5.1. Distribution of group A rotaviruses among human, bovine and porcine neonates.

The primary aim of the study was to detect rotaviruses from the neonates of human, cow and pig characteristically from a setting which is closely shared. Those settings significantly facilitate interspecies or zoonotic transmission that acts as key aspect for genetic reassortment within the virus genome producing new viable strains. In our study, sampling area demonstrated close proximity among humans and animals where barely hygienic conditions are upheld. The monoclonal antibody (mAb-EIA) and nucleic acid (RT-PCR) based detection allowed significant and rapid diagnosis of rotaviral infection from the clinical samples. When data were analyzed on the basis of sampling phase and faecal consistency, the infection was found more prevalent during the first week of life comparing to the late phase and the positivity rate was highest with watery/liquid faeces (33.3%). Group A rotaviruses were detected more recurrently and is consistent with the earlier reports (Martella et al. 2006; Ghosh et al, 2008; Miyazaki et al. 2011; Ganime et al. 2012). Maximum numbers of rotavirus positive cases were found in piglets followed by human and cow. Earlier studies (Neog et al. 2011) from the Brahmaputra valley solely on pigs also confirmed piglet rotavirus prevalence. In cows and humans, cases of positive rotavirus incidence, it is the first reported incidence from this region. These findings signify that rotavirus is highly circulating in human and animal in this vicinity.

The discontinuous RNA-PAGE was employed for rotavirus strain grouping. Studies reported that this technique is another mostly used gold standard test for rotavirus detection in faeces (Gatti et al. 1993; Jindal et al. 2000; Ramos et al. 2000; Bozdayi et al. 2008; Aminu et al. 2010). In this study, the electropherotypes of rotavirus isolates corresponded to the basic RNA migration pattern of 4:2:3:2 typical for group A rotavirus (RVA). The result of the present
study also depicts that electropherotype of few (6.7%) animal rotaviral isolates of similar epidemic demonstrated additional variation in the migration pattern of their RNA segments by representing both, longer and shorter electropherotype. This finding suggests that inside a single region, variant strains of rotavirus are circulating that most likely suggesting genetic diversity of rotaviruses. However, variations in the RNA profiles of human samples were not detected during the study but the entire phenomenon might be responsible for the circulation these variant strains within the whole community.

The VP7 gene, encoded by the 9th segment of rotavirus genome is a glycoprotein that forms the outer surface of the virion and induces neutralizing immune responses during rotavirus infections. The VP7 gene targeted by RT-PCR amplified an expected band of 1062 bp for pig and human RV’s; 1011bp for cow RV strains and further confirmed the presence of rotavirus in the studied species as been evidenced earlier too (Rao et al. 2000; Aminu et al. 2010). The VP4 protein (VP8*), encoded by the 4th segment of the RV genome too yielded expected band of size 880bp. Circulations of rotavirus in different susceptible host in a single geographical region, therefore generate a potential possibility of interspecies transmissions and thus provokes for reassortment of the viral strains consequently emerging a new variant strain.

Further, the RFLP assay was designed for identification and differentiation of such group A rotaviruses of human, porcine and bovine origin. Examination of VP7 and VP4 restriction profiles obtained after digestion with VspI, HaeIII, NlaIV, TaqI and HindIII revealed several interesting features of rotavirus diversity in Assam. The banding patterns showed isolates demonstrating a single enzyme profile, while others had a combination of enzyme profiles or a unique RFLP pattern. The generated enzyme profiles suggested that when a single G-type infection occurred there was an obvious similarity between the enzyme profiles. Interspecies transmission or sharing of serotypes within different hosts is being reported continuously by various workers (Steyer et al. 2008; Mukherjee at al. 2013; Degiuseppe et al. 2013). Our finding is consistent with those reports where
co-circulation or sharing of intrinsic G-types/ RFLP patterns in diverse hosts range suggests event of interspecies transmissions particularly during mixed infections and more generally, in the setting of proximal contact between humans and farm animals. The phenomenon could be evidenced from the study area where closer contacts between animals with their dwellers were recognized. In such circumstances, the fecal-oral route offers greatest occasion for viral transmission within the associated hosts. Reassortment of rotavirus genome occurring after co-infection of a host, has been shown to be an important mechanism to generate diversity on many occasions and moreover, other less important mechanisms such as inter- or intragenic recombination are believed to occur less frequently. The affinity of the Indian rotavirus isolates towards sharing of G-types could significantly boost evolution of the viruses and subsequent emergence of atypical or novel strains.

Comparison of RFLP data for Indian and global isolates suggested Indian rotavirus population is distinguishable from global strains and none, of their associated RFLP patterns were shared. The global isolates produced a consistent profile through empirical analysis but the Indian isolates exhibited a greater diversity by providing additional or no cleavage site. It could be assumed that there were some point mutations in the gene during replication and existing restriction enzyme sites disappeared or new sites generated. Restriction enzymes were also found potential to identify the presence of mixed infections in the circulating rotavirus strains by Halloran et al. in 2002. The combined analysis of the RFLP patterns proposed considerable occasions of mixed rotavirus infection within the hosts. It may be thus, reasonable to suggest that one of the co-existing G-serotype was dominant towards infection.

Since, rapid evolution of these viruses by the generation of reassortant in multiple infections is evident; molecular epidemiological surveillance of the rotavirus types co-circulating in the population is indispensable. Therefore, the present study could differentiate positive interspecies transmission cases and significant variations among the serotypes were also anticipated but the extents of
such mechanisms are ambiguous which are supposed to be established by analyzing respective sequence data. Thus, further, the rotaviral VP7 and VP4 genome segments were analyzed to establish the variations and assessing the genetic diversity to monitor emergence of any uncommon combinations.

5.2. Identification of unusual/rare genotypes accounting emergence of reassortants.

The sequence analysis of variant rotavirus strains isolated during the present study determined for the first time the G and P genotypes of circulating rotavirus strains from human and animals. Previous surveys on different geographical region of the world reported G1, G2, G3, G4, G9, P[8], P[4], and P[6] as dominant genotypes in human rotavirus episodes (Gentsch et al. 2005; Santos and Hoshino, 2005). Globally, the prevalence of common genotype combination G1P[8] is consistent in this study as well, since we found 25% of the rotavirus strains as G1P[8]. The genotype G1 detected more frequently in combination with P[8] constitutes one of the important component in a currently recommended vaccine, RotarixTm (Zeller et al. 2012). The G1P[8] strains also demonstrated epidemiologically relevant to infections in animal species, where remarkably one of the studied porcine specimen has been characterized for G1P[8] in the present study. Rotavirus strains of G1 specificity have been isolated worldwide from humans but few earlier studies report its incidence in porcine and bovine (Blackhall et al. 1992; Ciarlet and Liprandi, 1994). Therefore, evidence of G1 frequency in combination with a human P[8] VP4 specificity in porcine is rare.

G-type G1 in a combination with typical animals P-types P[7], P[13] and P[23] has not been reported in pigs and cattle previously from the country. Worldwide, common porcine rotavirus genotypes are G3, G4, G5, G11 and P[6], P[7], P[13], P[19], P[23], P[26], P[27] (Martella et al. 2010). Likewise, bovine rotavirus A have been classified as G6, G8 or G10 genotypes, associated with either P[1], P[5] and/or P[11] and genotypes G6P[5], G6P[1] and G10P[11] are
considered the most common ones. However, we detected a considerable prevalence of the rare G1P[7] genotype in animals. The common porcine P[13] and P[23] genotypes along with the human G1 genotype also established to be dominant in the animal populations. The presence of such unusual rotaviral strains among farm animals observed during the study may be the reason for their possible transmission among the proximal man and animals.

An emerging G9 strains associated with majority of human diarrheal episodes worldwide (Matthijnssens et al. 2010; Alam et al. 2013) was characterized from a human specimen in the present study. The finding concurred with studies from India as well reporting G9P[8] genotype in high frequency of the characterized rotavirus isolates (Gladstone et al. 2011; Miles et al. 2012). Rotavirus G5 strains are primarily porcine pathogens that are recovered sporadically from bovine and horses (Hoshino et al. 1996; Ha et al. 2009). The study reports an incidence of human diarrhea with G5 genotype specificity which is having strong identity with a porcine G5 Chinese strain as well as with few other Chinese human G5 strains included in the analysis. The VP4 sequence of the P[8] counterpart showed usual identity with VP4 sequences of human P[8] strains. Thus, G5P[8] is considered as an uncommon genotype combination and, there are rare or no reports of this genotype incidence in human from India previously except few in bovines (Dhama et al. 2009).

G3 is the only rotavirus G-type for which such a broad host range has been described (40, 41). We detected G3 strain with an unusual P[7] VP4 gene in a piglet whose VP7 sequence is mostly identical to recently prevailing G3 strain in China. This incidence concurred with the studies recently reported G3 strains, though in piglets but with P[13] specificity (Saikruang et al. 2013; Miyazaki et al. 2013). In India, a few studies reported G3 rotavirus prevalence in bovines and humans with P[8] specificity (Zade et al. 2009) but detection in combination with P[7] counterpart is fairly rare and not reported from any diarrheal episodes yet. Overall, the results highlight that animal is an important reservoir for thriving rotavirus infection, facilitating interspecies-transmission or sharing of genotypes.
within multiple hosts; particularly during mixed infection and more generally in setting of close contact between man and animals. The phenomenon can be well evidenced from the study sites where, backyard pig and cattle rearing is a habitual commotion of the people residing here. Animals are housed indoors in group-housing or straw-lines shed that allows easy contact with waste matter for the human handlers and vice-versa. It is thus reasonable to state that the environment contributes to the transmission of rotaviruses by infecting incoming animals and their human handlers. Incidence of few species-specific genotypes of animal origin in humans and vice-versa supports strong evidence of interspecies transmission during the study time period.

The unusual genotype combinations reporting for the first time from the region consequently advocate significant evidence of interspecies transmission. Thus, finding of uncommon genotypes/genotype combination of rotavirus adds to the global distribution of this strain and strengthens the need to continue strain surveillance in developing countries to further understand the extent of strain distribution and diversity. However, emergence of such unusual combinations warrants analysis of additional gene segments further, to determine whether it is an example of a strain that arose through direct interspecies transmission from a particular animal host, or by reassortment with heterologous rotavirus strains.

5.3. Evidence of rotavirus gene reassortment

Reassortment of the rotavirus genome occurring after co-infection of a host has been shown to be an important mechanism to generate diversity as well. The detection of few unusual rotavirus G-P combinations (G1P[7], G1P[13], G1P[23], G5P[8] and G3P[7]) in man and animals indicated a significant contribution of reassortants in causing diarrhoea. Analysis of such unusual genotypes revealed an example of porcine-like VP7 gene and an intrinsic human VP4 counterpart strain has been detected from a human of Nepali Basti, Guwahati. Moreover, during the course of characterizing the P and G types of rotavirus strains isolated from animals in, we again unexpectedly encountered
thirteen (13) such unusual rotavirus genotypes having the common animal VP4 genotype but whose VP7 genes belonged to human lineages. The factors that promote the unexpected detection of human/animal gene counterparts in a rotavirus strain isolated from varied hosts are poorly understood. It can be speculated that close contact of children with animals and vice-versa characteristically depicted by the studied sites may resulted in the interspecies transmission among animal rotaviruses and/or coinfections with human and animal rotaviruses, leading in the reassortant formation/events. Subsequent spread to an animal/human could then result in the establishment of these strains in population.

Studies on the diversity of animal rotavirus VP7 and VP4 genes are of ecological importance and can provide insight into the mechanism involved in rotavirus evolution, interspecies transmission and exchange of genetic material during reassortment. However, to depict the whole genetic constellation and the origin of the strains emerging subsequently, analysis of the rest of the segments is required so as to obtain conclusive data on ongoing genetic events. Thus, based on the two structural genes VP7 and VP4, the study provides novel information on the occurrence of the unusual animal rotavirus strains and direct evidence to support the interspecies transmission and reassortment of human and porcine/bovine rotaviruses in nature. Identification of such novel rotavirus strains of varied host origin is of epidemiological importance and can lead to serious implications regarding rotavirus vaccine development and implementation.

5.4. Genetic disparity in VP7 and VP4 antigenic epitopes between circulating Rotaviruses and strains in Rotarix™ and RotaTeq™.

The World Health Organization has recommended inclusion of rotavirus vaccines in national immunization programs worldwide, especially in countries like India where diarrhoea is responsible for ≥10% mortality in children [36]. Two vaccines, Rotarix and RotaTeq are currently licensed. In India, Rotarix was launched in 2008 and RotaTeq in 2011. Both vaccines are available through the
private sector and have not been introduced into the national immunization program yet [37]. Moreover, the parental strains of existing vaccines were isolated more than 20 years ago in France (G4 parental strain in RotaTeq) and the United States (all other parental strains). To interpret about the efficacy of the existing vaccines for providing optimal protection against diversified strains characterized in the study, we compared the VP7 and VP4 antigenic epitopes of the two licensed and available vaccines Rotarix™ and RotaTeq™ with those of the circulating rotavirus strains. By analyzing the ORFs of VP7 and VP4 genes, we identified important antigenic disparities between the vaccine and local strains. G1P[8] rotavirus strains are predominant in India and are represented in both the current vaccines. In particular, being components of existing vaccines, the G1P[8] strains displayed a large intragenotypic variety. Most of the G1 strains belong to lineage 1, 2 and 3, of which lineage 3 is the most distinct having Rotarix and RotaTeq clustered together. Up to seven amino acid differences were found when we compared the VP7 of local G2 strains to the G1 strain of Rotarix. Since G1 strains are generally associated with P[8] genotypes, protection against G1 viruses afforded by Rotarix would depend on both the components of the vaccine i.e G1 and P[8]. Similarly, protection against G1 strains afforded by RotaTeq would depend on the both the components of the vaccine i.e G1-G4, G6 and P[5]. However, the VP4 component of the local strains of genotype G1P[7], G1P[3] and G1P[23] does not conform to any of the VP4 constituent of RotaTeq and Rotarix. Moreover, huge extent of antigenic dissimilarity within their VP7 and VP4 antigenic epitope of these non-vaccine components is persisting. Therefore, antigenic drift within the rotavirus genome could reduce the vaccine efficacy against such variant strains much faster. However in-vivo protection study could give conclusive evidence on efficacy of the vaccines against circulating strains.

Similarly, for VP4 proteins, most variation was observed in P[8] strains as they showed genetic closeness to non-vaccine lineages. Significant amount of indels in 8-1 epitope by possessing an additional glycosylation site was found to be an exceptional mutation in the active VP4 protein antigenic epitope. These amino
acid changes could have emerged due to acquired mutations or, alternatively, could be the results of an *in-vivo* selection of minor variants already present in the vaccine. The mutations in the antigenic regions of rotavirus play an important role in the outcome of vaccine response. Among all the non-vaccine VP7 and VP4 components of local rotavirus strains, increased number of mutations in the antigenic regions of the respective genotype can modify the antigenicity of the respective region and might to play a significant role in antigenic recognition (Coulson and Kirkwood, 1991). Our results concurred with the finding of Jin et al in 1996 where they reported that most of the G1 strains identified during their study and the Rotarix™ vaccine RRV-S1 strain differ in their antigenic properties and mutations in these antigenic sites may ultimately cause vaccine failure (Jin et al. 1996). As during this study, six-seven provinces of NE India were explored which represents a small fraction of population. However, more samples encompassing diverse geographical areas can give clear picture about the true variability present in the rotaviruses prevailing in man and animals of NE India.

5.5. Conclusion

Till now, rotavirus surveillance has mostly been focused on human infection. The present study reinforces the need to continue surveillance program including farm animals from agrarian region because of (i) their close contact with humans, especially in developing countries; (ii) increased reports of the detection of strains common to animals in human populations; and (iii) the increasing spread and detection of strains with unusual G and P types and untypeable strains. The availability and affordability of sequencing methods now provide an alternative tool over PCR-genotyping for the characterization of unusual/non-typeable strains. In consequence, the surveillance programs and characterization of rotavirus strains shall continue specifically for these unusual rotavirus strain combinations lacking common rotavirus VP4 and VP7 proteins, which have been the focus of future rotavirus vaccine development.
Precisely, the results reported here reveal extensive genetic diversity of RVAs both in man and animals. The results also suggest either temporal or spatial fluctuations in RVA genotype distribution in addition to the consistent presence of RVAs in all the three hosts’ i.e man, cattle and pigs. The unusual genotype combinations from human and animal in close settings advocate significant evidence of interspecies transmission. Thus, finding of uncommon genotypes/genotype combination of rotavirus in agrarian region of Barak and Brahmaputra valleys adds to the global distribution of this strain and strengthens the need to continue strain surveillance in developing countries to further understand the extent of strain diversity.

Successive detection of porcine-like rotavirus strain isolated from a human and 13 rotaviruses of human lineages from animal species, clearly provided evidence of possible human-animal reassortment during the period. Thus, based on the two structural genes VP7 and VP4, the study provides direct evidence to support the interspecies transmission and reassortment of human and porcine/bovine rotaviruses. However, emergence of reassortant strains warrants analysis of additional gene segments further, to determine confirm the occurrence of reassortment with heterologous rotavirus strains.

The further comparisons of the rotavirus strains to the existing vaccines evaluated the antigenic relationship among themselves. Higher degrees of synonymous and non-synomous substitutions/indels in the antigenic regions of VP7 and VP4 proteins were observed that plays an important role in the outcome of vaccine response. Among all the non-vaccine VP7 and VP4 components of local strains, increased number of mutations in the active binding sites can modify the antigenicity of the respective region. The structural analyses of the VP7 and VP4 amino acid differences are yet to be done and the study of their role of influence in antigenicity of these proteins are lacking. The precise impact of amino acid changes in the antigenic epitope predicted during the study provided strong evidence regarding the differences of the currently circulating rotavirus strains to the existing rotavirus vaccines at amino acid level. The analysis thus,
provides an important finding of genetic and possible antigenic differences between the characterized rotavirus strains after several years of introduction of rotavirus vaccine Rotarix and RotaTeq. The study also provides an epidemiological data to the present National Surveillance Network System and further, providing a probable vaccine candidate to the rotaviral repository of this region. Thus, the work presents here a challenge to the efficacy of existing vaccines based on VP7 and VP4 genes that warrants the need to extend the vaccine coverage by novel rotavirus strains predominating all over the world, including the region under study.