The available rotavirus vaccines have been reported to be highly efficacious in reducing the rotavirus disease burden in developed countries (Ruiz-Palacios et al., 2006; Vesikari et al., 2006) but in low and middle income countries, the effectiveness of these vaccines were low (Madhi et al., 2010). These vaccines are available via private sector currently and the Indian Government has declared to include rotavirus vaccination national vaccination program. IAPCOI (Indian Academy of Paediatrics Committee on Immunization) has recommended both the vaccines and either of vaccine can be administered with the consent of parents (IAPCOI, 2014). However, the proportion of parents/guardians allowing rotavirus vaccine administration, was reported very less (9.7%) by a national survey (Gargano et al., 2012). This low coverage may be attributed to the prohibitive cost of these vaccines and most of the childhood vaccines are delivered by public sector.

The *Lancet* in 2014 reported the effectiveness and safety trial of the first Indian Rotavirus vaccine, Rotavac (116E) successful. In a multicenter study carried out in Delhi, Pune and Vellore, the first Indian vaccine to complete phase 3 clinical trials, exhibited efficacy similar to the existing International vaccines (Bhandari et al., 2014). However, a good quality epidemiological data is prerequisite for successfully controlling, eliminating or eradicating rotavirus in the public health domain. A problem cannot be worked out unless it could be estimated successfully. In reference to disease preventable by vaccines, routine surveillance activities are critical to measure the data to define epidemiological parameters of disease, predicting the genotypes/strains prevalent in India, inspecting the trends in disease burden and to determine whether any intervention like vaccine is economically viable. If decision of any vaccine induction into the national vaccination program is to be made, it becomes imperative to have continuous surveillance to determine effectiveness of vaccine, to demonstrate any decrease in vaccine preventable illness, to determine any side effect of vaccine & to demonstrate the development of herd immunity.

### 5.1 Sample screening analysis

In the present study, 410 diarrheic stool samples were collected over the period of two years (May 2012- October, 2014) from the infants and children below five years of age following inclusion and exclusion criterion with the consent of parents/guardians. Out of 410, ninety samples were found positive for group A rotavirus by ELISA based
Rotaclone kit. The percent positivity was estimated to be 21.95%. Although 5 more samples exhibited rotavirus positivity by ELISA but found negative after RT-PCR done for VP6 gene. This may be due to some cross reaction with other enteric pathogens. 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. Epidemiological studies conducted in Pune, Chennai, Vellore, Kolkata, Berhampur, Delhi, Lucknow and Manipur during 1999 to 2010 indicated human rotavirus prevalence in the range 12.58%-49% (Bahl et al., 2005; Baneerjee et al., 2006; Chakravarti et al., 2010; Das et al., 2002; Kang et al., 2002; Kelkar et al., 1999; Mukherjee et al., 2010; Samajdar et al., 2006, 2008; Saravanan et al., 2004). The results of the present study is lying in the medium range of rotavirus prevalence.

An epidemiological surveillance study conducted between July 2009 and June 2012 at two hospital settings in South India and one in North India revealed 39% sample positivity during 2009-2012 (Babji et al., 2014). Saluja and coworkers (2014) collected samples from 12 sites across India and reported 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. This low positivity in community cohort study may be attributed to association of mild diarrhea with other causative agents.

All these studies, including our study, indicated a significant disease burden of rotavirus diarrhea among Indian children from various regions. Minimum sample positivity was reported in Kolkata (12.58%) whereas maximum prevalence of rotavirus was found in Manipur region (49.9%). In Haryana, there is paucity of information about prevalence of Human Rotavirus although Bovine rotavirus were detected in 11-43% of diarrheic calves faecal samples (Singh and Pandey, 1988; Minakshi, 1999; Gulati et al., 1999). People from Haryana share a common socioeconomic fabric with animals as dairy farming is highly popular in this region. Due to this close proximity, there are high chances of interspecies transmission of rotavirus among human being and animals. However, no such event was reported in the present study.
5.2 Electropherotype analysis

In this study, viral genomic RNA extracted from ELISA positive samples by Trizol method was used to analyze the electrophoretic migration pattern of human rotavirus on RNA-PAGE. The segmented viral genome resolved into 11 segments with a unique banding pattern of 4:2:3:2 and exhibited both short and long migration pattern. Among ELISA positive stool samples, 86.66% exhibited long electropherotype due to faster migration of the 10th and 11th segment, but in one sample (HR-223), more than eleven segments were visible and the banding pattern was also different from group A rotavirus. It may be due to coinfection of more than one type of rotavirus. This short electropherotype phenotype (13.44% in the present study) is attributed to partial duplication of segment 11, which resulted in its slow migration (Matsui et al., 1990).

The most frequently detected rotavirus strains belong to two distinct genogroup: Wa genogroup & DS-1 genogroup. These unique genome constellations are very different to each other as described by the very high stringency hybridization experiments using probes for whole genome (Flores et al., 1982; Nakagomi et al., 1989). Wa genogroup strains carry subgroup II antigen (on the basis of VP6 epitopes) and typically exhibit long electropherotypes. Most commonly occurring G types, i.e. G1, G3, G4, G9 belongs to Wa genogroup. In the present study also, all the G1 strains exhibited long electropherotypes. Interestingly, G9P[8] strains exhibited long electropherotype whereas G9P[6] exhibited short electropherotypes. Strains belonging to DS-1 genogroup are usually of short electropherotypes and carry subgroup I antigen (Nakagomi et al., 1989). In the present study, all the G2 strains exhibited short electropherotypes. These G2 strains are members of DS-1 genogroup. No sample exhibiting non-group A migration pattern was observed in the present study. Several studies in India as well as abroad have detected group B & group C rotaviruses from human infants and children on the basis of RNA-PAGE migration pattern different from group A pattern (Krishnan et al., 1999; Saiada et al., 2011; Barman et al., 2006).
5.3 Semi-Nested PCR based Genotyping

The enzymatic amplification of a particular DNA segment by mean of polymerase chain reaction in an automated reaction has changed the face of molecular diagnostic and surveillance studies. Many other variants of PCR have had far reaching impacts in various avenues of biological sciences. PCR has become especially important in the cases where pathogens are difficult to cultivate, undergoing latency or produce very low antibody titer. The reverse transcription converts Rotaviral genomic RNA into cDNA and rapid amplification of this template by one million fold could be done in very small time and generate optimum concentration of DNA for further analysis such as gel purification, cloning, restriction profiling and nucleotide sequencing. Kang et al. (2004) observed that RT-PCR offers many advantages in addition to high sensitivity and specificity. This rapid amplification helps in detection of viral nucleic acid during the early stages of virus infection without the requirement of sufficient titer of antibodies or high virus titer (Fedorova et al., 2005).

For partial length amplification of VP7 gene, primers designed by Das et al. (1994) were used and amplified DNA product of 905 bp was obtained as described in other studies as well (Ray et al., 2007; Tiku et al., 2014). Partial length amplification of VP4 gene was carried out in this study by using the published primers (Gentsch et al., 1992) with change in annealing temperature to 50°C for 30 seconds. Open reading frame of VP6 and NSP4 were amplified by oligonucleotide primers designed in this study only and DNA products of size 1194 bp and 528 bp, respectively were observed. Seminested RT-PCR was utilized for G- (Das et al., 1994) and P- genotyping (Gentsch et al., 1992) of rotavirus using published primers with modification in the annealing temperature. DNA fragments of different length were observed in different samples, i.e. in HR-3 DNA product of 463 bp indicate G12 genotype, whereas DNA fragments of size 112 bp, 161 bp & 246 bp indicate presence of G9, G1 & G2 genotypes. For P- genotyping, different size indicates different P- genotype, i.e.- 483 bp, 267 bp and 345 bp indicate P[4], P[6] and P[8] genotypes. Our findings on the agarose gel were in coherence with Gentsch et al. (1992), Das et al. (1994), Ray et al. (2007), Sharma et al.(2009) and Tiku et al. (2014).

Nucleotide sequences were obtained after sequencing of recombinant plasmids containing VP4, VP6, VP7 & NSP4. All the sequencing were submitted to NCBI via
BankIt and are available on http://www.ncbi.nlm.nih.gov. The accession number of all the gene sequencing are given in table 4.1. Genotyping on the basis of nucleotide sequences was done by using RotaC [http://rotac.regatools.be/] software (Maes et al., 2009). The results obtained by RotaC tool were reconfirmed by Blastn (http://www.ncbi.nlm.nih.gov/BLAST).

5.4 Age-Wise Distribution of Affected Children

Most of the rotavirus illness in India occurs during first two years of life. In the present study, 75% of children suffering with acute rotavirus were under two years of age. In a number of hospital based surveillance studies (Kelkar et al., 1999; Kelkar et al., 2001; Saravanan et al., 2004; Bahl et al., 2005; Tiku et al., 2014; Babji et al., 2014) has indicated that 87% of all rotavirus gastroenteritis episodes in children below 5 years occurred during first 18 months of life. Intriguingly, rotavirus prevalence is comparatively very low in young children. In our study, only 3.33% children under 2 months and 8.88% children below 5 months of age suffered with rotavirus associated gastroenteritis. Prevalence in the age group 3-5 months was higher during 2013-14 (9.52%) as compared to 2012-13 (7.4%). However, in the age group 0-2 months, prevalence during both years was almost same (3.7% & 3.17% respectively). Therefore, 12.21% of rotavirus positive samples were from infants below five months of age. Other studies (Kelkar et al., 1999; Kelkar et al., 2001; Saravan et al., 2004; Bahl et al., 2005; Tiku et al., 2014; Babji et al., 2014) revealed that only 13% of children suffering with rotavirus gastroenteritis were under 6 months.

Surprisingly, community cohort studies (Kelkar et al., 2001; Banerjee et al., 2006; Paul et al., 2014) indicated a significantly higher prevalence (30%) in children under six months. This contrast may be attributed largely to the severity of disease. In young children, rotavirus gastroenteritis is often mild and this mild infection may not warrant a visit to hospital. It may be due to attenuation of rotavirus by the persistent high concentration of maternal antibodies. Paul et al. (2006) also reported that 18% of the infants suffered with mild rotavirus infection in first month of life, mostly by an unusual strain G10P[11]. In the present study, two neonates were found suffering with G9P [11], which are not normally reported. G9P[11] isolated from an asymptomatic infection in AIIMS, New Delhi has formed the base for Indian Rotavirus Vaccine-Rotavac (Bhan et al., 1993). Other studies (Vethanayagam et al., 2004; Banerjee et al.,
2007; Ramani et al., 2008) have also recorded high prevalence (22-73%) rotavirus in Indian children below five years.

Rotavirus infection in very early age is usually asymptomatic and 65-95% cases not showing signs of overt gastroenteritis. However, rotavirus detection was higher in the neonates having diarrhea as compared to those not having diarrhea (Vethanayagam et al., 2004), indicating neonates are not totally immune to rotavirus infection. This early age infection may be protective against future rotavirus diarrhea, some conflicting results have been reported. In Australia, neonatal infection was not found protective against subsequent rotavirus infection, but severity of subsequent illness was less (Bishop et al., 1983). Bhan and coworkers (1993) in a community cohort study based in New Delhi reported that neonatal infection resulted in 46% fewer incidence of rotavirus in future.

5.5 Seasonality of infection

Global expansion of public health network for surveillance of important disease has galvanized attention to fluctuation in seasonal occurrence of diseases. An in-depth knowledge of temporal pattern of disease prevalence is vital for designing the preventive measures for the disease. Many diseases exhibit a seasonal pattern in prevalence and this seasonal fluctuation can be characterized by timing and duration. Mukherjee et al. (2010) observed no association between rotavirus infection and season. Similar findings were also reported by other studies conducted in South India (Banerjee et al., 2006; Paul et al., 2014). However, in the present study, the seasonality association with rotavirus infection was conspicuous. An increase in the number of cases started from July onward and maximum number of cases were reported in September-November. Decline in the number of cases occurred from November onward till February. Small numbers of cases were reported in the month of April, May, and June.

Therefore, in winter season, maximum numbers of cases were observed in the current study. A number of 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) has reported a significant increase in rotavirus gastroenteritis during the cooler months (October to February) of the year. The percentage positivity in cooler months ranged from 59% to 72% (median 64%). Our findings are consistent with these studies. Kang et al. (2009)
observed that Northern India having more temperate milieu, exhibit strong seasonality as compared to Southern India (having more tropical environment). Nevertheless, several studies from Southern India have reported seasonality association with rotavirus infection (Kelkar et al., 1999; Saravanan et al., 2004; Nair et al., 2010). Therefore, the degree to which seasonality of rotavirus varies with geography is still not clear.

5.6 **Haryana Rotavirus strain diversity**

Reassortment events including gene encoding for outer capsid proteins, are of particular relevance to rotavirus disease epidemiology and vaccine effectiveness. Genotyping of rotavirus is done on the basis of the two outer capsid proteins VP4 (Protease sensitive, P- type) and VP7 (Glycoprotein, G- type). According to the Rotavirus classification working group, Rotavirus A genome segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are referred to as the Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx genotypes, respectively (Matthijnssens et al., 2008) and classification can be done on the basis of all the gene segments but most of the epidemiological studies employ genotyping on the basis of two outer capsid proteins only. A total of 27 G- & 37 P- genotypes have been identified in man and animals till date (Trojnar et al., 2013) however, only 10G- & 11P- genotypes have been found in Human being. Although reassortment of G and P genes could theoretically lead to hundred of G type and P type arrangement 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, significant differences in most prevalent circulating strains have been observed in different geographical and socioeconomic settings (Banyai et al., 2012).

In the present study also, G1P[8] was detected in maximum number (N=30, 33.33%) followed by G2P[4] (N=14, 15.55%) and it is concordant with the 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 strain and it increased dramatically during 2000-2003 (Banyai et al., 2012). Interestingly, third most common genotype combination found in our study was G12P[6] (N=10,
11.1%). The first detection of G12 was reported from Phillippines 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). The present study is also the first report of human G12 rotavirus strain from Haryana region.

All these cases reported were sporadic, 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). In the present study, G12 was found in combination with P[8] also (N=6, 6.66%). Other genotype combinations reported in our 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%). G9P[11] detection is very significant in the context that the forthcoming Indian Rotavirus vaccine also contains live attenuated rotavirus strain 116E (G9P[11]) which often cause asymptomatic or very mild disease. If G9P[11] detection frequency increases, it may result in the development of natural immunity in the children of Haryana region. But in the present study, only two cases (out of 410) of G9P[11] have been detected. Our results displayed concordance with that of a multicenter epidemiological study conducted in hospitalized children from 3 sites (Vellore, Trichi and Delhi) (Babji et al., 2014).

Babji et al. (2014) reported the prevalence of rotavirus strains as: G1P[8]=33%, G2P[4]= 17%, G9P[4]=11% G9P[8]=6%, G12P[6]=6%, G12P[8]=4% and mixed infection=9%. In our study, six rotavirus samples (6.66%) having more than one strain (mixed) were found. 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. Another multicenter surveillance study conducted at 12 sites across India observed relatively low prevalence of G1P[8] (24%) 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).

In the present study, genotype G1 was 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%) has surpassed the existing genotype G2 (15.55%). Detection of G9 in India happened approximately a decade after its
discovery in Philadelphia, USA. Intriguingly, G9 was first reported as G9P[11] in a neonatal outbreak in AIIMS, India (Bhan et al., 1993). Gradually, the strain may have reassorted with commonly occurring P types, i.e. P[6], P[8] and P[4]. In the past two decades, G9 has been found with an unusual number of P types and both VP6 subgroup I and II. Mukherjee et al. (2009) detected high similarity of G9P[6] with porcine P[6] which may be a result of reassortment between human and animal strains.

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 the emergence of G9 strains which reached the peak after 2000 and detected as 15% (Miles et al., 2012). These reports are in accordance with the findings of our study. In the present study, no G3, G4, G6 and G8 were reported. Change in century also witnessed the emergence of G12 strains which were not detected in earlier studies. Studies conducted from 2005 to 2009 indicated about 9% G12 strains in India, which reveal the steady state of rotavirus transmission (Kahn et al., 2012).

In our study, P[8] was reported to be the most predominant in the Haryana region during 2012-14. It is in accordance with the other studies done in different parts of the country (Babji et al., 2014; Saluja et al., 2014; Namjoshi et al., 2014). P[4] was found to be second most common P- genotype (25.56%) followed by P[6] (14.44%). A total of 6.66% of positive cases exhibited mixed genotypes and four samples (4.44%) could not be genotyped by any of the methods. An aberrant genotype P[11], normally not found in the human being was also reported in 2 cases. Our findings are in concordance with a report in which P[8] and P[4] genotypes were most prevalent in studies conducted before 1994 as well as post 2000. P[6] showed the most variation in prevalence (8.5%-18.9%) and mixed infection also detected (Miles et al., 2012). Bhan and coworkers (1993) reported P[11] genotype from neonatal outbreak, but its detection is rare and no sign of emergence was observed in any other study in India.
5.7 Molecular Characterization of Viral Proteins

Characterization of important viral proteins VP4, VP6, VP7 and NSP4 was carried out in the present study as VP4 and VP7 constitute the outermost layer of the virus capsid. These are directly exposed to the immune system. VP6 is the most abundant protein in the virus capsid and a high titer of antibodies are generated against VP6. NSP4 is a multifunctional protein, which acts as enterotoxin and cause diarrhea. Any significant change in nucleotide and deduced amino acid sequence of these proteins may alter the field strain pathogenicity as well as immunogenicity of vaccine strains. The forthcoming introduction of group A rotavirus vaccination in the national immunization program of India elicited the idea of determining the constitution of Rotaviruses prevalent in Haryana region and to establish the phylogenetic relationship of VP4, VP6, VP7 and NSP4 genes of local circulating strains with different geographical areas and available rotavirus vaccines. Moreover, the potential amino acid differences in epitopes of immunogenic proteins between these strains and vaccine strains were also investigated, in order to find their potential repercussion on efficacy of future rotavirus vaccination programs.

5.7.1 Viral Protein-7

In order to determine the phylogenetic relationship between VP7 gene of Haryana strain with that of Rotarix, RotaTeq and Indian vaccine 116E, dendrogram analysis was performed. Although earlier studies have been conducted to compare the phylogenetic relationship of local circulating strains from Belgium, Tunisia & India (Zeller et al., 2012; Fredj et al., 2013; Kulkarni et al., 2014; Mullick et al., 2014) with commercially available rotavirus vaccine (Rotarix and RotaReq) but this is the first study comparing the phylogenetic relationship of local circulating Haryana rotavirus strains with soon to be commercialized Indian rotavirus vaccine- Rotavac (116E), in addition to Rotarix and RotaTeq. G1P[8] was the most predominant combination found in our study. Dendrogram analysis of Haryana G1 strains with vaccine strains indicated that all the local circulating G1 strains are closely related and form a separate clade in lineage 1 except HR-21/MDU/Rohtak/2012/G1P[8] & HR-30/MDU/Rohtak/2012/G1P[8] strains.

These Haryana strains exhibited maximum similarity with THA/CU1024-KK, AUS/CK00037 at nucleotide similarity level (90.7-99.4%) while at amino acid level,
maximum similarity was observed with THA/CU1024-KK, AUS/CK00037, PAK/3083, PAK/NIBGE-32, SKR/KR/Seoul-677, ZAF/MRC-DPRU83 (91.3-98.7%). Rotarix formed lineage 2 and RotaTeq 179-9 formed lineage 3. Although G1P[8] is present in Rotarix and RotaTeq but Haryana G1P[8] strains displayed, comparatively less similarity (88.4-97% at nucleotide level and 88.9-96% at amino acid level) with these vaccines. This distance is evident in phylogenetic tree where both these vaccines are present in separate lineages as compared to Haryana G1 strain. Indian vaccine, Rotavac (G9P[11]) was found to have much more distance with local circulating G1 strains. Maximum similarity between Rotavac (116E) and Haryana G1 strains was found to be 65.7% at nucleotide level & 71.4% at amino acid level. The Haryana G1 lineage 1 & 2 strains are more similar with Rotarix (97% at nucleotide and 96% at amino acid level) as compared to lineage 3 RotaTeq 179-9 strain (90.7% at nucleotide level and 92.3% at amino acid level). 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). In order to ascertain the possible effect on the efficacy of rotavirus vaccination program, the antigenic difference between recent rotavirus strains from Haryana and vaccine strains were investigated by detecting any difference in amino acids at important antigenic regions of VP7 protein. 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 into 7-1a & 7-1b (Aoki et al., 2009).

In our study, most of the variation was reported in 7-1a domain. Five sites (Glutamine at 104 & 201, Glycine at 264, Tryptophan at 98, Lysine at 291) were
completely conserved among all the Haryana strains. In a similar study, only three sites (98, 104, 201) were found conserved among all 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. It is interesting that number of conserved sites are different in different geographical areas.

In all the Haryana G1, G2 and G12 strains asparagine was found at 238 position, which is an important site of glycosylation (Aoki et al., 2009). Since VP7 is a glycosylated protein and the absence of glycosylation at important sites can alter the immunogenicity of vaccine strain and escape mutant may arise in vaccinated population. The tripeptide Asn-Xaa-Thr/Ser at position 238-240, is a significant site for glycosylation. In all of the Haryana G9 strain, Aspartic acid is present in place of Asparagine which may alter the glycosylation process. Mutants at this site have 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).

Haryana G1 strains exhibited only three amino acid difference with Rotarix (Asn94Ser, Ser123Asn, Met217Thr) whereas with RotaTeq five differences were observed (Asn94Ser, Asp97Glu, Ser123Thr, Ser147Thr & Met217Thr). Aoki and coworkers (2009) reported that the variations in position 94 and 217 could be significant as these positions are part of recognition site of antibody. However, 14 differences were found between Haryana G1 strains and Indian Vaccine 116E strain (Ile87Thr, Gly94Ser, Gly100Asp, Asp123Asn, Ile129Val, Thr212Val, Ala213Asp, Asp238Asn, Asn242Thr, Asn145Gln, Ser146Gln, Thr147Asn, Glu217Thr). These differences also include sites 94, 217 & 238 and most of variations lie on the surface of the VP7 molecule. Such a large difference may decrease the reactogenicity of field strains with the vaccine induced humoral response.

G2P[4] strains were second most common G-P- combination (15.55%) detected in the present study. According to phylogenetic analyses, the Haryana G2 Rotavirus strain was distantly related to SC2-9 strain (G2P[5]) of RotaTeq (91.6% similarity at
nucleotide and 93.2% similarity at amino acid level) as compared to circulating G2 strains in Canada, India, Zimbabwe and Mauritius. However, this similarity was more than that exhibited with Rotarix (61.4% & 62.9% at nucleotide & amino acid level, respectively) and 116E (66.6% & 66% at nucleotide and amino acid level). Similar results were reported by studies conducted in India and Tunisia (Kulkarni et al., 2014; Fredj et al., 2013) where local circulating G2 strains exhibited more similarity with the field rotavirus G2 strains and showed distance with the RotaTeq SC2-9 strain.

Most of the times, G2 strains are found in combination with P[4] genotype and protection against this kind of constellation by RotaTeq (G2P[5]) would mainly depend on the VP7 component (G2) of the vaccine strain. Furthermore, the occurrence of antigenic drift at certain amino acid positions between vaccine and local circulating strains could lead to diminished vaccine effectiveness against these G2 strains. 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). Variation at this position was also reported in Haryana HR-353 strain (Asp96Asn) as compared to RotaTeq SC2 strain in the present study.

Variations were observed at 5 positions between Haryana G2 strain and RotaTeq G2 strain (Thr87Ala, Asp96Asn, Asn99Lys, Ser213Asp & Ser242Asp) whereas a large number of differences were observed with Rotarix (18 residues) & 116E strains (20 residues). Moreover, post-vaccination reports from Brazil and Belgium has indicated a substantial rise in the 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). Therefore, it has generated a lot of debate in scientific fraternity whether this increase in the number of vaccine escape mutants is due to the introduction of the vaccine or it is spontaneous mutations in the local circulating strains (Matthijnssens et al., 2012). No G3, G4, G6 & G8 were reported in the present study.

During the present study, we observed remarkable diversity in the rotavirus strains circulating in Haryana, with several new G-P- combinations emerging in this geographical area, i.e. G9P[8], G9P[4], G9P[6], G12P[8] & G12P[6]. This emergence of the new genotype may be the result of natural reassortment and antigenic drift or due to the introduction of rotavirus vaccine through the private sector. Haryana G9 strains
(HR-48, HR-49 & HR-90) shared 98.2-99.4% and 98.2-99.1% similarity at nucleotide and amino acid level with each other. But, Haryana G9 strain shared 83.5-87.5% similarity at nucleotide level and 88.4-92.3% similarity at amino acid level with Indian vaccine 116 strain. However, it was significantly higher than similarity shared by Haryana G9 strains with Rotarix (63.3-67.2% & 69-73.1% at nucleotide and amino acid level) and RotaTeq strain W178-8 (72.1-73.4% & 80.3-82.9% at nucleotide and amino acid level). This study revealed that local circulating Haryana G9 strains exhibited maximum similarity with Indian vaccine 116E and amino acid variations were observed only at 4 positions (87, 96, 100 & 145).

Very low nucleotide and amino acid similarity was observed after comparing Haryana G12 strains with vaccine strains. These Haryana G12 strains exhibited maximum similarity with Indian 116E vaccine (67.8% at nucleotide and 74.4% at amino acid level). In the present study, an increase in G12 prevalence was reported which is in coherence with the studies conducted in India as well as other part of the world (Castello et al., 2004; Das et al., 2003; Pongsuwanna et al., 2002; Griffin et al., 2002; Shinozaki et al., 2004). There is a global increase in G12 detection rate and it may affect the effectiveness of rotavirus vaccine in the future.

5.7.2 Viral Protein-4

VP4 protein is cleaved into VP5* & VP8* by trypsin in the 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). No Haryana strain formed a cluster with RotaTeq strains W178-8, BrB-9, W179-9, SC2-9 and these vaccine strains form a separate cluster together. Indian vaccine Rotavac 116E also could not cluster with any of the Haryana strain and exhibited significant distance in both the phylogenetic tree. All the Haryana VP8* (VP4) P[8] proteins formed a cluster in lineage 3. These strains displayed distance with lineage 1 P [8] of Rotarix but comparative closeness with P [8] lineage 2 of RotaTeq. When compared with rotarix, the local P[8] strains exhibited maximum similarity of 90.4% at nucleotide level and 91.5% similarity at amino acid level, whereas with RotaTeq-W-179-4 these strains exhibited more similarity (91.8-93.8% similarity at nucleotide and 94.1-95.9% at amino acid level).
Local P[8] strains shared very low similarity with Indian vaccine 116E (44% & 25% at nucleotide and amino acid level). Our findings are in coherence with observation of Fredj and coworkers (2013) that all Tunisian 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. Out of 25 amino acid residue of VP8* epitopes, only two loci (Glutamic acid at 180, Asparagine at 132) remained completely conserved among all the Haryana strains. Haryana P[8] strains displayed less difference with RotaTeq (at 2 amino acid sites) as compared to Rotarix (6 amino acid sites). A very high divergence was observed (22 residue) with Rotavac 116E.

Haryana P[4] strains displayed maximum similarity with Rotarix (87.6-88.4% at nucleotide & 85.1-86.1% at amino acid level) and RotaTeq W179-4 (88.4-89.5% at nucleotide and 87-87.9% at amino acid level). However, similarity exhibited with 116E strain and other strains of RotaTeq was comparatively very low. Haryana P[4] strains also exhibited variations in comparison to the vaccine strains. When compared with Rotarix, eleven variations were observed, whereas 10 amino acids in Haryana P[4] strains didn’t match with any of RotaTeq strain. Intriguingly, Haryana P[6] genotypes (HR-67, HR-192, HR-218) displayed low similarity with all the existing vaccine strain. It is significant as there is a global increase in G12P[6] and G9P[6] detection and it may further increase under vaccine selection pressure. In turn, it may alter the effectiveness of existing vaccines in the future. The VP8* antigenic epitopes of Haryana P[6] and P[4] exhibited largest number of differences (20 and 19 amino acid differences respectively) with 116 vaccine strain. This indicates that Rotavac 116E VP4 didn’t share significant similarity with any of the P genotype prevalent in Haryana region and very unlikely will generate protective immune response based on VP4 protein.

Review of literature indicated that variation at position 190 may be of great importance in virus entry in cell as it has been found to influence sialic acid binding of 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).
5.7.3 Viral Protein-6

The VP6 protein in this study exhibited a significant amount of genetic variations compared to Rotarix and 116E strain. The fact that the I2 genotype Haryana strains were more conserved with RotaTeq vaccine strains as compared to other vaccine strains available. Rotarix and RotaTeq vaccine have been introduced in India and indigenous 116E will be available commercially very soon. Role of antibodies against VP6 is not confirmed, although some workers have reported protective role of anti-VP6 antibodies (Burn et al., 1996). High percentage of amino acid substitution at antigenic region in circulating strains may affect the efficacy of these vaccines. Haryana strains share high amino acid identity with bovine strains of Indian origin as compared to other Indian human strains. This may suggest that the reassortant process has taken place due to close proximity of human and bovine in the socioeconomic fabric of Haryana.

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 the 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. Alanine residue at position 172 also attribute to the formation of 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). Our findings are in accordance with the earlier report as amino acid variation has been reported between Haryana strains and Rotarix strain at position 305, 310, 315. The possible effects of these reported genetic changes on the host disease susceptibility demands further understanding.

The VP6 genes from these circulating strains may be gradually evolving at different rates compared to other rotavirus gene segments, due to accumulation of antigenic changes caused by point mutations and reassortments. This was clearly shown by clustering of Haryana human strains with bovine strains of Indian origin. However, amino acid changes reported here were in accordance with different studies (Ghosh and Kobayashi, 2011; Matthijnssens et al. 2012). These amino acid variations may lead to change in VP6 epitopes. These changes could affect the potency of the
Rotavirus detection assays (Kerin et al., 2007). Although the number of VP6 nucleotide sequences was small, the findings were in consistence with those reported elsewhere (Kerin et al., 2007; Matthijnssens et al., 2012). The current VP6 detection methods should work expeditiously as the changes ascertained within the antigenic regions of these Haryana strains looks subtle. These results suggest that rotavirus investigators ought to continually take into account this continuous variation and update the primers needed for the detection and characterization of the major capsid protein accordingly.

5.7.4 Nonstructural Protein-4

Nonstructural protein 4 (NSP4) is the first viral enterotoxin described. This 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 chloride ion (Ball et al., 1996). NSP4 is also reported to induce 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). In this study, six of our strains (HR-23, HR-30, HR-15, HR-45, HR-49, HR-34) clustered in a large group designated as genotype E1 which also contained 116E Indian vaccine strain and Rotarix vaccine strain. It was also observed that Rotavirus genotypes G1P[8], G9P[6], G9P[11] & G12P[6] are more closely related to NSP4 genotype E1 whereas G9P[4], G2P[4] are more closely related to genotype E6. Genotype E2 was found to correlate with G9P [8], G2P [8] and G1P[7] genotypic constellation.

The amino acid sequence alignment indicated substitutions in several important regions of NSP4. These variations at important antigenic domains may affect the efficacy of vaccines as it has been reported that anti-NSP4 antibodies may neutralize the enterotoxin during illness, thus diminishing the severity of disease (Ball et al., 1996). HR-34 exhibited differences with other E1 genotype strains in VP4 binding domain at various sites: 133 (Asn>Lys), 135 (Ile>Ala), 136 (Thr>Val), 141 (Ile>Asp), 142 (Val>Ile), 145 (Thr>Ser). Rotarix & 116E vaccine exhibited more closeness to E1 genotype, which is more prevalent in this region and amino acid difference were observed only at one position (Val141Ile) with Haryana strains. Two sites found
Different by comparing 116E and Haryana strains were 141 (Val>Ile) (in VP4 binding domain) & 160 (Lys>Glu) (in tubulin binding region).

Difference in sequences increased immediately downstream from the DIR (Diarrhea inducing region) and remained high toward the C-terminal of the NSP4 protein with the greatest amino acid sequence variation between 135-141. Ball et al. (1996) found that the presence of tyrosine at position 131 is strongly associated with the enterotoxigenic property of NSP4. Three of Haryana strains (HR-11, HR-15, HR-45) possessed tyrosine at position 131 while rest seven strains have histidine at this position. Kirkwood and coworkers (1996) contrasted symptomatic and asymptomatic rotavirus isolates from Australian neonates and found that NSP4 amino acid residue 135 was having a correlation with virulence. He observed that isoleucine at position 135 is associated with asymptomatic infection with strain G3P[6]. Contrary to this, present study reported the presence of Isoleucine at position 135 in most of symptomatic rotavirus strains.

VP4 binding domain C-terminal half contained the patch of highest amino acid variations while its N-terminal half is highly conserved. PMDD (Plasma membrane destabilizing domain) N-terminal end, tetramerization domain (TD), double layered particle (DLP) binding domain & tubulin binding domain were found highly conserved. Thus we observed that these conserved regions have more important biological functions as compared to the highly variable region. It is still unclear whether the high amino acid variation domains are due to lack of important biological role constraint or result of humoral immune response pressure against NSP4 domains.

5.8 Quantitative Analysis

One of the objective of present study was to develop a simple, rapid and sensitive qRT-PCR assay for detection of a broad range of Rotavirus genotypic constellation to be applied in clinical settings as well as in case of environmental samples. Number of Rotavirus genotypes are increasing in both animals and human being and water samples in environment contains a broad range of rotavirus genotypes (Trojnar et al., 2013). Conventional diagnosis of rotavirus is based on the detection of VP6 antigen in stool samples using enzyme linked immunosorbent assay (Beard et al.,
Polyacrylamide gel electrophoresis of genomic RNA is used commonly for differentiation of different groups of rotaviruses on the basis of their electropherotype.

Reverse transcription polymerase chain reaction (RT-PCR) has been reported to be more sensitive than ELISA for detection of rotavirus in stool samples of diarrheic children (Wilde et al., 1991). In the present study also, the reverse transcription-PCR proved to be 4 logs more sensitive than ELISA and RNA-PAGE. Despite the advantage of more sensitivity, RT-PCR has not been widely used for clinical diagnosis of rotavirus diarrhea, as it is a complex multistep protocol and it is more prone to cross contamination.

As compared to classical RT-PCR, real time qRT-PCR has been reported to be more sensitive and rapid for diagnosis and quantification of rotavirus in clinical samples (Kang et al., 2004). The present study reconfirms this fact, as qRT-PCR was found 2 logs more sensitive than classical RT-PCR. 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. qRT-PCR assay developed in this study was able to detect G1, G2, G9 and G12 genotypes. Genotype G9 and G12 have emerged very rapidly in the late 1990s and the first decade of this century (Ray et al., 2007). However, this assay was not able to detect a unique reassortant G9P[11]. It may due to some sequence variations at the site of primer binding. A confirmed rotavirus positive sample (HRV-271/CMBT/MDU) could be detected in 10^{-8} dilution also. It indicated the sensitivity of the assay. The range of Rotavirus copy number detected was 2.17 \times 10^6 to 3.65 \times 10^9 copies in clinical samples. Recently, a real time RT-PCR based assay was tested to differentiate wild type and vaccine strain group A rotaviruses (Gautam et al., 2014). Since, India is also in a pre- vaccination stage of rotavirus immunization, it will be important to differentiate wild type and vaccine strains in acute gastroenteritis cases. This will require development of another sensitive and specific assay.

In spite of low efficiency of rotavirus vaccine in low and middle income countries, the available vaccines are predicted to decrease the morbidity and mortality due to rotavirus infection. Introduction of Indian rotavirus vaccine ‘Rotavac’ at national level can prevent 27,000 deaths, 2,91,000 hospitalizations and 6,86,000 outpatient visits yearly (John et al., 2014). Our results underscore the huge diversity of circulating
rotavirus strains in Haryana and these surveillance studies is the need of hour to better understand the variations in the strain distribution before and after introduction of vaccination. It is proposed that serotype/genotype specific immune response plays an important role in protection against rotavirus gastroenteritis. Therefore, molecular surveillance of G and P genotypes of circulating strains will generate a unique knowledge database which could be used for determining the effectiveness of rotavirus vaccine.