Human group A Rotavirus continues to be the most important etiological agent of severe gastroenteritis in infants and children below five years. The present study was envisaged to determine the disease burden of rotavirus in Haryana children. A total of 410 diarrheic stool samples of children below 5 years of age were collected from different medical colleges, private clinics and diagnostic labs. After screening by qualitative ELISA for group A rotavirus, viral genomic RNA was analyzed on native PAGE to determine electropherotype. Semi-nested multiplex PCR was done for G- and P-genotyping. The nucleotide sequencing of VP4 and VP7 gene of untypeable samples along with VP6 and NSP4 of representative samples was also carried out. These nucleotide sequences were analyzed by RotaC for determining genotypes. This data was used for epidemiological analysis depending on age- and season-wise distribution of disease. Phylogenetic relationship of VP4, VP7, VP6 and NSP4 was carried out to ascertain the relative distance between Haryana strain and with Rotarix, RotaTeq and Rotavac (116E) strains. Comparison of immunogenic epitopes of VP4, VP7, VP6 and NSP4 gene of Haryana strains with vaccine strains was carried out to predict any potential mutation which can escape vaccine induced immunity. In addition, Real Time PCR assay based on conserved VP6 gene was also standardized in order to detect rotavirus in very small concentration. The comparative analysis of various rotavirus detection tests with qRT-PCR assay was also carried out. The summary of the present work is presented below:

**Qualitative analysis**

- Typical positive reaction (blue colour) detected in 95 samples but 5 samples were found negative by RNA-PAGE and RT-PCR of VP6 gene. About 22% sample positivity for group A rotavirus was detected in this study.
- A total of 89 samples exhibited a segment clustering pattern of 4:2:3:2 indicating group A rotavirus. One sample (HR-223) exhibited more than 11 segments and could not grouped.
- Long electropherotype was found in 78 RNA samples, whereas 12 RNA samples displayed short electropherotype.
G- & P- Genotyping and ORF amplification

- A total of 67 samples could be G- genotyped. For rest of samples, VP7 segment of 905 bp was amplified, cloned and sequenced.
- Out of 90, only 64 samples could be P- genotyped. Highly variable segment of 877 bp of VP4 was cloned and sequenced after amplification.
- Full length ORF (1194 bp) of VP6 gene was amplified.
- The enterotoxin (NSP4) gene ORF (528 bp) was also amplified using the in house designed primers.

Sequence Based Genotyping

- VP4, VP6, VP7 and NSP4 genes of Haryana rotavirus were cloned.
- A total of 22 VP4, 21 VP7, 11 NSP4 & 3 VP6 nucleotide sequences were deposited in NCBI with the accession number given in Table 4.1.
- Genotyping based on nucleotide sequences was done using RotaC tool (Table 4.1). A total of 15 P[8], 4 P[4] & 3 P[6] genotypes was detected on the nucleotide sequence basis.
- Sequence based G- genotyping detected 13 G1, 1 G2, 5 G9 & 2 G12 strains.
- On the basis of VP6 nucleotide sequence, all the three strains were found to be of I2 genotype.
- A total of 7 E1, 3 E6 & 1 E2 genotype was detected on the basis of NSP4 sequences.

Age-wise distribution of Affected Children

- Rotavirus occurrence was found very low (3.33%) during first two months of life and slightly higher during subsequent 3-5 months (8.88%).
- Incidence of rotavirus diarrhea was maximum (32.22%) in the age group 6-11 months.
- It remain high (31.1%) during 12-23 months of age, but declined after 2 years of age gradually till 5 years of age.
Seasonality of Infection

- A clear association of seasonality with rotavirus infection was observed in the present study. An increase in the number of cases started from July onward and maximum number of cases was reported in September-November (about 32% increase).
- Decline in number of cases occurs from November onward till February. Small numbers of cases were reported in the month of April, May, and June.
- In the months of high temperature (May, June & July), the incidence of rotavirus diarrhea was very less.

Diversity of Rotavirus strains in Haryana

- Genotype G1P[8] was identified in maximum number of cases (N=30, 33.33%) followed by G2P[4] (N=14, 15.55%).
- Intriguingly, G12P[6] was found to be the third most common genotype combination during present investigations (N=10, 11.1%).
- This seems to be the first detection of G12 rotavirus strain from Haryana region.
- In addition to P[6], G12 was also found in combination with P[8] also (G12P[8], N=6, 6.66%).
- Other G- and P- genotype combinations detected in the present study were G9P[4] (N=9, 10%), G9P[8] (N=5, 5.55%), G9P[6] (N=3, 3.33%) & G9P[11] (N=2, 2.22%).
- A total of six rotavirus samples (6.66%) were found to have more than one strain. Three samples could not be typed for P- genotype, one sample could not be typed for G- genotype and one sample for both G- and P- genotype.
- G1 was found to be predominant G- genotype (36.67%) and second most commonly found genotype was G9 (in 21.12% positive cases). Proportion of emerging G-genotype G12 (17.78%) was found more than genotype G2 (15.55%).
- Genotype P[8] was detected in maximum number of cases (46.67%) in the Haryana region during 2012-14.
- P[4] was found to be the second most common P- genotype (25.56%) followed by P[6] (14.44%).
• An unusual genotype P[11], normally not reported in human being was also found in 2 cases.

Molecular Characterization of Viral Proteins

Molecular characterization of four viral proteins (VP7, VP4, VP6 and NSP4) was carried out as described below:

Viral Protein-7

• The present study seems to be the first report of the phylogenetic relationship of Haryana rotavirus strains with Indian rotavirus vaccine- Rotavac (116E) along with Rotarix and RotaTeq.
• Haryana G1P[8] strains was found to be less similar with Rotarix and RotaTeq strain than with other local strains but it was more than that with Rotavac (116E).
• Five amino acid sites of 7-1a, 7-1b, and 7-2 epitopes were completely conserved among all the Haryana strains.
• All of Haryana G9 strains have aspartic acid is present in place of asparagine at position 238 which may alter the normal glycosylation process.
• Haryana G1 strains exhibited only three amino acid difference with Rotarix whereas with RotaTeq, five differences were observed. A maximum difference of 14 amino acids was observed with Rotavac (116E) strains.
• Haryana G2 strains exhibited maximum similarity (91.6% & 93.2% at nt and aa level) with SC2-9 strain of RotaTeq and high amino acids differences with Rotarix and Rotavac (116E).
• Haryana G9 shared maximum similarity (87.5% & 92.3% at nt and aa level) with Rotavac (116E) and significantly high amino acids differences with Rotarix.
• The emerging G12 Haryana strains exhibited very low similarity and high amino acids differences with all the existing vaccine strains.
Viral Protein-4

- Local P[8] strains exhibited maximum similarity with RotaTeq W179-4 strain (91.8-93.8\% & 94.1-95.9\% similarity at nt and aa level) followed by Rotarix (90.4\% & 91.5\% similarity at nt and aa level) but very less similarity with Rotavac (116E).
- RotaTeq strains W178-8, BrB-9, W179-9, SC2-9 and Rotavac (G9P[11]) could not cluster with any of the Haryana strain and presented as separate clade.
- Haryana P[4] also shared maximum similarity with Rotarix (87.6-88.4\% at nt & 85.1-86.1\% at aa level) and RotaTeq W179-4 strain (88.4-89.5\% at nt and 87-87.9\% at aa level) and high distance with Rotavac (116E) but local P[6] exhibited low similarity with all the existing vaccine strains.
- Out of 25 amino acids residues at important antigenic sites of VP8* (VP4), only two sites were conserved among all the Haryana strains.
- Haryana P[8] exhibited amino acids differences of 6, 2 and 22 residues at antigenically important epitopes with Rotarix, RotaTeq and Rotavac, respectively.

Viral Protein-6

- All the three Haryana rotavirus VP6 genes shared maximum similarity with animal reference strains and bovine field isolates (98.4-99.6\% aa similarity) as compared to human strains (97.7-98.8\% aa similarity).
- Haryana rotavirus VP6 shared maximum similarity with RotaTeq strains (98.8-99.6\% aa similarity) as compared to Rotarix (91.6-94.9\% aa similarity) and Rotavac strains (92-94.1\% aa similarity).
- Variations at VP6 protective region, variable region and subgroup determining region of Haryana strains were highest with Rotarix and Rotavac.
Nonstructural Protein-4

- RotaC analysis indicated dominance of E-1 among genotypes detected on the basis of NSP4 gene.
- Genotype E2 was found to have more proximity with G9P[8], G2P[8] and G1P[7] genotypic constellation.
- Local circulating Haryana strains belonging to E1 genotype exhibited high degree of conservation in plasma membrane destabilising domain (PMDD), tetramerization domain (TD), double layered particle domain (DLPD) and tubulin binding domain (TBD) of NSP4.
- Haryana rotavirus strains exhibited very low amino acids variations with Rotarix and Rotavac (116E) while high variations with RotaTeq.
- NSP4 domains having important biological functions were found conserved and variations were observed in less important domains.

Quantitative and comparative analysis

- G1P[8], G2P[4], G12P[6], G9P[8] genotypes could be detected within copy number ranging from $2.17 \times 10^6$ to $3.65 \times 10^9$ but G9P[11] could not be detected by this assay.
- qRT-PCR developed in present study could detect the presence of rotavirus in $10^{-8}$ dilution of a confirmed rotavirus positive sample (HR-271) while ELISA, RNA-PAGE and reverse transcription PCR could detect positivity in $10^{-2}$, $10^{-2}$ & $10^{-6}$ dilutions, respectively.
- It indicated the high sensitivity of the assay. Specificity was checked by using DNA of *E. coli*, *Salmonella* and *Shigella* as negative control. Negative results with these samples indicated specificity of the assay.
Conclusions

- Rotavirus exerts a huge disease burden (21.95% sample positivity) in children of Haryana below the five years of age. Long electropherotypes predominate (86.66%) in this region of the country.
- Children of age 6 to 23 months (>65% of disease burden) were found to be most susceptible to rotavirus infection. A conspicuous association of seasonality (cooler months) with rotavirus infection was observed in this region.
- G1P[8] is predominant genotype combination (33.33%) in Haryana followed by G2P[4] (15.55%), G12P[6] (11.11%) & G9P[4] (10%). Unusual strains like G9P[6] (5.5%) & G9P[11] (2.2%) were also observed in this study.
- G1 was most commonly detected (36.67%) G- genotype followed by G9 (21.12%), G12 (17.78%) & G2 (15.55%). This study seems to be the first report of G12 genotype from Haryana. P[8] was predominant P genotype (46.67%) followed by P[4] (25.56%) & P[6] (14.44%)
- Haryana G1P[8] strains exhibited more amino acid variations with 116E as compared to Rotarix and RotaTeq.
- Haryana G9 strains displayed maximum similarity (87.5% & 92.3% at nt and aa level) and less amino acid variations with 116E vaccine.
- Intriguingly, Haryana rotavirus VP6 gene displayed high similarity with bovine rotavirus strains (98.4-99.6% aa similarity) as compared to human strain, indicating occurrence of reassortment.
- Haryana NSP4 gene displayed high amino acid difference with the RotaTeq NSP4 gene as compared to 116E & Rotarix.
- qRT-PCR was found 2 log more sensitive than reverse transcription PCR and 6 log more sensitive than ELISA & PAGE.
Proposed future investigations

With the results from the present study, the following future studies will be carried out in order to detect any change in genotype distribution after introduction of rotavirus vaccine in India and the emergence of other enteric viruses:

1. To analyze the distribution of Rotavirus genotypes in post-vaccination period.
2. To evaluate the efficacy and safety of newly introduced Rotavac 116E vaccine.
3. Comparative analysis of pre & post vaccination circulating rotavirus genotype.
4. To analyze selection pressure due to vaccine induced immunity.
5. To detect emergence of any other enteric virus (i.e. Norovirus, Adenovirus, Calicivirus) in case of rotavirus decline.