains and rotavirus vaccine strains has been carried out. Zeller and coworkers (2012) observed a significant difference between P[8] circulating in Belgium with P[8] of Rotarix vaccine. Fredj and coworkers (2013) reported that all Tunisian P[8] strains formed a separate clade within lineage 3 and exhibited relative distance with Rotarix and RotaTeq. Similar findings were also reported by Kulkarni et al. (2014) in Pune, India.
Mutation at amino acid position 190 may be of great importance in virus entry in cell as it has been found to influence sialic acid binding of the virus. Difference at this site may affect the efficiency of virus host interaction (Dormitzer et al., 2002a). A recent finding mentioned the difference between circulating Tunisian strains and vaccine strains (Fredj et al., 2013) and described an amino acid difference pattern similar to our study. Another study also indicated a similar pattern of mutation in Belgian rotavirus strains (Zeller et al., 2012). Sequence alignment with earlier published sequences of human group C rotavirus indicated that the VP4 encoding gene was uniquely conserved in structure with only few mutations. VP4 protein sequence of rotavirus isolate from goat GRV was found to be 96% similar with simian rotavirus (RRV) VP4 and comparatively less similar with Simian Rotavirus (SA11). Another new rotavirus genotypic constellation G6P[6] was observed by Rahman and coworkers (2003). Till date, a total of 37 P- genotypes have been identified by nucleotide sequence based method (Trojnar et al., 2013).

### 2.4.3 VP6 protein

Viral protein 6 constitutes the intermediate capsid layer of rotavirus and it contributes more than 51% of total virion protein. It is encoded by genomic segment 6. A high titer of antibodies is generated against VP6 during natural infection and due to this property, it is targeted in most of the antibody based rotavirus detection assay. Role of antibodies against VP6 is not confirmed, although some workers have reported protective role of anti-VP6 antibodies (Burn et al., 1996). In addition, VP6 also contains group and subgroup specific antigen (Yolken et al., 1978) and on the basis of these antigens rotaviruses have been classified into 8 groups (A-H) (Matthijnssens et al., 2008; Trojnar et al., 2013). Group A rotaviruses have been further subdivided into four subgroups on the basis of reactivity with subgroup specific mAbs: (a) Subgroup I (b) Subgroup II (c) Subgroup I & II (d) non I + II. Although both subgroup I and II have been found among human rotaviruses, subgroup II was detected more frequently. The majority of animal origin rotaviruses has subgroup I antigen except pig strain Gottfried. Mouse EDIM rotavirus didn’t exhibit reactivity with either of mAbs (non I + II) (Greenberg et al., 1983). Some of the horse rotaviruses were found to have both subgroup I & II antigen specificities (Hoshino et al., 1987). Most of subgroup II and I +II rotaviruses exhibited long electropherotypes whereas majority of subgroup I
rotaviruses displayed short electropherotypes (Kapikian et al., 1981). Nucleotide sequencing of human and animal rotaviruses indicated that 90% of amino acids are conserved and most of variations were detected at amino acid regions: 39-62, 80-122 & 281-315 (Both et al., 1984; Cohen et al., 1984; Estes et al., 1984).

Among 8 groups, group A is the major cause of disease in human beings (Estes et al., 2001). Subgroup I specificity was mapped to amino acid position 305 while subgroup II specificity was mapped to amino acid position 315. Substitution at these positions may affect the binding of monoclonal antibodies 255/60 (for SG-I) & 631/9 (for SG-II). Earlier reports of VP6 protein using site directed mutagenesis (Tang et al., 1997) or recombination (Lopez et al., 1994) have demonstrated that substitutions at positions 296 to 299, 305, 306, 308, and 315 could change the reactivity to the SG I- and SG II-specific MAbs. It has been found that alanine residue at position 172 also attribute to the formation of the SG-I specific epitope; however, point mutations at this position resulted in low reactivity in immunoprecipitation assay with SG –I specific monoclonal antibodies (Tang et al., 1997). Changes in amino acid sequences at important epitopes could affect the potency of the Rotavirus detection assays (Kerin et al., 2007).

VP6 protein was shown to be trimeric as VP6 proteins extracted from virus with calcium chloride displayed sedimentation coefficient as that of trimeric form in sugar density gradient purification (Gorziglia et al., 1985). Recombinant VP6 expressed in insect cell line under baculovirus expression vector spontaneously assemble into polymorphic forms (Estes et al., 1987). Recombinant VP6 produced alone or in combination with VP2 spontaneously assembled into single layered capsid like structure (Gonzalez and Affranchino, 1995). Virus like particles (VLPs) formation was also reported when recombinant viral proteins were coexpressed: VP6+VP7, VP2+VP4+VP6, VP2+VP6+VP7 & VP2+VP4+VP6+VP7 (Sabara et al., 1991; Crawford et al., 1994).

Strongest humoral immune response is generated against VP6 protein during rotavirus infection both in humans and animals (Svensson et al., 1987a, 1987b; Ishida et al., 1997; Colomina et al., 1998). Humoral immune response to this protein was quick, increase in antibody titer was detected during 19-40 days after infection and remained high even four months after infection when detected with immunoblotting by
using sera of rotavirus diarrhea infected children. Tc-cell epitopes domain (Franco et al., 1994) and Th-1 cell epitopes domain (Banos et al., 1997) have been detected for VP6 protein. A stretch of amino acid from 283 to 307 having a Th-1 cell epitope could provide complete immunity in BALB/c mice (Choi et al., 2000). It has been demonstrated that VP6 can prime an augmented neutralizing humoral response, and thus Th cells particular to VP6 provide inducing help to VP7 and VP4 specific B cells for neutralizing antibodies (Esquivel et al., 2000).

2.4.4 NSP4 protein

Nonstructural protein 4 (NSP4) is the first viral enterotoxin described (Ball et al., 1996). The activity of enterotoxic region was mapped to domain 114-135 and binding to enterocyte start a signal transduction pathway which results in increased intracellular calcium concentration. It leads to decreased absorption of sodium ion & water and increased secretion of chlorine ion (Ball et al., 1996). NSP4 has been shown to produce a heterotypic antibody response during rotavirus illness (Ray et al., 2003). NSP4 play an important role in rotavirus morphogenesis as it acts as intracellular receptor for budding of DLP in to the endoplasmic reticulum (O’Brien et al., 2000). It is a 175 amino acids protein having multifunctional domains and encoded by segment 10. Synthesis of NSP4 takes place in endoplasmic reticulum as transmembrane glycoprotein and it have 3 hydrophobic domains (H1 to H3) (Bergman et al., 1989; Chan et al., 1988).

The optimum concentration of calcium was found mandatory in the budding process in the endoplasmic reticulum and subsequent morphogenesis, as Poruchynsky et al. (1991) reported accumulation of enveloped virus intermediates in calcium deficient cells infected with rotavirus. Due to calcium deficiency, change in glycosylation (N-linked) was found in NSP4 and especially in VP7. Svensson and coworkers (1994) observed that disruption of disulphide bond by reducing agents (like dithiothreitol) also leads to the formation of enveloped intermediate states of rotavirus. Due to inhibition of glycosylation, monoclonal antibody epitopes of VP7 disappeared, but not of VP4. Insect’s cells expressing NSP4 were found to have fivefold increase in calcium concentration (Tian et al., 1994). Knocking out of NSP4 gene by RNAi has demonstrated that it is essential for replication of rotavirus and morphogenesis, still the exact mechanism of NSP4 role to the above mentioned process is not clearly
understood. This protein is able to differentially interact with other cellular and viral protein: Calnexin (Mirazimi et al., 1998), Integrin I domain (Seo et al., 2008), laminin 3, fibronectin (Boshuizen et al., 2004) and tubulin (Xu et al., 2000).

It may be due to the presence of NSP4 to different compartment of the cellular machinery (Berkova et al., 2006). Although various polymeric form of NSP4 (dimeric, tetra and pentameric) including association with VP4 and VP7, the structural information is in paucity to the greatly constant patch of aa 95-135. This stretch of amino acid which form diarrhea inducing region from aa 114 to 135 constitutes a tetrameric parallel coiled coil in association with core (hydrophobic) interspersed with 3 hydrophilic amino acids at position 109 (Gln), 120 (Glu) & 123 (Gln) (Bowman et al., 2000; Chacko et al., 2011). A total of 2 of four glutamic acid residues (position 120) and four Glutamine residues (at position 123) interact with centrally located calcium ion (Bowman et al., 2000; Chacko et al., 2011). NSP4 polymerize to form a pentameric coiled structure when purified under acidic conditions and interaction with calcium at position 120/123 is absent in this condition. This indicates that NSP4 exhibit different morphology under different conditions (Chacko et al., 2011).

2.5 Replication cycle of Rotavirus

Rotavirus replicates in the cytoplasm of differentiated enterocyte in small intestine. The different steps of virus replication were studied in MA104 cell lines (Monkey Kidney Cell Line). The replication cycle is fairly quick with high yield of virus after 10-12 hours of carbon dioxide incubation at 37°C. The replication cycle of rotavirus (Figure 2.5) consists of following steps:

1) Attachment of Rotavirus to intestinal epithelium via VP4 & VP7 proteins
2) Penetration and uncoating
3) Synthesis of mRNA mediated by VP1, VP2 & VP3 protein and early translation
4) Viroplasm formation, RNA replication (minus strand synthesis) and formation of DLPs
5) Maturation of virus particles to TLP and release
1. Attachment of Rotavirus to intestinal epithelium via VP4 & VP7 proteins

This is the first step of rotavirus replication and it is a complex process. The infectious rotavirus particle interact by the spikes of VP4 (VP8\*) with the receptor present on host cell (López and Arias, 2004). The type of glycoprotein that will function as receptor for a given strain of rotavirus depends on the strain and type of host cell. Two methods of virus adhesion have been reported: sialic acid dependent and sialic acid independent method of virus adhesion to host cells. The exact sialic acid binding site for Rhesus rotavirus VP4 has been identified (Dormitzer et al., 2002a). Rotaviruses exhibiting sialic acid dependent entry infect only the apical surface of polarized enterocyte cells, whereas viruses which are sialic independent in attachment, infect apical as well as basolateral surface of nonpolarized enterocytes (Isa et al., 2006). The virus attachment with receptor is mediated by VP8\* subunit of VP4 protein and it requires proteolytic cleavage of VP4 protein. Recently, it has been found that some rotaviruses like G10P[11] bind to histo blood group antigen (HBGA) instead of sialic acid via the Gal β-1–4GlcNAc motif (Hu et al., 2012; Ramani et al., 2013).

2. Penetration and uncoating

Binding of VP4 protein to cellular receptor results in conformational change in such a way that the hidden lipophilic domains of VP5* are exposed. It results in the formation of a post penetration umbrella like conformation. Exposure to trypsin treatment probably favour this change in conformation. Certain rotavirus strains resistant to trypsin treatment exhibited delay in cellular entry as well as replication, compared to wild type strains (Trask et al., 2013). The exact mechanism of virus entry in the cell is still not clear. It is proposed that it may be receptor mediated endocytosis or direct membrane penetration by the virus. The whole genome RNAi analysis has revealed the involvement of ‘endosomal sorting complex for transport’ components (Silva-Ayala et al., 2013). Diaz-Salinas et al. (2014) reported that some rotaviruses have requirement of Rab7 mediated transport for the usage of endosomal pathway.

3. Synthesis of mRNA mediated by VP1, VP2 & VP3 protein and early translation

Once inside the cell, the synthesis of mRNA and negative strand is initiated by the viral RNA-dependent RNA polymerase (RdRp). The virus carries its own transcription complex which include VP1, having property of RdRp and VP3, the
capping enzyme (having property of methylase, phophodiesterase and guanylyltransferase). The transcription complex is located at the inner surface of core formed by VP2 at the 5 fold symmetry axes (Jayaram et al., 2004). Every viral RNA segment is complexed with separate transcription complex (Periz et al., 2013). Rotaviral double layered particle (DLP) produce capped non-polyadenylated, single stranded RNA transcripts from the template (-) strand of the viral genomic RNA, released through class I channels. This (+) sense RNA can serve as mRNA for translation process or can act as a template for the synthesis of (-) sense RNA.

4. Viroplasm formation, RNA replication (minus strand synthesis) and formation of DLPs

Because of the consistent length of rotavirus double stranded RNA, It cannot be accommodated into the core. Instead, 11 dsRNA fragments are reasserted, associate with the core protein of the virus and then packaged. Trask et al. (2012) reported that the initial replication assembly of ssRNA with VP1/VP3 associated with VP2. This step most likely involves NSP2 and NSP5 protein (Berois et al., 2003). The negative charge present on RNA has to be balanced during this process either by divalent cations or trivalent cations from cellular origin (Desselberger et al., 2013). Boudreaux et al. (2013) observed that for attachment of ssRNA and for encapsulation of VP1, VP2 (N-terminal domain) play an important role. Association with VP2 is prerequisite for RNA dependent RNA polymerase activity of VP1 (Zeng et al., 1998). The exact molecular process of rotavirus early morphogenesis is still not clear. Particularly, it is not well understood that how only 11 set of dsRNA segments are packaged in a single virion particle. Once packaging of viral genome in core particle occur, the VP6 rapidly transcapsidate the core and form double layer particle (Desselberger et al., 2013).

5. Maturation of RV particles and release

Double layered particle of rotavirus leave the viroplasms and moves toward endoplasmic reticulum for budding. NSP4 play a very crucial role in this process (Taylor et al., 1996) as it serves as an intercellular receptor for binding with VP6 protein. It has been reported that the interaction of NSP4 with double layered particle is a very important step and silencing of NSP4 expression by siRNA leads to maturation defects in rotavirus particle and in turn resulted in inhibition of rotavirus replication.
This lipid enveloped is acquired by the nascent rotavirus particle temporarily and it is lost when rotavirus acquire another layer of 60 trimers of VP4 & 260 trimers of VP7. The exact mechanism of origin, role and loss of temporary lipid envelope is not known (Estes and Greenberg, 2013). Furthermore, it is proposed that the spike protein, VP4 interact with VP6 initially. After this process, VP7 trimers form the outermost layer (Trask and Dormitzer, 2006). Release of mature virus particles occurs via cell lysis from non-polarized cells and via a kind of budding from polarized cells (Gardet et al., 2006). In case of immunocompromised patients and cell culture, rotavirus can cause persistent infection (Mrukowicz et al., 1998). The detailed mechanism of this persistent infection is poorly known.

2.6 Rotavirus genetics and reverse genetics

Rotavirus genetics has been thoroughly studied to have in-depth knowledge of various combinations of G- and P- genotype, to better understand the phenomenon of reassortment between different strains and to allocate different gene function. Rotavirus genetic studies are mainly based on temperature sensitive (ts) mutants grown at non permissive temperatures (Criglar et al., 2011). A total of 10 out of eleven reassortant groups have been recognized. Nine of them have been allocated dsRNA genome segments (Vande et al., 2013). A number of other ts mutants of rotavirus strains have been identified for rotavirus genetics studies. The observed phenotypes of these temperature sensitive mutants were, according to the role allocated to that specific genome segment. Early phase of retrovirus replication gave the maximum number of reassortant at non-permissive temperature and these mutants can intervene in the replication of wild kind rotavirus (Estes and Desselberger, 2012). Reassortment of temperature sensitive mutants has been successfully carried out in vitro to obtain progeny of the required set of genome combinations. These mutations have been used to assign the temperature sensitivity to a specific segment and to extrapolate the biological role of particular gene fragment (Desselberger, 2014). Several systems for reverse genetics studies have been developed and all of these systems require helper virus and stringent selection criteria. Viral protein-4 gene synthesized from recombinant VP4 plasmid was rescued into the mature virion particle due to the presence of specific antibodies against helper virus VP4 (Komoto et al., 2011). A mechanism based on dual selection criterion (siRNA against the helper virus gene and
temperature sensitive mutant at non-permissive temperature) has been utilized to study single gene reverse genetics (Trask et al., 2010). Till date, no rotavirus reverse genetics study mechanism which is helper virus free, reproducible, universally utilizable, has been developed. But the same system has been developed and working successfully for Orthoreovirus (Richards et al., 2013).

2.7 Molecular pathogenesis and manifestation of rotavirus illness

Rotavirus primarily affects the mature intestinal lining cells (enterocytes) present at the top of the small intestinal villi. A characteristic vacuolization and loss of epithelium is observed, followed by hyperplasia of crypt cells. Though rotavirus has been detected in extra-intestinal locations, but the importance of these findings in healthy individual is still contentious (Blutt and Conner, 2007). In contrary to a healthy individual, rotavirus can multiply in the liver, pancreas and biliary system of immunocompromised patients and may lead to pancreatitis and biliary atresia (Feng et al., 2008). A very surprising finding in animal model is the occurrence of diarrhea at early point of time when no histopathological change appears in small intestine (Ward et al., 1996). It may largely due to secretion of viral enterotoxin (NSP4) which usually form polymers of different sizes (Estes and Greenberg, 2013). The pathogenesis of rotavirus is attributed to many factors: heterogenicity of viral-host interaction, age of patient, viral gene products (VP4, VP7, VP6 & NSP4; Ball et al., 1996; Estes and Greenberg, 2013).

Rotavirus disease mechanism may involve many factors as: disruption of normal absorption, shortening & stuntin of intestinal villi, action of viral enterotoxin (NSP4). It also involves action of intestinal ganglia stimulation (Lundgren et al., 2000). Recently it has been reported that rotavirus also multiply in enterochromaffine cells in the small intestine. This multiplication causes the release of serotonin from these cells, which in turn stimulate the emetic center present in the brain stem (Hegbom et al., 2012). Mohanty and coworkers (2013) reported that infection of rotavirus in newborn mice can lead to biliary atresia, which in turn can result in obstructive cholengitis. The severity of this illness depends on age of newborn mice and innate immune system developed. Hertel et al. (2013) that these new born mice can be protected from obstructive cholengitis and biliary atresia by either vaccinating the dam with
recombinant rotavirus like particles or by administering polyclonal sera against rotavirus to the newborn mice prior to infection.

A very interesting area of scrutiny of intestinal disease is virome analysis by metagenomic approach. This virome data can be correlated with metagenomic data of other microbes and severity of disease (Minot et al., 2013). By this method, viruses which are uncultivable or not previously reported can be discovered. Till date, DNA viruses (mostly bacteriophage) have been analyzed by this method. For analyzing DNA and RNA viruses in stool samples, protocols have been standardized (Sachsenroder et al., 2012). These protocols have been used in the analysis of human intestine virome (Ryan et al., 2013). It will be interesting to observe that up to what extent the intestinal virome affect the severity of disease in combination with other microbes.

Rotavirus is the most important cause of acute viral gastroenteritis in children below 5 years of age. It causes death of 4,53,000 children each year worldwide and this mortality rate represent 37% of global diarrhea related mortality in children and 5% of the annual death rate in children below five years (Tate et al., 2012). More than half of rotavirus associated mortality occur in five countries: Congo, India, Ethiopia, Pakistan and Nigeria. India alone contributes more than 22% of global rotavirus associated mortality as India have 11% of its population under five years of age (John et al., 2014). The important facts of rotavirus gastroenteritis in India (John et al., 2014) are as follows:

- 11,373,098 cases of diarrheic episodes.
- 3,271,187 outpatient visits to various government and private hospitals.
- 8,72,315 cases of hospitalization due to severe diarrhea, vomiting and dehydration.
- 78,583 cases of death due to severe dehydration and shock.
- 4,53,000 rotavirus associated mortality worldwide (Tate et al., 2012).

2.8 Age of patients affected by rotavirus infection

Although rotaviruses have been reported to cause gastroenteritis in children above 5 years of age, adults and old age people, but the severity and percentage affected is much more in children below five years of age. Children below 6 months also exhibited rotavirus gastroenteritis in milder forms and very less percentage. It may
be attributed to the presence of maternal antibodies. However, they are not completely immune and neonate infections have been reported most commonly from nurseries, crutches in many countries including India (Bishop, 1996; Estes and Greenberg, 2013; Desselberger, 2014). The major rotavirus disease burden has been reported in the age group 6-23 months of age (John et al., 2014; Saluja et al., 2014; Tiku et al., 2014). The risk of rotavirus infection gradually decreases after 2 years of age.

Rotavirus infects adults and other age groups also quite frequently, but these infections are mostly asymptomatic as a humoral immune response developed due to previous exposure, protect from severe rotavirus illness. Several studies indicated that 55% of adults have immunological evidence of rotavirus infection during childhood (Anderson et al., 2004; Kapikian et al., 1983). Another interesting report from India revealed transmission of rotavirus from children to older persons and infection exhibited in the form of diarrhea (Awachat and Kelkar, 2006). Few outbreaks of rotavirus have been reported in Finland army recruits and in old age wards (Anderson et al., 2004). Almost every child gets rotavirus exposure upto age of 5 years. Therefore, immunocompetent adults acquire asymptomatic or milder form of rotavirus gastroenteritis whereas immunocompromised patients may exhibit mild to severe rotavirus diarrhea. Group B rotaviruses (GBR) have been found associated with outbreak of diarrhea in adults in China, Bangladesh and India (Krishnan et al., 1999; Yang et al., 2002; Barman et al., 2006; Saiada et al., 2011).

2.9 Hospital borne infection

Several studies have reported the occurrence of hospital borne rotavirus infection in neonates and children below five years of age (Bishop et al., 2001; Gelber and Ratner, 2002). During a 10 year study in Germany, it was reported that hospital borne rotavirus infection in premature infants contributed 26% of rotavirus disease burden (Piednoir et al., 2003). Another study conducted in a hospital paediatric ward in France revealed that the occurrence of nosocomial acquired rotavirus infection was 6.6% during a winter diarrhea outbreak (Piednoir et al., 2003). These reports of hospital borne infection clearly point towards the substantial risk and increased cost of treatment due to nosocomial infection.
2.10 Rotavirus disease transmission

Transmission of disease occurs mainly by ingestion of contaminated food and water (fecal-oral route), although fomites have also been found to cause disease transmission in some cases. In the initial studies, when rotavirus contaminated water was given by oral route in volunteers, diarrhea disease occurrence was reported (Bernstein et al., 1986; Kapikian et al., 1983). However, Jian and coworkers (1991) reported detection of rotavirus particles from the upper respiratory mucosa. It may be indicating involvement of respiratory route in rotavirus transmission, but such study were very few and didn’t provide substantial evidence. Due to absence of lipid envelope, rotavirus can survive in environment for a longer time at optimum temperature and humidity. In an interesting study, it was reported that some rotavirus (positive) stool samples left unintentionally outside in the environment due to armed conflict in West Africa for 2.5 months, exhibited positive reaction by ELISA and RT-PCR and retained infecting capability (Fischer et al., 2002). In contrast, some studies revealed that rotavirus cannot survive for long period under high humidity (80%) conditions (Ansari et al., 1991; Estes et al., 2001). It has been found that efficient disinfection and following good hygienic and sanitary protocols drastically reduce the rotavirus disease burden in hospital settings, nurseries and crutches (Estes and Greenberg, 2013; Desselberger, 2014).

The importance of animals in the spread of this zoonotic disease cannot be undermined in India, as people share the same socioeconomic environment with animals due to agriculture based farming. It is supported by the studies indicating natural occurrence of animal strains in human diarrhea disease or reassortment formation with human strains (Das et al., 1993; Nakagomi and Nakagomi, 1993; Midgley et al., 2012). Although this reassortant phenomenon is sporadic, human-porcine or human-bovine reassortant strains were found at a higher frequency in Brazil and India (Das et al., 1993; Midgley et al., 2012).

2.11 Clinical features and treatment

The incubation period for the disease has been reported to be about 2 days (Ramig, 2004). In immunocompetent persons, the disease is either asymptomatic or mild and it is self limiting. The incubation period may be 1 to 4 days in adults (Ramig,
2004; Yolken and Wilde, 1994). Although rotavirus cause disease in all age groups, but children are at highest risk of developing rotavirus diarrhea. The disease may be asymptomatic or acute gastroenteritis may lead to mild to severe dehydrating diarrhea and vomiting. Onset of diarrhea may be accompanied by fever (37.9°C or above). Other symptoms may include anorexia, letharginess, irritability, pharyngeal erythema, swollen cervical lymph nodes. In immunocompromised, bone marrow transplant and hepatitis patients, rotavirus was shown to produce chronic infection (Yolken and Wilde, 1994; Patel et al., 2010) and during chronic infection, more number of atypical reassortant strains was reported.

Interestingly, rotavirus infection was found associated with intestinal intussusception in Japan, where 11 out of thirty intussusception cases were rotavirus positive. Another investigation revealed rotavirus infection in 33% of intussusception cases (Konno et al., 1978; Mulcahy et al., 1982). However, these studies failed to establish any correlation of rotavirus infection with intestinal intussusception (Mulcahy et al., 1982; Staatz et al., 1998; Ward et al., 1996). Furthermore, Simonsen et al. (2005) reported a strong correlation between first rotavirus vaccine RotaShield licensed in USA in 1998 with intestinal intussusception in vaccinated children and due to this vaccine was withdrawn from the market one year later. Death due to rotavirus infection occurs due to severe dehydration, electrolyte imbalance and cardiovascular shock. Mortality due to rotavirus diarrhea is rare in industrialized countries due to availability of medical facilities, but death is a frequent consequence of rotavirus diarrhea in less industrialized countries, i.e. Sub-Saharan countries and South-east Asian countries (Tate et al., 2012).

Most effective treatment of rotavirus diarrhea is timely rehydration (Atia and Buchman, 2009; Guarino et al., 2012). Different compositions of oral rehydration solution have been tested and advised (Guarino et al., 2012). Rice water has also been used successfully to treat the dehydration condition (Pizarro et al., 1991). Administration of anticholinergic compounds and loperamide has not been advised. Encephalinase inhibitors (like Racecadotril) can be used for decreasing the enteric nervous activities, increased due to rotavirus infection (Lundgren et al., 2000). In some cases, a serotonin receptor antagonist was also found useful in decreasing the severity of rotavirus diarrhea. Rotavirus VP6 specific single chain antibody fragments derived
from llama were found protective due to cross reaction in vitro and decreased shedding of rotavirus particles from infected neonatal mice (Garaicoechea et al., 2008; Aladin et al., 2012). These single chain antibody fragments were found effective in decreasing virus shedding from gnotobiotic piglets (Vega et al., 2013) and also proved to have some efficacy against human infection of rotavirus (Sarker et al., 2013; Kang, 2013).

2.12 Methods of Rotavirus Diagnosis

Proper and timely diagnosis of rotavirus is very important for the formulation of appropriate disease control plan. A number of rotavirus diagnosis methods have been developed and all the following methods have some advantages and disadvantages, but the ideal diagnostic procedure should have high specificity, sensitivity and should have reproducibility:

1. Electron Microscopy
2. Cell culture based detection
3. Enzyme linked immunosorbent assay (ELISA)
4. Latex Agglutination
5. RNA-PAGE (Polyacrylamide gel electrophoresis)
6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)
7. Nucleic acid hybridization
8. Restriction Fragment Length Polymorphism
9. Real Time Reverse Transcription PCR

1. Electron Microscopy

Rotavirus shedding occurs in high quantity from first to fourth day of disease. Virus particles can be directly visualized in stool samples after negative staining using electron microscopy as rotavirus particle has a distinctive morphology (Figure 2.1). The capsid consists of three parts: Outer shell, intermediate shell and core. Outer and intermediate shell can be clearly visualized under cryomicroscopy (Prasad et al., 1988). The whole rotavirus particle is icosahedral in symmetry, non-enveloped and 75 nm in
Immuno electron microscopy is more sensitive (100 times) than negative staining electron microscopy and can detect approximately $1 \times 10^6$ particles/ml which is equivalent to ELISA detection limit (Rubenstein and Miller, 1982). The main problem with the immune-electron microscopy is difficulty in standardization as composition of antibodies may differ from one laboratory to other place. These antibodies may not be able to detect all genotypic combinations of rotavirus. In addition, if the quantity of rotavirus in stool sample is low, the antigen-antibody aggregates may be missed on the grid (Dennehy et al., 1990).

2. Cell culture based detection

Although culturing of wild strains of Rotavirus in cell culture is difficult but then also, the virus particles can be recovered from the samples by cell culture with an appropriate success rate. It is the most conventional way of Rotavirus confirmation in the stool sample. It may require initial adaptation of wild strain in primary monkey kidney cell line before growing in continuous type of monkey kidney cell line. Trypsin treatment is required for growing rotaviruses in MA-104 cell line and virus presence can be detected by characteristic cytopathic effects (CPE) which include rounding of cells, detachment from substrate etc. However, this method cannot be applied for large epidemiological studies as it is cumbersome, expensive, labor intensive and time consuming process. Some rotavirus strains, i.e. SA11 can be easily cultivated in the cell culture system and rescuing of VP7 gene by reassortment can be used for adaptation of wild type virus (Greenberg et al., 1982). Success rate of this method is also limited. Cell culture adaptation of wild type rotavirus strains has made serotype identification more feasible.

3. Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) has become the standard test for rotavirus diagnosis. Earlier polyclonal serum was used for antigen detection and percentage of false positive results was high. However, this deficiency was rectified after development of confirmatory/blocking ELISA (Kok and Burell, 1989). Monoclonal antibody (Mabs) directed against VP6 was used for development of ELISA which was more sensitive and specific than polyclonal antibody based ELISA (Dennehy et al., 1988 and 1990). But Mabs based assay is more expensive than
polyclonal antibody based assay. When different commercial ELISA based kits were compared (according to manufacturer’s instructions), significant difference was found in sensitivity and specificity (Kok and Burell, 1989; Gomara et al., 2001). Monoclonal antibodies have also been used for subgroup determination within group A Rotaviruses. Enzyme immunoassay has also been developed for diagnosis of group B & group C Rotaviruses (Coulson et al., 1987). An antibody based assay for G- and P- genotyping has also been developed (Coulson, 1996).

4. Latex agglutination test

This test is based on macroscopic detection of agglutination reaction due to antigen-antibody interaction. Latex particles coated with anti-rotaviral antibodies interact with specific antigens present in stool sample to produce agglutinates which are visible macroscopically. This method is used in hospital settings for quickly diagnose the cause of diarrhea. Although this method is simple, quick and easy to perform, but chances of false positive are more due to non specific reaction against other viruses. Samples positive by latex agglutination test need to be confirmed by ELISA or RT-PCR.

5. RNA-PAGE (Polyacrylamide gel electrophoresis)

The segmented ds RNA genome of Rotavirus can be separated by native polyacrylamide gel electrophoresis. Each kind of group gives a characteristic signature pattern of migration. This unique pattern of migration can be visualized by silver nitrate or ethidium bromide staining (Coulson et al., 1987; Dunn et al., 1993). RNA-PAGE has been used as a diagnostic assay for detection and identification of segmented RNA viruses (Rotavirus, Blue tongue virus, Birnavirus etc.). The RNA-PAGE method is highly specific and used worldwide for epidemiology of this pathogen in a community. Genetic differences between strains of a genotype can be directly correlated with the similarity and difference in migration pattern (Dunn et al., 1993; Gouvea et al., 1991).

The significance of RNA-PAGE lies in its capability to separate the rotavirus segmented genome into 11 bands. This method has been used both in poor, developing and developed countries during large rotavirus outbreaks. Espejo and coworkers (1977) reported the use of electropherotyping as an epidemiological tool for the first time. In that study, two kinds of RNA migration patterns, ‘short’ (due to slow migration of 10th
and 11th segment) and long (due to faster rate of migration of 10th and 11th segment) were reported. This assay can be used as a detection method of rotavirus disease (Kasempimolporn et al., 1988) as well as for studying its molecular epidemiology (Estes et al., 1984). But its importance in subgroup analysis is subtle except in some association of some serotypes and electropherotype i.e. Human subgroup I rotaviruses are mostly associated with short electropherotype whereas human subgroup II are associated with long electropherotypes (Kalica et al., 1981).

The detection limit of RNA-PAGE is about $10^8$-$10^{11}$ viral particles shedded per gram of stool sample. The main advantage of this technique lies in the fact that it can be used to differentiate virus subgroup, reassortant and non-cross reactive strains (which are missed in serological assays) (Pereira et al., 1983). However, variations in compositions and conditions of polyacrylamide gel electrophoresis can also result in change in electrophoretic pattern (Espejo et al., 1984). Therefore the method is not completely reliable. In addition, this is time consuming, labor intensive and requires expert personnel.

6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Since the invention of Polymerase Chain Reaction by Kary Mullis and coworkers (1986) for in vitro amplification of a specific segment of DNA by using specific primers and Taq DNA polymerase, the utilization of this technique in molecular diagnostics has increased several folds. It is now treated as indispensible for molecular diagnostic procedures and has attained paramount importance due to very high sensitivity, specificity and low cross reactivity. Reverse transcription polymerase chain reaction is currently used for diagnosis and genotyping of rotavirus isolates in several countries. Gouvea and coworkers (1990) described the method of VP7 gene based G- genotyping of rotavirus for the first time using genotype specific primers. In another study, six VP7 genotypes (G1-G4, G8 and G9) were reported in human group A rotaviruses (Nakagomi and Nakagomi, 1991) and genotyping could be done for 89% of the samples using genotype specific primers. Viral protein 4 based genotyping (P-genotype) was described for the first time by Gentsch et al. (1992), describing five genetically distinct P genotypes. Another protocol developed by Taniguchi et al. (1992) for human as well animal rotaviruses, was reported to have more sensitivity (93%) than the ELISA (82.6%). In India, Minakshi et al. (2001) developed protocol for genotyping
of bovine group A rotaviruses. RT-PCR is 100,000 times more sensitive than that of RNA-PAGE and ELISA (Coulson et al., 1999).

7. Nucleic acid hybridization

Nucleic acid hybridization techniques have played very important role in establishing genetic relationship of genome segments between various rotavirus genotypes, even before the invention of reverse transcription polymerase chain reaction and nucleotide sequencing technology. This technique is specific and sensitive similar to RNA-PAGE, direct electron microscopy or ELISA (Eiden et al., 1987). It can be used for detection of non-expressed genes (defective genes) as it is less dependent on expression of genome. This technique employs in situ immobilization of denatured dsRNA genome on nitrocellulose membrane. Dot blot hybridization was reported to be 10 to 100 times more sensitive than ELISA (Greenberg et al., 1981; Flores et al., 1983). Viral genomic RNA concentration as low as 8 picograms of purified homologous viral RNA can be detected by using this method. Heterologous viral RNA detection limit has been found to be 0.5 to 31 ng (Dimitrov et al., 1985). However, variation in type of probe and hybridization stringency conditions resulted in change in sensitivity and specificity of dot hybridization technique (Pedley and McCrae, 1984; Dimitrov et al., 1985; Broor et al., 1995).

8. Restriction Fragment Length Polymorphism (RFLP)

Analysis of restriction endonuclease digestion of wild type rotaviruses is a powerful technique to get a fine understanding of genome diversity of rotavirus strains prevalent in a community. It can be used for establishing genetic and antigenic relationship between wild type rotaviruses in different geographical sites (Palombo et al., 1997). In addition to monitoring of genetic difference between different rotavirus strains within a niche, RFLP can also be used as a tool for examination of cross species transmission and evolution of rotavirus strain. Restriction enzyme profiling of complete length as well as partial length PCR products of VP7 and VP4 genes have been used to examine intratypic variations between bovine rotaviruses (Minakshi, 1999).
9. Real Time reverse transcription – polymerase chain reaction (qRT-PCR)

Although rotavirus shedding occurs in large quantity during first to fourth day of infection, but in very early infection as well in environmental samples (drinking water, sewage water, medical waste, food products etc.), the quantity of virus particles may be very low. The conventional rotavirus detection assay cannot be used for detecting rotavirus in these conditions. Real time amplification of cDNA (from reverse transcription of viral genomic RNA) gives accurate results about the quantity of rotavirus particles in the stool as well as environmental samples. Some of the earlier reports published could not succeed in detecting more than one (Kang et al., 2004) or three (G1, G2 and G4) (Pang et al., 2004) G- genotypes. In India, closely shared habitat of human and animals has increased the chances of genetic reassortments among different rotaviruses. It will further increase the genotype complexity in this region.

2.13 Epidemiology of disease

Rotavirus associated acute gastroenteritis accounts for one third of these deaths in this age group (Parashar et al., 2009). In developed as well developing countries, nearly every child up to the age of five year will contact rotavirus infection (WHO, 2013). About 86.6% of rotavirus associated deaths occur in these settings (Parashar et al., 2009; WHO, 2013). A large proportion of the Indian population is prone to acute rotavirus gastroenteritis as 11% of the population is below 5 years out of 1.2 billion people. India, alone accounts for one fifth (22%) of global rotavirus mortality (Parashar et al., 2009). This huge burden of disease also affects the economy of the country in an adverse way. Tate et al. (2009) reported that average money spent per case in the treatment of severe gastroenteritis in Vellore, India come to 5.8% & 2.2% of house annual income in large and small hospital settings, respectively. According to latest estimates, rotavirus diarrhea is responsible for 78,500 deaths, 8,72,000 hospitalizations and outpatient Paediatrician visits of 3.2 million cases below five year of age. About 59366 deaths out of 78,500 occur in the first two year of life (John et al., 2014).

A number of surveillance studies were carried out in other parts of India showing varying percent positivity for group A rotavirus. Most of these studies used VP6 antigen-antibody reaction for rotavirus detection. Kelkar and coworkers (1999) reported rotavirus positivity in 266 (28.15%) out of 945 diarrheic stool samples
collected in the time span between July 1992 and June 1996 from the children below five years of age in Pune. In a similar study carried out in Chennai found that out of 745 stool samples screened, 168 (22.55%) were found to be positive for RVA (Saravanan et al., 2004). A four year (1995-1999) surveillance study in Vellore which included screening of 602 diarrheic stool samples, reported 126 samples positive (21%) (Kang et al., 2002). Das and coworkers (2002) conducted a two year surveillance (1998-2000) in Kolkata and screened 2114 diarrheic stool samples from the children below 4 years of age at B. C. Roy Children’s Hospital, Kolkata. A total of 266 stool samples was found positive for group A rotavirus.

Epidemiological study conducted in Delhi during 2000-2001 involved screening of 584 diarrheic stool samples and reported 137 cases (23.5%) exhibiting presence of group A rotavirus (Bahl et al., 2005). A similar study carried out in Vellore during 2002-2003 indicated 27.4% positivity for rotavirus (Baneerjee et al., 2006). An increase rate of detection was reported from Kolkata and Berhampur during a two year study (2003-2005) in which 36.3% of diarrheic stool samples were found positive (Samajdar et al., 2006). Mishra and coworkers (2010) found 79 diarrheic stool samples positive out of 412 recruited patients under 3 years of age in Lucknow. Surveillance studies carried out in Kolkata (2005-06) and Delhi (2005-07) revealed 37.3% & 36.9% sample positivity (Samajdar et al., 2008; Chakravarti et al., 2010) which were higher as compared to that of Lucknow. In an epidemiological study done in the Northeast Indian city, Manipur, very high sample positivity (49%) was observed during 2005-2008 from children below five years of age (Mukherjee et al., 2010). In a multicenter Indian Rotavirus surveillance network study conducted in 4 laboratories and 10 hospitals in 7 different regions of India during 2005-2007, it was reported that 39.2% of diarrheic stools samples were positive for group A rotavirus from children below five years of age (Kang et al., 2009). Tiku and coworkers (2014) reported 38.4% sample positivity from two hospitals (AIIMS from South Delhi & Kalawati Saran Children’s Hospital from Central Delhi) out of 756 samples collected during 2007-2012.

An epidemiological surveillance study conducted between July 2009 and June 2012 at two hospital settings in South India (Christian Medical College, Vellore; Child Jesus Hospital, Trichy) and one in North India (St. Stephen’s Hospital, Delhi) revealed 39% sample positivity out of 1191 diarrheic stool samples during 2009-2012 (Babji et
al., 2014). Saluja and coworkers (2014) collected 2051 samples from 12 sites across India (Ludhiana, Lucknow, New Delhi, Chandigarh, Jaipur, Hyderabad, Mysore, Bangalore, Manipal, Goa, Kolkata, Bhubaneshwar) and report 26.4% positive samples. In addition to hospital based studies, community based studies were also conducted in various parts of India i.e. Pune (Kelkar et al., 2001), Vellore (Banerjee et al., 2006). These community based cohort studies indicated relatively low sample positivity (15.5% & 7.1% in Pune and Vellore respectively) as compared to hospital setting studies.

2.14 Strain diversity and antigenic epitope difference

Due to antigenic drift, mutations are accumulating in the rotavirus strains and reassortment between different genotypes lead to formation of new reassortant. Till date, a total of 27 G- & 37 P- genotypes have been identified in man and animals (Trojnar et al., 2013; Matthijnssens et al., 2014) however only 10G- & 11P- genotypes have been reported commonly in Human being. Furthermore, reassortment of different G and P genes could theoretically lead to hundred of G type and P type constellations, but only five strain combinations (G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]) are associated with 80-90% of rotavirus disease in children worldwide (Gentsch et al., 2005; Santos et al., 2005; Banyai et al., 2012). However, substantial differences in most prevalent circulating strains have been observed in different geographical and socioeconomic settings (Banyai et al., 2012). G1P[8] was most commonly detected genotype combination in India followed by G2P[4] according to reports from other parts of India (Banyai et al., 2012; Kang et al., 2013; Tiku et al., 2014, John et al., 2014; Saluja et al., 2014; Mullick et al., 2014; Namjoshi et al., 2014). G1 strains were most prevalent worldwide from 1996-2007, although relative prevalence has decreased during this period. Furthermore, in Indian subcontinent G2 strains were most prevalent during 1996 to 1999. During the 1990’s, there was emergence of G9 strains and it increased dramatically during 2000-2003 (Banyai et al., 2012).

and the appearance of unusual strains like G1P[6] (4%) and G2P[6] (4%). A very high proportion of mixed infection (23%) was also reported in this study (Saluja et al., 2014). Change in century also witnessed the emergence of G12 strains which were not detected in earlier studies. Studies conducted between 2005 to 2009 indicated about 9% G12 strains in India, which reveal the steady state of rotavirus transmission (Kahn et al., 2012).

The genotypes G1 and G2 were the most frequently detected strain in the studies conducted from 1983 to 2009 in India. The average prevalence of G1 and G2 during this period was 31.4%, 29.4% respectively. Non G1, G2 strains detection rate varied over the time. G3 has transcended from 4th most commonly detected strain before 1994 to the least common in the recent studies (1.2%). Similarly, G4 genotype also transitioned from 31% detection rate before 1994 to 4% in 2005-2009. This drastic decline in G3 and G4 strains was accompanied by emergence of G9 strains which reached the peak after 2000 and detected at 15% (Miles et al., 2012).

The position of antigenic domains was defined by mapping escape mutants for neutralization and determination of surface exposed amino acids that exhibited intergenotypic variations among common G genotypes (Aoki et al., 2009; Dormitzer et al., 2004; Dormitzer et al., 2002b; McDonald et al., 2002). The glycoprotein VP7 contains two structurally defined domains- 7-1 & 7-2. The 7-1 is further subdivided in to 7-1a & 7-1b (Aoki et al., 2009). VP4 protein is cleaved into VP5* & VP8* by trypsin in small intestine and this step is a prerequisite for entry of rotavirus in the enterocyte (Estes and Desselberger, 2012). The VP8* (VP4) region consists of four antigenic domains which are surface exposed (8-1, 8-2, 8-3, 8-4) and VP5* consists of five domains (5-1, 5-2, 5-3, 5-4, 5-5). Difference at site 90 may affect the efficiency of virus host interaction (Dormitzer et al., 2002a). A recent finding mentioned the difference between circulating Tunisian strains and vaccine strains (Fredj et al., 2013) and described an amino acid difference pattern. Another study also indicated a similar pattern of mutation in Belgian rotavirus strains (Zeller et al., 2012).
2.15  Emergence of G9 and G12 genotype worldwide

Genotype G9 and G12 are emerging genotype reported worldwide in a number of studies. The G9 genotype was first reported in 1983 from Philadelphia, Pennsylvania, United States of America, but it was not detected frequently after that for 13 years. Unicomb et al. (1999) reported increase in prevalence of G9 in Bangladesh for the first time. Before that sporadic cases of G9 detection were reported worldwide. Now the G9 strain has been reported to be endemic in USA, Thailand, Bangladesh and India (Jiraphongsa et al., 2005; Ramachandran et al., 1998; Ray et al., 2007; Unicomb et al., 1999) and has become the third most commonly detected genotype worldwide. Bhan et al. (1993) detected G9 for the first in India from an asymptomatic neonate during a rotavirus outbreak in AIIMS, New Delhi. This strain G9P[11] was subsequently replaced with a more common strain G9P[6] and it may have further reassorted with P[8] genotype to for G9P[8] reassortant. This G9P[8] reassortant is more virulent than G9P[11] and G9P[6]. In India also, G9 has become third most commonly detected G genotype (Miles et al., 2012).

In the last sixteen years, G9 has been found in combination with various P types (P[11], P[6], P[8], P[10]), VP6 subgroup I antigen, VP6 subgroup II antigen, long as well as short electropherotype. On the basis of this, it has been proposed that G9 possesses a clear biological advantage over other G- genotypes in making association with various other strains (Mukherjee et al., 2009). Phylogenetic analysis of G9P[6] strain from Kolkata revealed closeness with porcine P[6]. It may indicate either a complete virus transmission across animal to human being or a reassortment event has taken place (Mukherjee et al., 2009). The first detection of G12 was reported from Phillipenes in 1990 (Taniguchi et al., 1990). Presence of G12 has been reported from USA (Griffin et al., 2002) and Thailand (Pongsuwanna et al., 2002). Review of literature revealed reports of G12 detection from India in 2003 (Das et al., 2003), from Japan in 2004 (Shinozaki et al., 2004) and from Argentina in 2004 (Castello et al., 2004). Mukherjee et al. (2010) reported G12 for the first time in Manipur. The predominant strains in his study were genotypes G1P[8] and G2P[4] (58% of the positive strains). All these cases reported were sporadic cases but findings from India have indicated the emergence of G12 in significant proportion of rotavirus causing gastroenteritis in infants and children (Samajdar et al., 2006). Primers specific for G12
were introduced in semi-nested RT-PCR kit for G- genotyping in 2000 and it will help in gathering of good epidemiological information about G12 prevalence. Continuous molecular surveillance is necessary for ascertaining the trends in the G12 distribution in the coming years.

2.16 Effect of season on rotavirus infection

Rotavirus is disseminated throughout all the countries and cause infection in almost every child below 5 years of age. In temperate weather, a characteristic pattern of increased incidence with cases of outbreaks has been observed in cooler months and that’s why it is also known as ‘winter diarrhea’. However, this typical pattern of incidence is not observed in other climatic conditions, especially tropical climate where the disease is observed throughout the year with any peak season. In a study carried out in Southern India, no association between rotavirus infection and season was observed (Mukherjee et al., 2010). Similar findings were also reported by other studies conducted in South India (Banerjee et al., 2006; Paul et al., 2014).

However, many studies (Kelkar et al., 1999; Saravanan et al., 2004; Kang et al., 2009; Ray et al., 2007; Mishra et al., 2010; Tiku et al., 2014) have reported a significant increase in rotavirus gastroenteritis during the cooler months (October to February) of the year. The percentage positivity in these studies during cooler months ranged from 59% to 72% (median 64%). It was observed that Northern India having more temperate milieu, exhibit strong seasonality as compared to Southern India (having more tropical environment) (Kang et al., 2009). Nevertheless, there are several reports from Southern India, which have reported seasonality associations with rotavirus infection (Kelkar et al., 1999; Saravanan et al., 2004; Nair et al., 2010). Therefore, the exact pattern with which seasonality of rotavirus varies with geography is still not clear.

2.17 Rotavirus vaccines

Up to late 2014, a total of 72 countries has introduced rotavirus vaccination in their national immunization program. The first rotavirus vaccine, RotaShield (quadrivalent reassortant vaccine) was found very effective in large clinical phase III trials (Santosham et al., 1997) but this vaccine was found to be highly correlated with occurrence of intestinal intussusceptions (Simonsen et al., 2005). RotaShield was
voluntarily withdrawn by the manufacturer after this finding. There are two vaccines available commercially in the international market: Rotarix and RotaTeq. However, an indigenous vaccine, Rotavac (116E) has also been licensed in India recently and will be available commercially soon. Rotavac has been reported to provide, similar to international vaccines in low income settings (Bhandari et al., 2014).

Rotarix is a monovalent vaccine containing G1P[8] which is an attenuated strain having highly antigenic characteristics (Ruiz-Palacios et al., 2006). It was licensed in India in 2008. This vaccine was highly efficacious against homologous strains and to some lesser extent to the heterologous strains like G3P[8], G4P[8] and G2P[4] (Vesikari et al., 2007). RotaTeq (Merck, USA) is a pentavalent vaccine containing reassortant from human and animal origin. Its G1, G2, G3, G4 & P[8] components are from Human rotavirus and G6P[5] strain is from bovine rotavirus origin. The vaccine has exhibited high efficacy in clinical phase III trials (Vesikari et al., 2006) and licensed in 2006 onwards in many countries across the world.

Both these vaccines were found to be highly effective in industrialized countries (Patel and Parashar, 2009; Vesikari et al., 2010, Tate et al., 2013; Rha et al., 2014) in decreasing the number of rotavirus episodes, number of outpatient visits, hospitalization and in some reports even number of deaths due to rotavirus diarrhea (Tate et al., 2013; Rha et al., 2014). No difference was observed in efficacy of Rotarix and RotaTeq in developed countries. However, very small risk of intestinal intussusceptions (1:50,000 or less) is still associated with these vaccines, but the benefit of vaccination outweighs the risk (Glass and Parashar, 2014; Yih et al., 2014). Contrary to high efficacy in developed countries, in some less industrialized countries (Mexico, Malawi, South Africa, Mali, Bangladesh, India), the effectiveness of these vaccines was reported to be significantly (20-30%) lesser (Armah et al., 2010; Zaman et al., 2010).

The exact cause of this lower efficiency was not fully understood. One reason proposed was malnourishment (Vitamin A deficiency) which has been proven on gnotobiotic pigs (Kanasamy et al., 2014). The number of rotavirus associated deaths is maximum in these less industrialized countries (Tate et al., 2012). Therefore, WHO has recommended inclusion of rotavirus vaccination in the national immunization program of all these countries since 2009 (WHO, 2013). Although Rotarix provide heterotypic immunity against G2P[4] to some extent, Matthijnssens et al. (2014) reported higher
occurrence of G2P [4] in a vaccinated population as compared to unvaccinated population. However, other studies were not able to find any such correlation (Pelaez-Carvajal et al., 2014; Donato et al., 2014).

Indian vaccine – Rotavac (116E) is a monovalent vaccine and contains G9P[11] which was isolated from a neonatal outbreak in AIIMS, New Delhi (Bhan et al., 1993). This vaccine has completed phase III trials successfully and was found to exhibit 56% efficacy which is comparable to Rotarix and RotaTeq under low income country setting (Bhandari et al., 2014). It will be commercialized by Bharat Biotech, India. Some other indigenous vaccines are also in clinical trials. Bovine human reassortant pentavalent (G1, G2, G3, G4 and G9) vaccine (to be commercialized by Serum Institute of India) has completed phase I and phase II trials and phase III trial has been planned for it (Zade et al., 2014). A tetravalent bovine human reassortant (G1, G2, G3, G4) vaccine (to be commercialized by Shantha Biotech) has successfully completed phase I and II trials (Dhingra et al., 2014).

2.18 Need for continuous molecular surveillance

In summary, Rotaviral gastroenteritis continues to be a major cause of global morbidity and mortality among children below five years. In India, 11% of the population is under 5 years of age and are susceptible to rotavirus illness. As calculated by John et al. (2014), rotavirus causes diarrheic episodes in 1 of every 2 children, outpatient visits in 1 in every 8 children, hospitalization due to severe gastroenteritis in 1 in every 31 children and death of 1 in every 345 children in India. This indicates a huge disease burden of rotavirus gastroenteritis in India. In addition, the country has to spend 10.28 billion rupees for treatment and care of rotavirus infected patients. This disease burden can be substantially reduced by the introduction of an effective rotavirus vaccine. A problem cannot be addressed until it could be estimated. The decision to introduce a vaccine depends on the kind of genotype prevalent in different regions of the country. The emergence of novel genotype constellations can pose challenge to the existing vaccine and decrease the vaccine effectiveness. Some rare reassortant may escape the vaccine induced immunity as it has happened in many countries (Brazil, Australia and Taiwan) and prevalence of these strains may increase due to vaccine pressure. Therefore, in nut shell, a rigorous molecular surveillance of prevalent rotavirus genotypes is prerequisite for a successful vaccination program in India.
Gaps in existing knowledge

The above-said review of the literature highlights some gaps in the existing knowledge as follows:

1. No systemic epidemiological study on Human Rotavirus in Haryana was found where age distribution, climate variation and genotype distribution across time frame were studied.
2. No earlier study was found where phylogenetic comparison of circulating Haryana strains with vaccine strains was carried out.
3. No earlier study comparing the important epitopes of Haryana Rotavirus strains with vaccine strains was found.
4. No earlier study describing the sensitive and specific detection of Haryana circulating strains with real time reverse transcription PCR and comparison with conventional methods of rotavirus diagnosis was found.
Objectives

Keeping the above perspective in consideration and to fill the gap in existing knowledge, the molecular characterization of circulating Haryana Rotavirus strains was carried out with the following objectives:

1. Isolation of group A rotaviruses from diarrheic children faecal samples.
2. Direct detection and G and P genotyping of group A rotavirus in faecal samples by RT PCR.
3. Cloning and sequencing of predominant G and P genotypes (VP7 and VP4 genes) isolated.
5. Real-time PCR based detection of rotavirus.