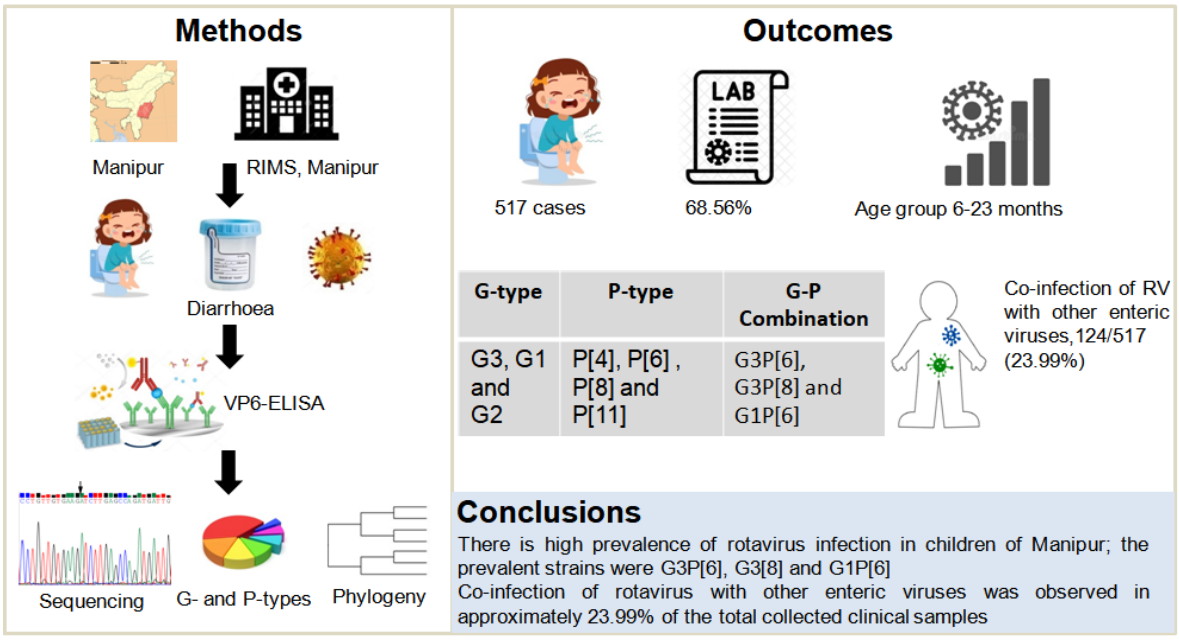
CHAPTER 3

MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES IN CHILDREN UNDER-FIVE-OLD IN IMPHAL, MANIPUR, INDIA

Graphical Abstract



3.1. Introduction

Rotavirus (RV) is the leading cause of acute gastroenteritis in children worldwide. Human rotavirus was first identified in 1973 by electron microscopy of thin sections of duodenal mucosa from infants suffering from diarrhoea [1]. Globally, the most common RVA genotypes are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] [2,3]. In India, in the pre-vaccine era, the prevalent strains were G1P[8] followed by G2P[4] (8%), G9P[4] (7.5%), G9P[8] (4.5%), and G12P[6] (3.8%) accounting 49.5%. In the post-vaccine era, G3P[8] (44.3%) was the most prevalent genotype, followed by G1P[8] (15.4%), G2P[4] (7.4%), G9P[4] (4.9%), and G1P[6] (3.7%) [4]. Apart from the common strains, there have been occurrences of rotavirus strains which are unusual, rare, and novel variants due to the various processes such as antigenic drift and antigenic shift [5,6,7].

As mentioned in Chapter 2, Rotavac and RotaSIIL have been introduced in the national immunization programs and Rotarix and RotaTeq are available since 2006 in India [8,9, 10]. According to the published report of NRSN, rotavirus causes approximately 34% of all diarrhoeal deaths in children under five-year-old [11,12,13]. This suggests the need for continuous surveillance pre- and post-vaccination to measure the efficacy of the rotavirus vaccines in the population.

In Manipur, during the year 1979 to 1988, few rotavirus outbreaks were reported with the evidence of zoonotic transmission [14,15]. A hospital-based surveillance study conducted in Manipur by Mukherjee et al. (2010) in children with acute diarrhoea have found the prevalence of genotype G12 strains [16]. There is a scarcity of research on diarrhoea and comprehensive epidemiological considerations pertaining to such investigations as relevant to northeast India require urgent attention. This emphasizes the need to understand the rotavirus strain diversity, the mechanism by which these strains emerged over time and their long-term impact on vaccination.

The current study examines the incidence of circulating rotavirus strains in Manipur, a state in northeastern India, before the introduction of an indigenous rotavirus vaccine. The findings will help in implementation of programs for improvement of child health, and it also provides a reference to measure the impact of vaccination in post-vaccination era in Manipur and other northeastern states. To further understand the effectiveness of vaccines in children under five-year-old in Northeast India, extensive studies of the prevalence of four common enteric viruses will be important after the introduction of vaccines for rotavirus-led diarrhoea.

3.2. Materials and methods

A schematic flowchart showing G-P typing of rotavirus positive stool specimens is given in Figure 3.1.

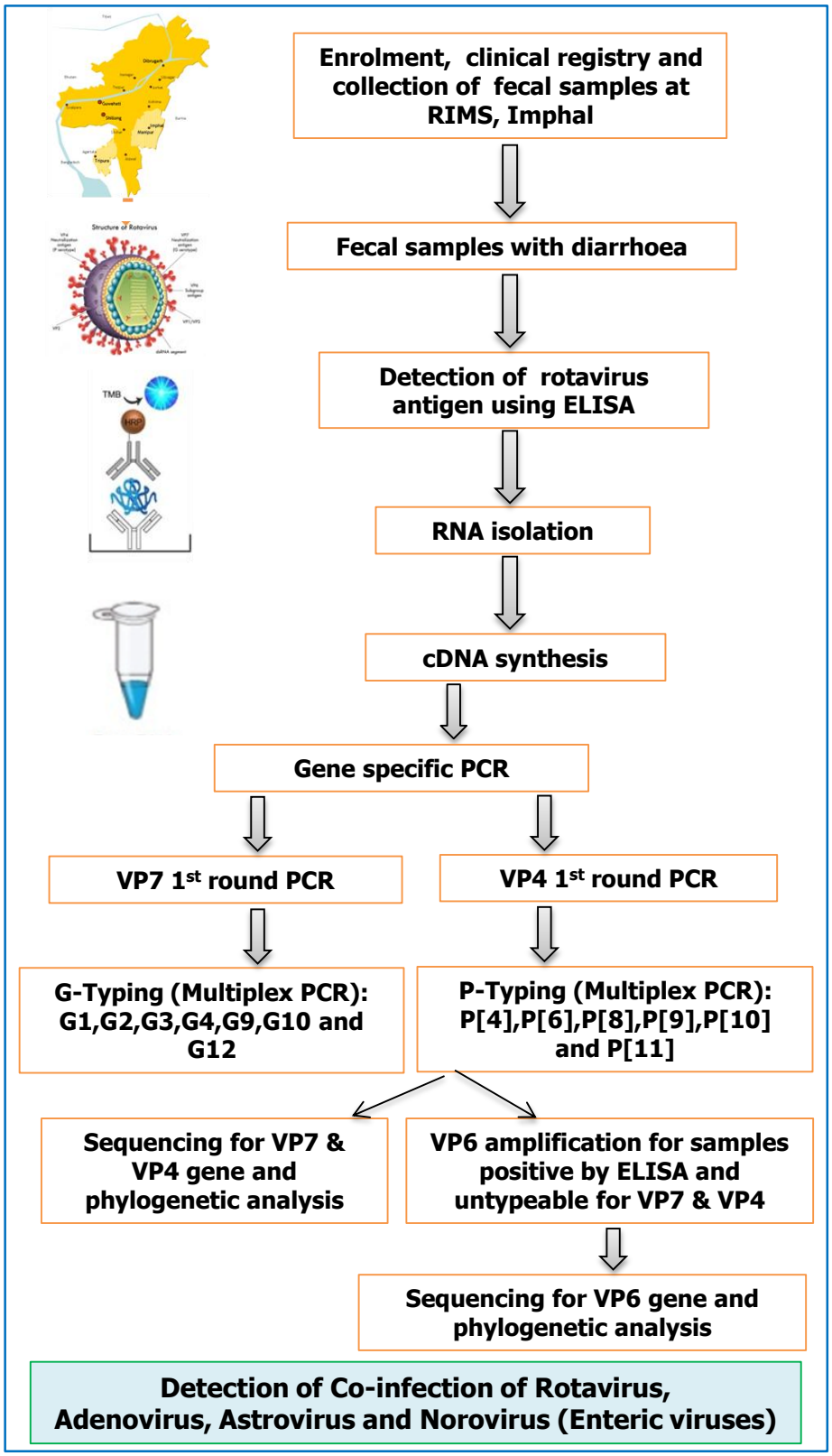


Figure 3.1. Schematic flowchart showing G-P typing of rotavirus positive stool specimens.

3.2.1. Enrolment and data collection

The inclusion criteria for children in the study was from 1 to 60 months who visited Regional Institute of Medical Sciences (RIMS), Imphal, Manipur, having acute gastroenteritis (AGE), < 7 days duration, with or without vomiting, mild fever and abdominal pain, body temperature, dehydration, oral rehydration therapy (ORT), rotavirus vaccination dose received (Rotateq/Rotarix). Children with compromised immune systems, people who are severely malnourished, and people who are extremely ill with other diseases—including severe sepsis, bleeding, acute respiratory failure, cardiac failure, meningoencephalitis, and coma were excluded. If the patient or the patient's mother is positive for HIV, hepatitis B or C children were excluded from the study. Healthy children were enrolled for the study to serve as control and the criteria was children of the same age group 1 month to 60 months without having fever, diarrhoea, dehydration, etc. Children that did not receive rotavirus vaccination were preferred for healthy control specimens (Figure 3.2). After consent, clinical data was recorded and stool specimens were collected from children enrolled at RIMS, Imphal, for laboratory confirmation of rotavirus within 48 hours after admission.

Table 3.7. Enrolment criteria for collection of stool specimens from RIMS, Imphal, Manipur.

Sl. No.	Inclusion criteria
1	Age of patient: 3 months to 60 months
2	Acute watery diarrhoea of < 7 days duration Patients with or without vomiting
3	Mild fever and abdominal pain and body temperature.
4	Dehydration and Oral rehydration therapy (ORT)
5	Rotavirus vaccination dose received (Rotateq/Rotarix)

Sl. No.	Exclusion criteria
1	Immunocompromised patients
2	Severely malnourished
3	Severely sick patients with other diseases- severe sepsis, bleeding, acute respiratory failure, cardiac failure, meningoencephalitis, coma, etc.
4	If the patient or the patient's mother is positive for HIV, hepatitis B or C.

Sl. No.	Inclusion criteria for healthy controls
1	Age of patient: 3 months to 60 months,
2	without having fever, diarrhoea, dehydration, etc.
3	Children who did not receive rotavirus vaccination prefer for healthy control specimens.

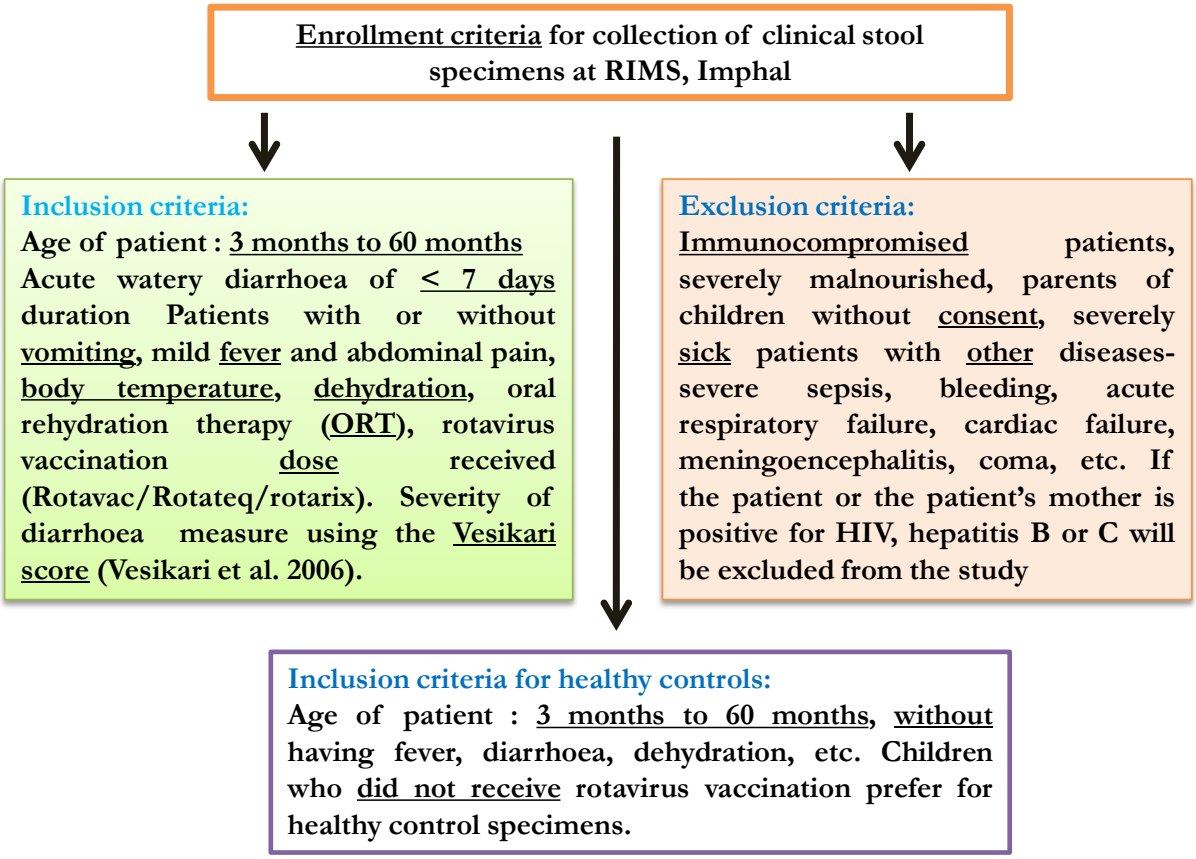


Figure 3.2. Schematic flowchart showing enrolment criteria for collection of stool specimens from RIMS, Imphal, Manipur.

3.2.2. Study samples

Fecal specimens were collected, stored at -20ºC and shipped to Tezpur University for laboratory analysis. A total of 527 human stool specimens (both in-patient and out-patients) excluding the control samples, were collected from the children presented with acute gastroenteritis visiting RIMS, Imphal, during the study period from December 2015 to March 2019.

3.2.3. Rotavirus detection in stool specimens

The diarrhoeal stool samples were primarily screened to detect the presence of RVA antigen by solid-phase sandwich enzyme immunoassay, Premier Rotaclone® kit (Meridian Bioscience, USA) according to the manufacturer's protocol. The fecal samples were diluted with sterile 1X phosphate-buffered saline (PBS) to prepare 30% viral suspension and clarified by centrifugation at 10,000 rpm for 10 min at 4ºC. The

supernatants were collected and stored at −20 ◦C until use. Clarified supernatant of 10% fecal suspension was used for ELISA.

3.2.4. Rotavirus RNA extraction and polyacrylamide gel electrophoresis

Viral RNA was extracted from 10% fecal suspension in duplicates using PureLink® viral RNA/DNA mini kit (Invitrogen, USA) following manufacturer's protocol. The migration patterns of 11-dsRNA segments of rotavirus were analyzed on 14% polyacrylamide gel stained with silver nitrate using one aliquot of the extracted RNA.

3.2.5. Co-infection of rotavirus, adenovirus, astrovirus and norovirus

To investigate the co-infection of rotavirus with other enteric viruses namely, adenovirus, astrovirus and norovirus, Rota+Adeno+Astro+Noro EIA Combo Card (Certest Biotec, S.L. Spain), a qualitative immunochromotographic assay was used. The 30% viral suspension samples were applied to the specimen port of the combo card and the test was performed according to the manufacturer's protocol.

3.2.6. Genotyping of rotavirus

Other aliquots of the extracted RNA by PureLink® viral RNA/DNA mini kit was used as a template for reverse transcription PCR (cDNA synthesis). RT-PCR was carried out by using the SuperScript™ III reverse transcriptase (Invitrogen, USA) and random hexamers (Invitrogen, USA). The dsRNA was denatured at 95°C for 5 min and reverse transcription reaction was performed with thermal cycle steps at 25ºC for 10 minutes and 55°C for 60 min.

The cDNA as template, VP7 & VP4 genes (first strand synthesis) were amplified using consensus primers mentioned in Table 3.3. The PCR reaction was set using DreamTaq DNA Polymerase (Thermo Fisher Scientific, USA). The amplification was performed in a MasterCycler PCR System (Eppendorf, Germany) with an initial denaturation at 94°C for 3 min, 35 cycles of amplification (1 min at 94°C, 1 min s at 42°C, 1 min at 72°C), and a final extension for 7 min at 72°C. The amplicons were analyzed on 1% agarose gel stained with ethidium bromide (EtBr) and visualized under UV light.

Next, the VP7 first strand PCR amplicon in the ration 1:10 was used as template and subjected to semi-nested multiplex PCR amplification with a mixture of G type-specific primers mentioned in Table 3.3, and a conserved reverse primer VP7-R. The thermal

cycle followed was initial denaturation of 94°C for 4 min, followed by 30 cycles of amplification (94°C for 1 min, 42°C for 2 min and 72°C for 1 min), and the final extension at 72°C for 10 min. It was followed by analysis of the PCR amplicons on agarose gel as mentioned above. Same protocol was followed for P-genotyping PCR for VP4. Similarly, semi-nested multiplex PCR was performed for VP4, using VP4 first strand PCR product as template and using P-type specific primers and consensus primers Fcon3 as forward primer given in Table 3.3. For samples which were positive by ELISA but VP7 and VP4 could not be amplified, amplification of VP6 was performed to rule out false ELISA positivity. If VP6 gene also could not be amplified, other non-structural RV genes such as NSP4 and NSP5 amplification was performed using primers given in Table 3.3.

3.2.7. PCR for adenovirus, astrovirus and norovirus genes

To validate and confirm the results of adenovirus, astrovirus and norovirus, PCR amplification of viral specific genes were also performed using the gene specific primers (Table 3.6). The cDNA synthesis protocol was the same as mentioned above. The PCR condition was kept for initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 35 sec, and 72°C for 1 min 30 sec), and the final extension at 72°C for 10 min.

3.2.8. Sanger sequencing of VP7, VP4 and VP6 genes and phylogenetic analysis

The PCR products were purified using PCR KlenZol™ (Merck Millipore, USA) according to manufacturer's protocol and sequenced on an ABI PRISM 3100 DNA analyzer (Applied Biosystems, CA) using BigDye Terminator Sequencing Kit (Applied Biosystems, CA). The primers used were VP7-F and VP7-R primers for VP7 gene and for VP4; it was Fcon3 & Rcon2 primers (Table 3.3). The sequences obtained from Sanger sequencing method were examined using BLASTN tool and deposited in the nucleotide database, GenBank. Sequence data analysis and the construction of a phylogenetic tree were performed using MEGA X (www.megasoftwares.com) [17]. The phylogeny was analyzed using the maximum likelihood method and was inferred from 1000 replicates. Molecular Evolutionary Genetics Analysis (MEGA) is an integrated tool for a large repertoire of programs namely, sequence alignments, evolutionary analysis, genetic distances and diversities analysis, ancestral sequence reconstruction, computing time trees, and testing selection [17].

3.2.9. Statistical analysis

Data gathered from the enrolled diarrhoea patients were analyzed using RStudio (RStudio, United States) and Microsoft office excel 2007. Chi-square and Wilcoxon ranksum test were used for statistical analysis and $p<0.05$ value was considered significant. Differences in age groups, clinical symptoms, and disease severity using Vesikari severity scoring system [18] among RVA positive patients were summarized (Table 2.2a&b). Continuous variables with non-normal distributions were compared using the Wilcoxon rank sum test while categorical variables were compared using the chi-square test (Table 3.1). The mean and 95% confidence interval (CI) of continuous variables were calculated from the observed value.

3.3. Results

3.3.1. Detection of rotavirus antigen

During the study period, a total of 527 stool specimens were collected from children with acute gastroenteritis at RIMS, Imphal from December 2015 to March 2019 and the clinical parameters were recorded. The fecal specimens were examined for RVA antigen by enzyme immunoassay. 217 control samples from healthy children were taken while performing ELISA. Of the total 527 samples, rotavirus was detected in 69.25% (358/527) of diarrhoeal cases (Table 3.1). The percentage of rotavirus-positive samples varied from year to year; it was lowest in 2017 (56.25%) and highest in 2019 (80.44%).

Table 3.1. Numbers and proportions of children enrolled, tested for rotavirus and having rotavirus-positive stool specimens, December 2015 – March 2019.

Note:

* 1 out of 1 was positive for RV ** 1 out of 2 was positive for RV *** 2 out of 2 were positive for RV

Year	No. (%) of children enrolled and stool specimen tested for rotavirus	Premier Rotaclone™ (VP6-ELISA)				CerTest
		No. (%) of children with rotavirus positive stool specimen	No. (%) of children with false negative rotavirus stool specimen	False Positive ELISA	No. (%) of children above ≥5 years	No. (%) of children with rotavirus positive stool specimen
2015	40	27 (67.50%)	1 (2.5%)	24	1	30 (75.00%)
2016	215	157 (73.02%)	1 (0.47%)		4	159 (73.95%)
2017	112	72 (64.29%)	-		1*	68 (60.71%)
2018	96	72 (75.00%)	-		2**	71 (73.96%)
2019	46	38 (82.61%)	-		2***	34 (73.91%)
Missing Clinical Data	18	12 (66.67%)	-		-	12 (66.67%)
Total	527	378 (71.73%)	2 (0.38%)	24/ 378 (6.35%)	10 / 527	374 (70.97%)
Total number of rotavirus positive stool specimen		356/517 (68.86%)				374 (70.97%)

3.3.2. Seasonality and clinical manifestations

A peak increase in occurrence of RVA diarrhoea was observed mainly in the winter months during the study period (Figure 3.3 and Figure 3.4). Most hospitalized children were in the age group 6 months to 2 years and rotavirus-positive acute watery diarrhoea (AWD) in this age group was 77.09%. We also detected rotavirus-related diarrhoea in infants younger than 6 months old (9.22%) and children between the ages of 2 and 5 years (9.5%) (Table 3.2). Twelve of the 18 samples that did not have clinical data (age, patient name, symptoms, etc) also tested for rotavirus positivity. Ten samples were taken from children older than five years old out of the total 527 samples, and four of those samples tested positive for RV (Table 3.1). Children who tested positive for the rotavirus experienced more diarrhoeal episodes than those who tested negative (mean 7

versus 6 times), while those who tested negative for the rotavirus experienced more incidences of vomiting (74.86% versus 83.65%). Disease severity was estimated by vesikari severity scoring system and found no significant difference in the severity of the disease between rotavirus infected children and non-infected children (Table 3.2).

Table 3.2. Characteristics of rotavirus-infected and -uninfected children (≤5 years) hospitalized with acute gastroenteritis.

Note:

* Out of 517, clinical data was missing for 18 samples.

** Actual Value is 0.000022.

Variable	Total number of children (517*)	Rotavirus infected children (n = 376)	Rotavirus uninfected children (n = 138)	P value	Test
Gender Male Female	499	 245 117	 83 54	0.127	Chi-square test
Age, mean months (95%CI)	499	12.65651 (11.4 - 14)	12.6058 (11.7 - 13.5)	0.094	Wilcoxon ranksum test
Age (months) 0–5 (n = 55) 6–11 (n = 183) 12–23 (n = 211) 24–59 (n = 50)	 55 183 211 50	 36 (9.58%) 136 (36.17%) 154 (40.96%) 39 (10.37%)	 19 (13.77%) 47 (34.06%) 57 (41.30%) 11 (7.97%)	0.496	Chi-square test
Clinical symptoms Number of diarrhoea episodes within 24h (mean, 95%CI)	 499	 7 (6.62 - 7.38) times	 6 (5.44 - 6.56) times	 0.001 **	 Wilcoxon ranksum test
Vomiting	488	285 (75.80%)	115 (83.33%)	0.400	Chi-square test
Disease severity by Vesikari score					
Mild (<7) Moderate (7-10) Severe (≥11) Total	1 143 355 499	0 (0 %) 107(28.46%) 254 (67.55%) 361	1 (0.73%) 36 (26.09%) 101(73.19%) 138	0.205	Chi-square test

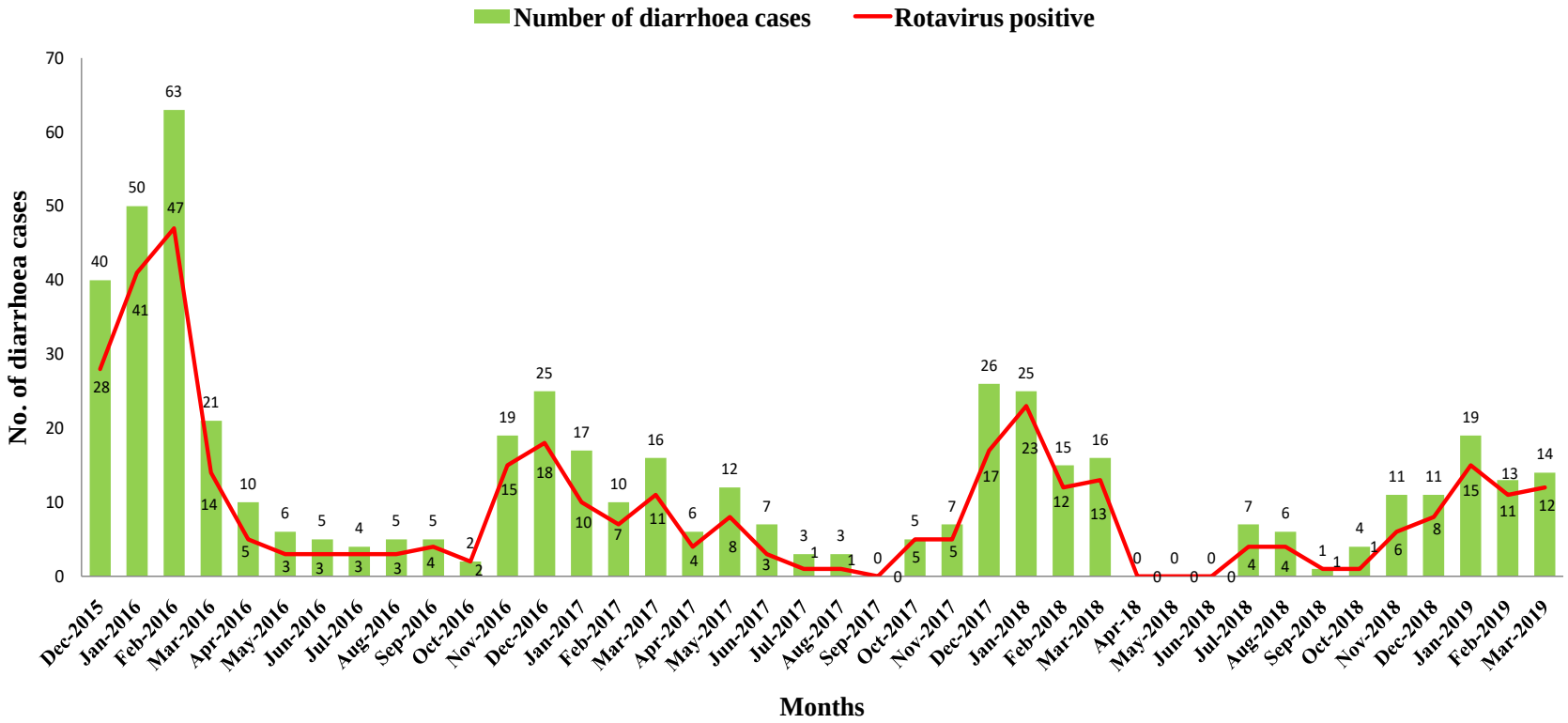


Figure 3.3. Bar graph showing seasonal distribution of rotaviral gastroenteritis from December 2015-March 2019.

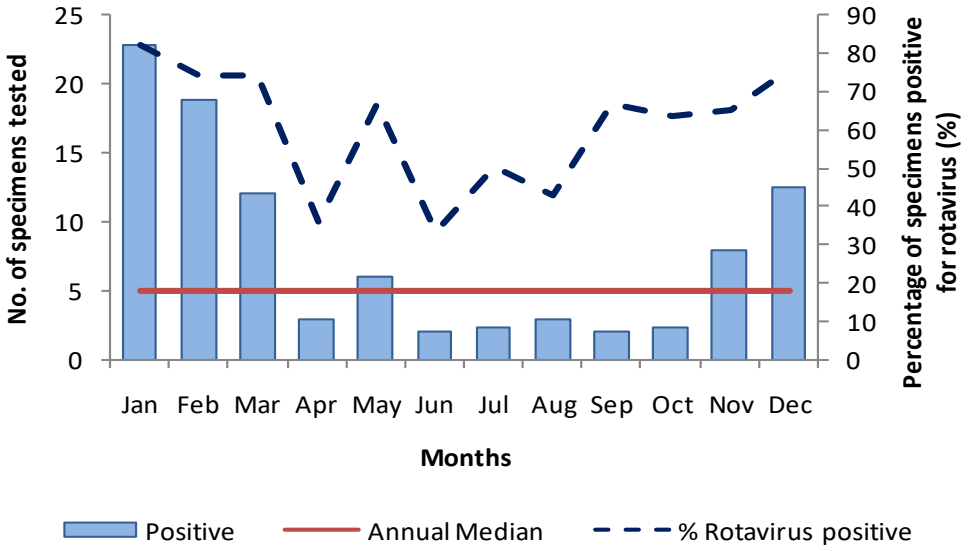


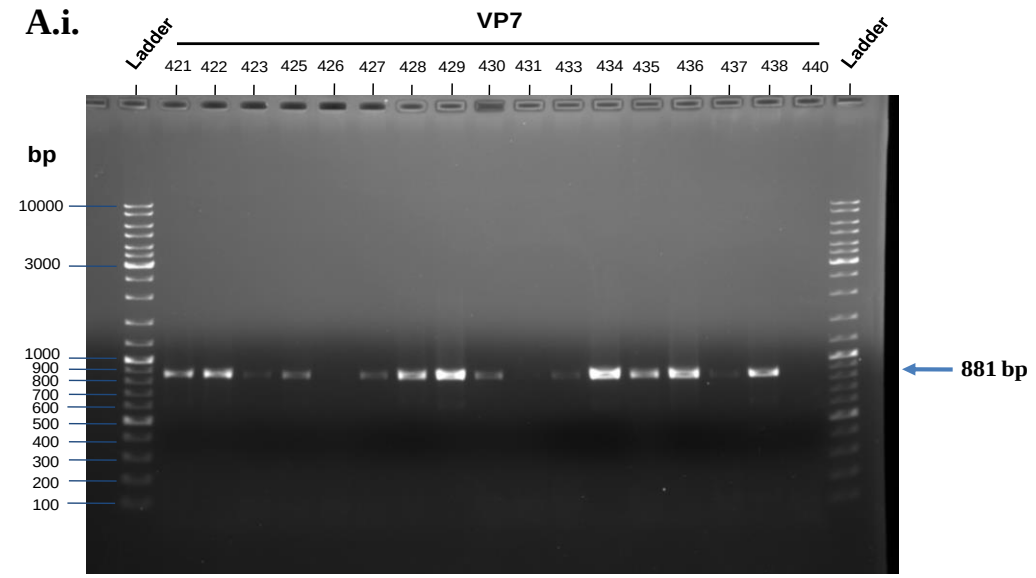
Figure 3.4. Rotavirus infection trends among hospitalized children <5 years from Imphal, Manipur, India visited RIMS, Imphal during December 2015 to March 2019.

Table 3.3. Consensus and G/P typing primers used for the amplification VP7, VP4, VP6, NSP4 and NSP5 genes.

Primer Name	Sequence (5'-3')	Position (nt)	Target gene	Product length (bp)	Full genome size (bp)	Reference
Oligonucleotide primers used for amplification of Rotavirus VP4, VP6, VP7 , NSP4 and NSP5 genes						
VP7-F	ATGTATGGTATTGAATATACCAC	51-71	VP7	881	1059	[47]
VP7-R	AACTTGCCACCATTTTTTCC	914-932				
VP6-F	GACGGVGCRACTACATGGT	747–766	VP6 (Partial)	382	1356	[48]
VP6-R	GTCCAATTCATNCCTGGTG	1126–1105				
Fcon3	TGGCTTCGCCATTTTATAGACA	11–32	VP4	876	2359	[47]
Rcon2	ATTTCGGACCATTTATAACC	868–887				
VP6-F	GGCTTTWAAACGAAGTCTTC	1–20	VP6 (Full)	1356	1356	[49]
VP6-R	GGTCACATCCTCTCACT	1340–1356				
NSP4-F	CTTTTAAAAGTTCTGTTCCGAGAG	3–26	NSP4	739	750	
NSP4-R	AAGACCATTCCTTCCATTAAC	721–741				
NSP5-F	GGCTTTTAAAGCGCTACAGT	1–20	NSP5	663	663	
NSP5-R	GGTCACAAAACGGGAGTGGGGA	642–663				
Oligonucleotide primers used for Rotavirus G-typing multiplex PCR						
G1-F	CAAGTACTCAAATCAATGATGG	314-335	VP7	618	-	[47]
G2-F	CAATGATATTAACACATTTTCTGTG	411-435	VP7	521	-	
G3-F	ACGAACTCAACACGAGAGG	250-269	VP7	682	-	
G4-F	CGTTTCTGGTGAGGAGTTG	480-498	VP7	452	-	
G8-F	GTCACACCATTTGTAAATTCG	178-198	VP7	754	-	
G9-F	CTTGATGTGACTAYAAATAC	757-776	VP7	179	-	
G10-F	ATGTCAGACTACARATACTGG	666-687	VP7	387	-	
G12-F	TAACGCTAATGAATTTTGGTACTG	498–nt 475	VP7	464	-	[50]
Oligonucleotide primers used for Rotavirus P-typing multiplex PCR						
P4-R	CTATTGTTAGAGGTTAGAGTC	474–494	VP4	483	-	[47]
P6-R	TGTTGATTAGTTGGATTCAA	259–278	VP4	267	-	
P8-R	TCTACTGCRTTRCANTGC	339-356	VP4	345	-	
P9-R	TGAGACATGCAATTGGAC	385–402	VP4	391	-	
P10-R	ATCATAGTTAGTAGTCGG	575–594	VP4	594	-	
P11-R	GTAAACATCCAGAATGTG	305-323	VP4	312	-	

3.3.3. Genotype distribution

Amplification of the VP7 and VP4 genes was performed by RT-PCR followed by G and P genotyping as shown in Figure 3.5. G3 accounted for highest prevalence (43%) in Imphal followed by G1 (16%), G2 (8%), G9 (5%), G8 (3%), G10 (1%) and G4 (1%), while G12 (0.26%) was rarely detected (Figure 3.6). G-types mix infection were 15%, while 13% were non-typeable with the primers used in the study. Among P-types, P[6] was the leading strain (22%) followed by P[8] (11%) and P[4] (4%), P[11] (4%), P[10] (3%) and P type mix infection 3% while 53% were un-typeable. Mix infection of P-types (P[4]/P[6], P[6]/P[10] and P[4]/P[6]/P[8]) was observed in 3% of the children (Figure 3.7). The age-wise prevalence of rotavirus G/P genotypes in children (≤5 years) hospitalized with acute gastroenteritis was analyzed; the prevalent strains were found in all the age groups (Table 3.4). Year-wise distribution of rotavirus genotypes was also assessed and the proportion of G3P[nt] was found high in all the years (Figure 3.8). In G/P combinations, 22 different rotavirus strains combinations were detected. Global strains G3P[8] and G1P[8] were observed in the study. G3P[6] was the most predominant rotavirus strain followed by G3P[8], G1P[6], G1P[8] and G9P[6]. The regionally common rotavirus strains, G1P[8], G2P[4], G9P[4] and G1P[4] were also observed. It is interesting to note that a large number of unique strains, notably G9P[4], G1P[11], G2P[11], G3P[10], G3P[11], G4P[11], G9P[10], G9P[11], and G10P[6], G10P[8,] are reported in this study (Table 3.5). Of note, non-typeable G-types were detected at low frequency (13%), but P-types were detected at high frequency (53%) suggesting there might be mutation in the primer binding regions (Figure 3.6 and 3.7).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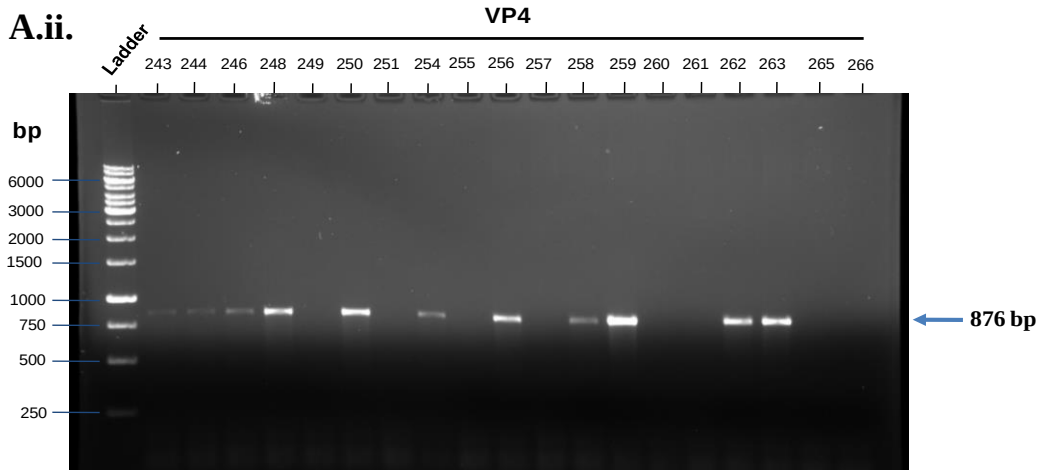
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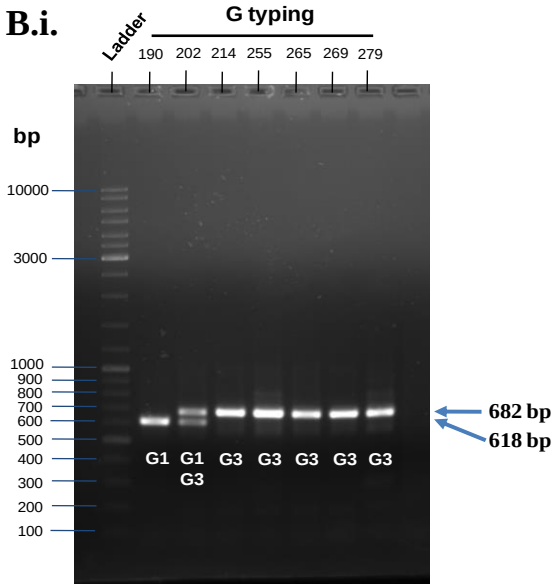
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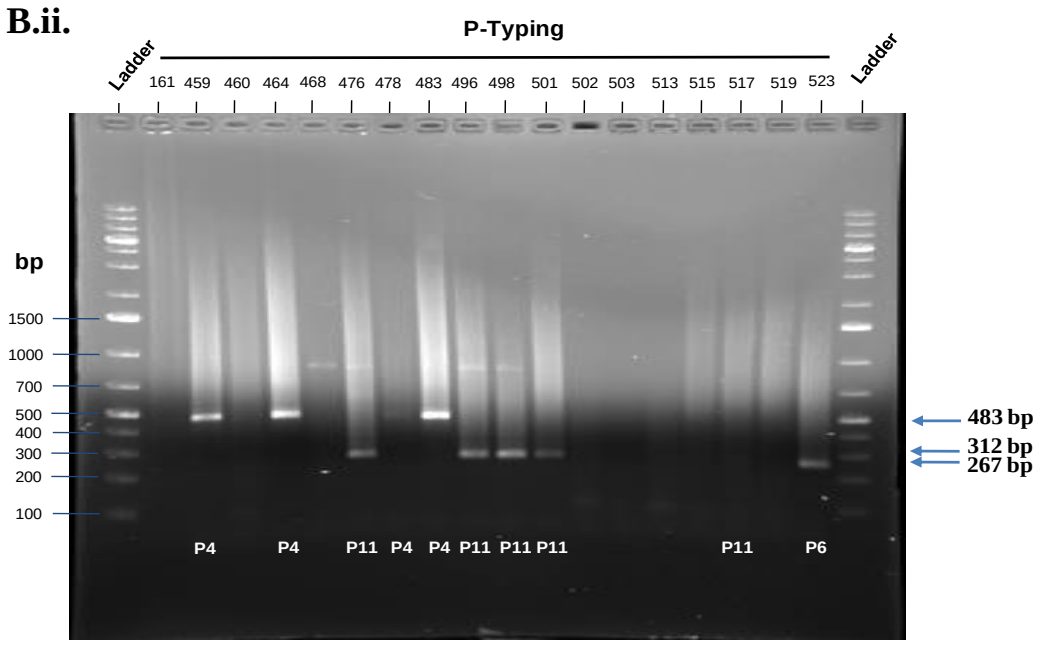


Figure 3.5. Amplification of the VP7 and VP4 genes by RT-PCR followed by G and P genotyping. A.i. Amplification products corresponding to VP7 (881bp), A.ii. Amplification products corresponding to VP4 (867bp), B.i. G genotyping by semi-nested multiplex PCR, and B.ii. P genotyping by semi-nested multiplex PCR. lane 1 in all the gel images are DNA ladder (Thermo Scientific-SM0333, SM1163 & SM1333).

Table 3.4. Age wise prevalence of rotavirus in children (≤5 years) hospitalized with acute gastroenteritis.

Age (months)	Total number of children (527)	Rotavirus infected children (n=380)	Rotavirus uninfected children (n=147)	G-genotypes (%)	P-genotypes (%)
0–5 (n = 55)	55	36 (9.47 %)	19 (12.93%)	G1=4/36(11.1%), G2=3/36(8.3%), G3=13/36(36.1%), G4=1/36(2.8%), G8=1/36(2.8%), G9=3/36(8.3%), G10=1/36(2.8%), Gmix=4/36(11.1%), Gnt=6/36(16.7%)	P[6]=6/36(16.7%), P[8]=4/36(11.1%), P[10]=1/36(2.8%), P[11]=2/36(5.6%), P[mix}=3/36(8.3%), P[nt]=20/36(55.6%)
6–11 (n = 183)	183	134 (35.26%)	49(33.33%)	G1=15/134(11.2%), G2=7/134(5.2%), G3=58/134(43.3%), G4=2/134(1.5%), G8=2/134(1.5%), G9=7/134(5.2%), G12=1/134(0.8%), Gmix=22/134(16.4%), Gnt=20/134(14.9%)	P[4]=7/134(5.2%), P[6]=26/134(19.4%), P[8]=15/134(11.2%), P[mix]=2/134(1.5%), P[10]=3/134(2.2%), P[11]=5/134(3.7%), P[nt]=76/134(56.7%)
12–23 (n = 211)	211	154 (40.53%)	57 (38.78%)	G1=27(17.5%), G2=14(9.1%), G3=62(40.3%), G8=4(2.6%), G9=4(2.6%), G10=1(0.7%), Gmix=23(14.9%), Gnew=1(0.7%), Gnt=18(11.7%)	P[4]=7(4.6%), P[6]=30(19.5%), P[8]=15(9.7%), P[10]=5(3.3%), P[11]=6(3.9%), P[mix]=4(2.6%), P[nt]=87(56.5%)
24–59 (n = 50)	50	40 (10.53%)	11 (7.48%)	G1=7(17.5%), G2=5(12.5%), G3=15(37.5%), G4=1(2.5%), G8=2(5%), G9=1(2.5%), Gmix=5(12.5), Gnt=4(10%)	P[4]=2(5%), P[6]=11(27.5%), P[8]=2(5%), P[nt]=22(55%), P[11]=1(2.5%), P[mix]=2(5%)
Above 5 yrs	10	4 (1.05%)	6 (4.08%)	G1=1(25%), G3=2(50%), Gnt=1(25%)	P[6]=1(25%), P[8]=1(25%), P[nt]=2(50%)
No age data	18	12 (3.16%)	6 (4.08%)	G1=3(25%), G2=1(8.3%), G3=3(25%), G8=1(8.3%), G9=3(25%), Gnt=1(8.3%)	P[nt]=6(50%), P[6]=3(25%), P[8]=2(16.7%), P[nt]=1(8.3)

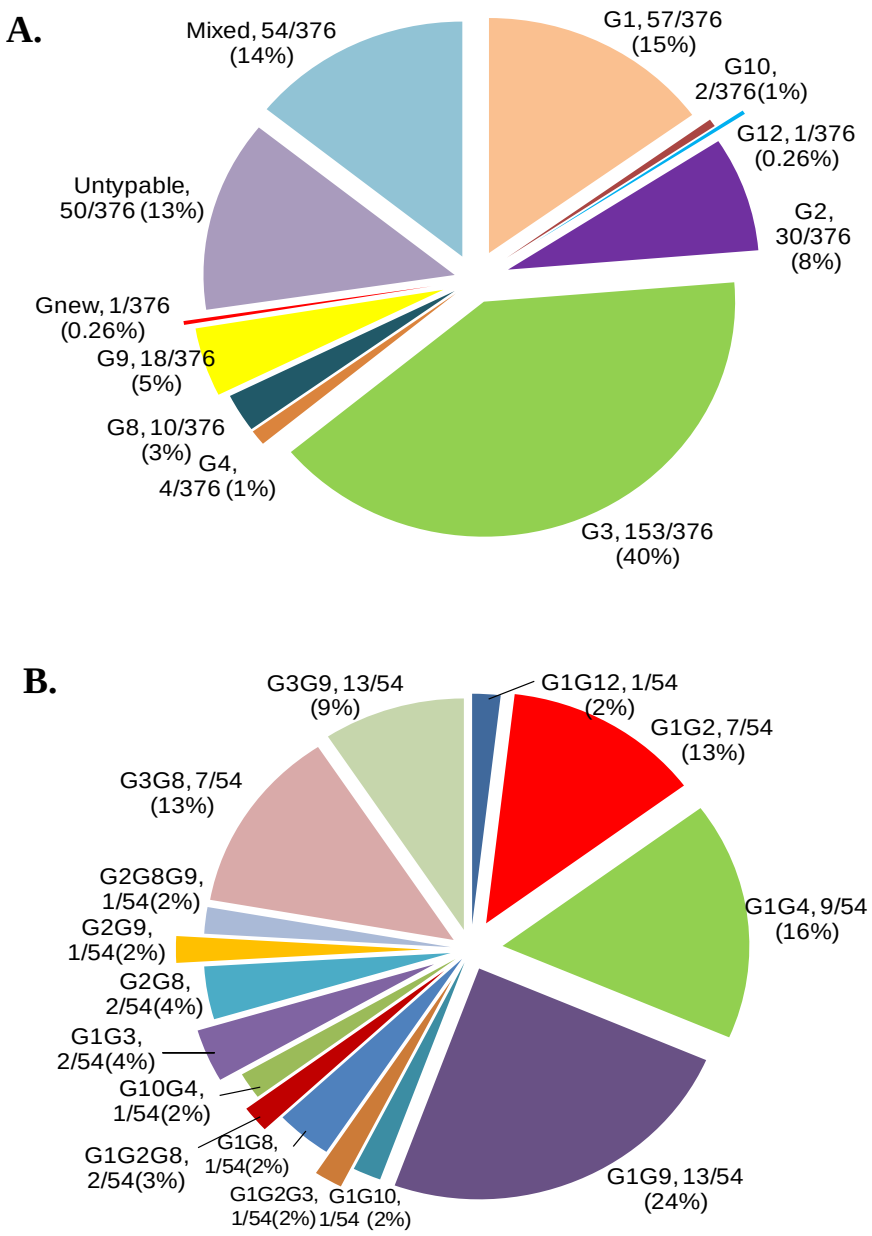


Figure 3.6. Frequency of rotavirus G and P genotype distribution isolated from stool specimens positive for rotavirus from children <5 years hospitalized for acute watery diarrhoea (AWD) during December 2015 – March 2019. A. Distribution of rotavirus G type strains in the region, B. Distribution of rotavirus mix G type strains.

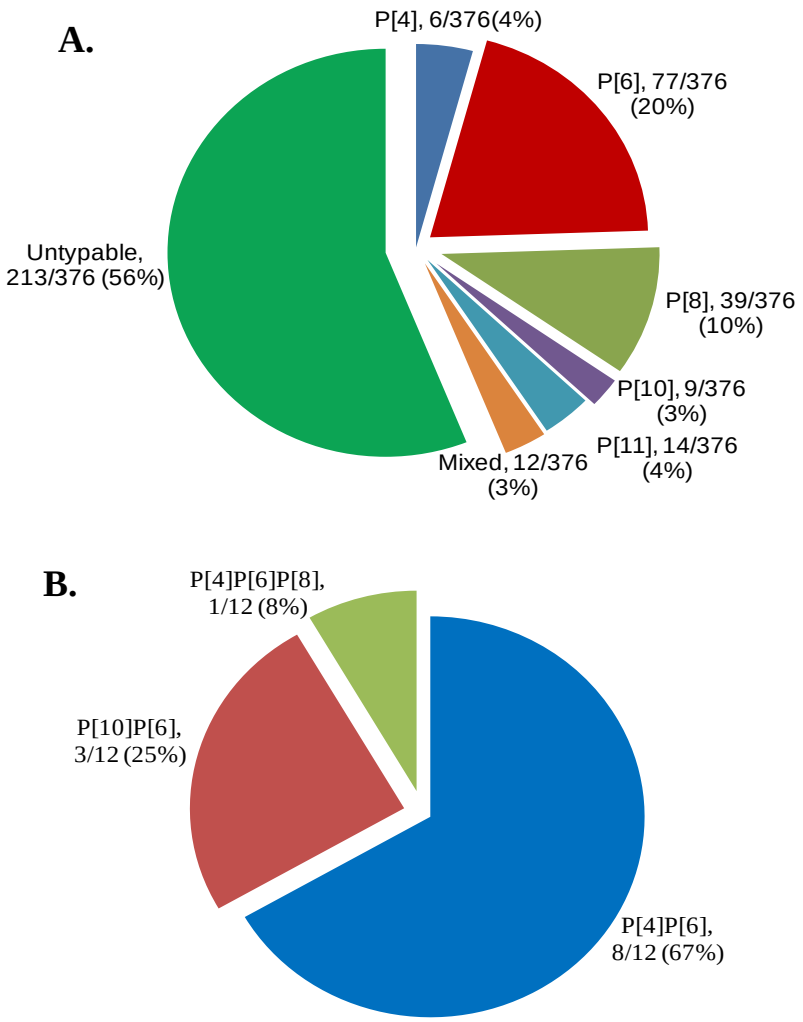


Figure 3.7. Frequency of rotavirus G and P genotype distribution isolated from stool specimens positive for rotavirus from children <5 years hospitalized for acute watery diarrhoea (AWD) during December 2015 – March 2019. A. Distribution of rotavirus P types in the region, and B. Distribution of rotavirus mix P type strains.

Table 3.5. Genotype distribution at the surveillance site during the 3 years study period in children ≤5 years.

	G1	G2	G3	G4	G8	G9	G10	G12	Mixed	New	Nontypeable	Total (%)
P[4]	1	8	3	0	0	2	0	0	2	0	0	16 (4)
P[6]	14	9	21	3	0	6	1	0	16	0	6	77 (20)
P[8]	8	1	16	0	0	0	1	0	9	0	3	39 (10)
P[9]	0	0	0	0	0	0	0	0	0	0	0	0
P[10]	0	3	1	0	0	2	0	0	2	0	1	9 (3)
P[11]	2	2	1	1	0	1	0	0	4	0	3	14 (4)
Mixed	2	2	3	0	0	1	0	0	4	0	0	12 (3)
Nontypeable	29	5	106	0	10	6	0	1	17	1	36	213 (56)
	57 (15%)	30 (8%)	151 (40%)	4 (1%)	10 (2.7%)	18 (4.8%)	2 (0.5%)	1 (0.3%)	54 (14.4%)	1 (0.3%)	49 (13%)	376

Above 5 years	**G1P[6]**	**G3P[nt]**	**G3P[nt]**	**G3P[nt]**	**GntP[nt]**	**Total**
	1	**1**	**1**	**1**	**1**	**4**

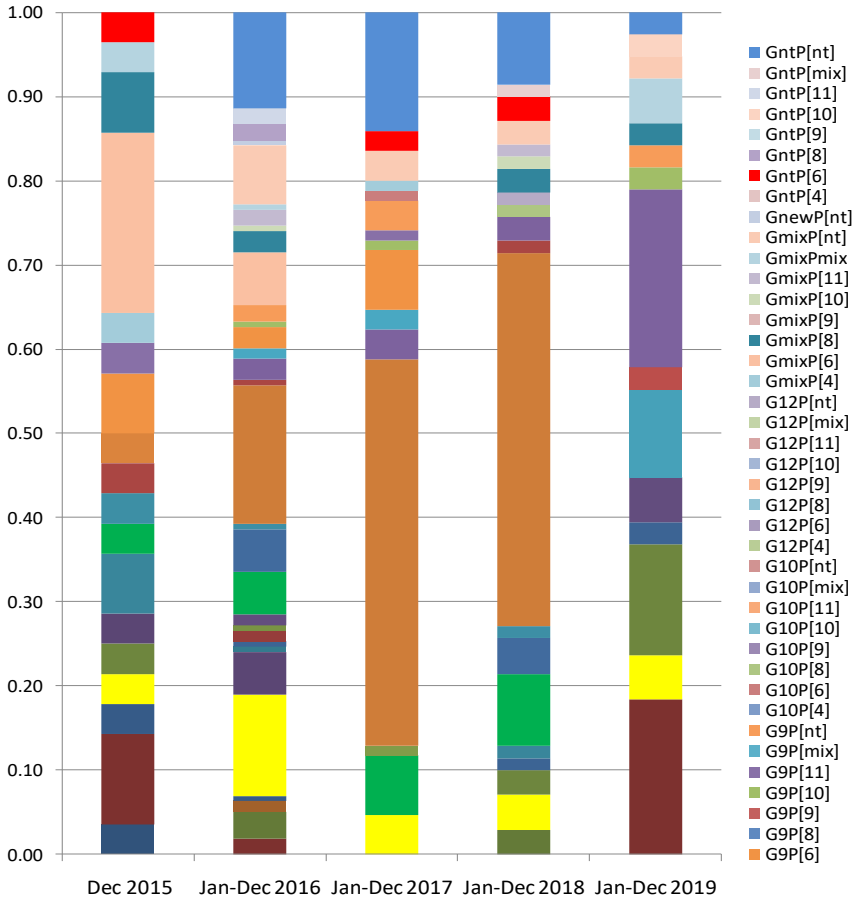


Figure 3.8. Year-wise distribution of rotavirus genotypes in < 5 years children admitted with acute gastroenteritis, December 2015 – March 2019.

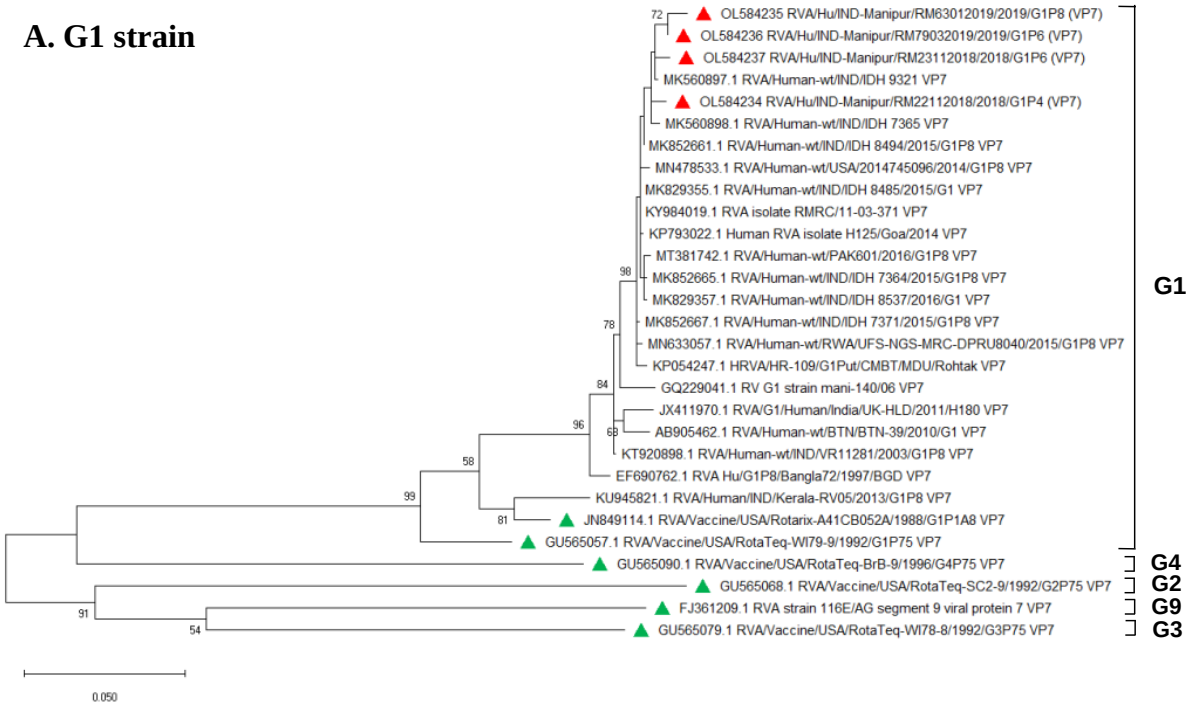
* P[nt] = P [non typable] * Gmix = G mixed infection
* P[mix] = P[mixed infection] * Gnew = G new type

3.3.4. Phylogenetic analysis

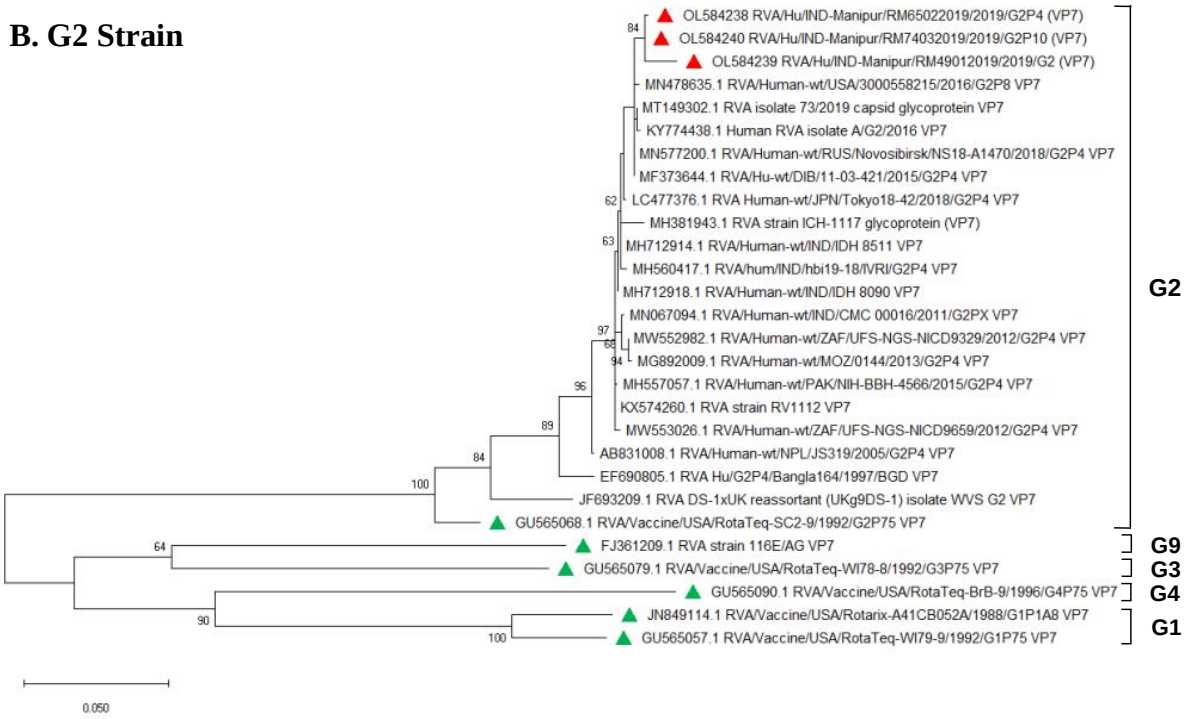
Phylogenetic analysis of the rotavirus G- and P-strains found in the study was constructed using the maximum likelihood method of MEGAX.0 software and the trees were inferred from 1000 replicates (Figure 3.9 (A-D) and Figure 3.10 (A & B). The template sequences were those sequences isolated from the northeastern region, different parts of India and other countries viz US, South Africa etc. The G1 strain in this study with Genbank accession nos.; OL584234, OL584235, OL584236 and OL584237 were found clustered together with G1 strains of Rotarix (accession no.: JN849114) and RotaTeq vaccine strains (accession no.:GU565057) (Figure 3.9A). While G2 strain from Imphal with Genbank accession nos.; OL584238, OL584239 and OL584240 were found clustered

together with RotaTeq G2 strain (accession no.:GU565068) (Figure 3.9B); G3 strains with accession nos.; OL584241, OL584242, OL584243, OL584244 and OL584245 were found clustered with RotaTeq G3 strain (accession no.:GU565079) (Figure 3.9C); In this study, G9 strain (Genbank accession number: OL584246) was found clustered closely with the Rotavac strain(accession no.:FJ361209) (Figure 3.9D). However, genetic closeness of VP4 sequences in this study and vaccine strains were found less as compared to VP7 sequences. Among the P-type strains in this study, P[8] (accession no:OL584248) was found genetically more related to RotaTeq P[8] strain (accession no.:GU565044) than P[6] (OL584247). VP6 sequences were also sequenced for confirmation of PCR result and deposited to NCBI Genbank with accession numbers; OM287605, OM287606, OM287607, OM287608, OM287609, OM287610, OM287611, OM287612, OM287613, OM287614, OM287615, OM287616, OM287617, OM287618, OM287619, OM287620 and OM287621. The sequences were found clustered together showing identity among the sequences and RM410032018 strain showed genetic relatedness with RotaTeq G3 VP6 sequences (Figure 3.9C).

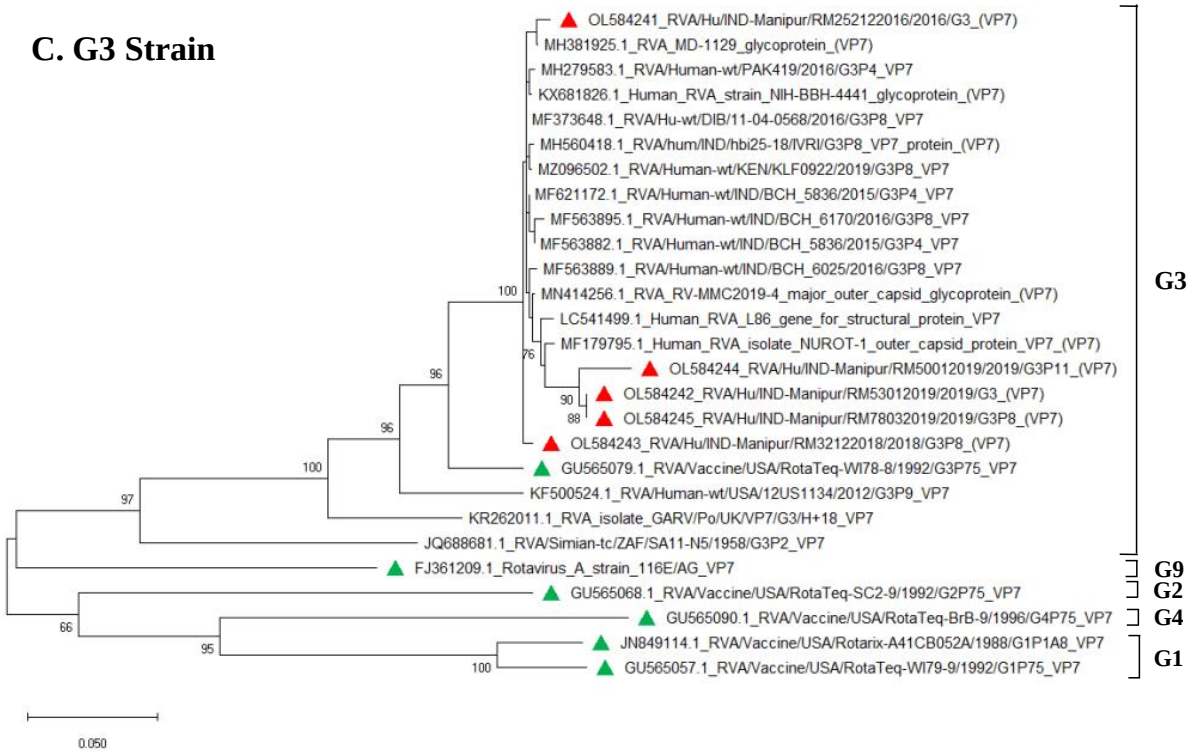
A. G1 strain

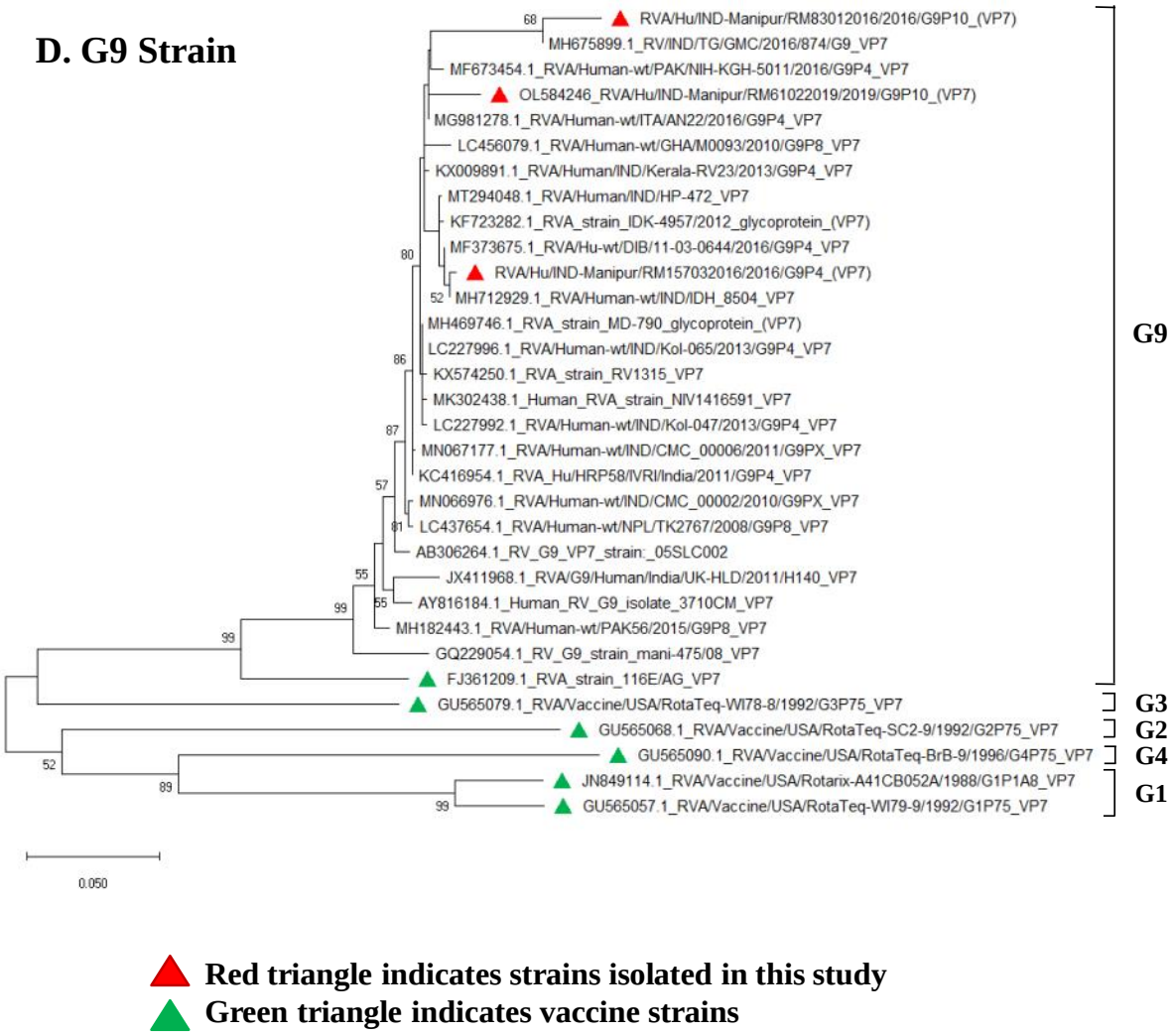


B. G2 Strain



C. G3 Strain

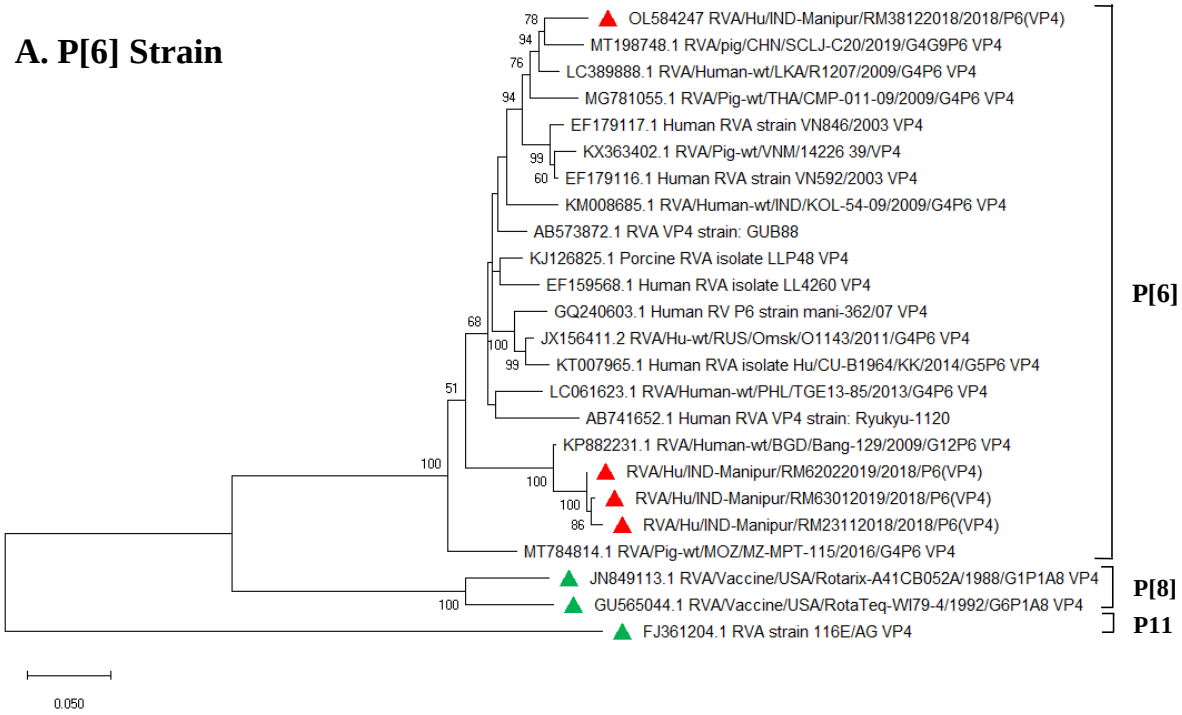




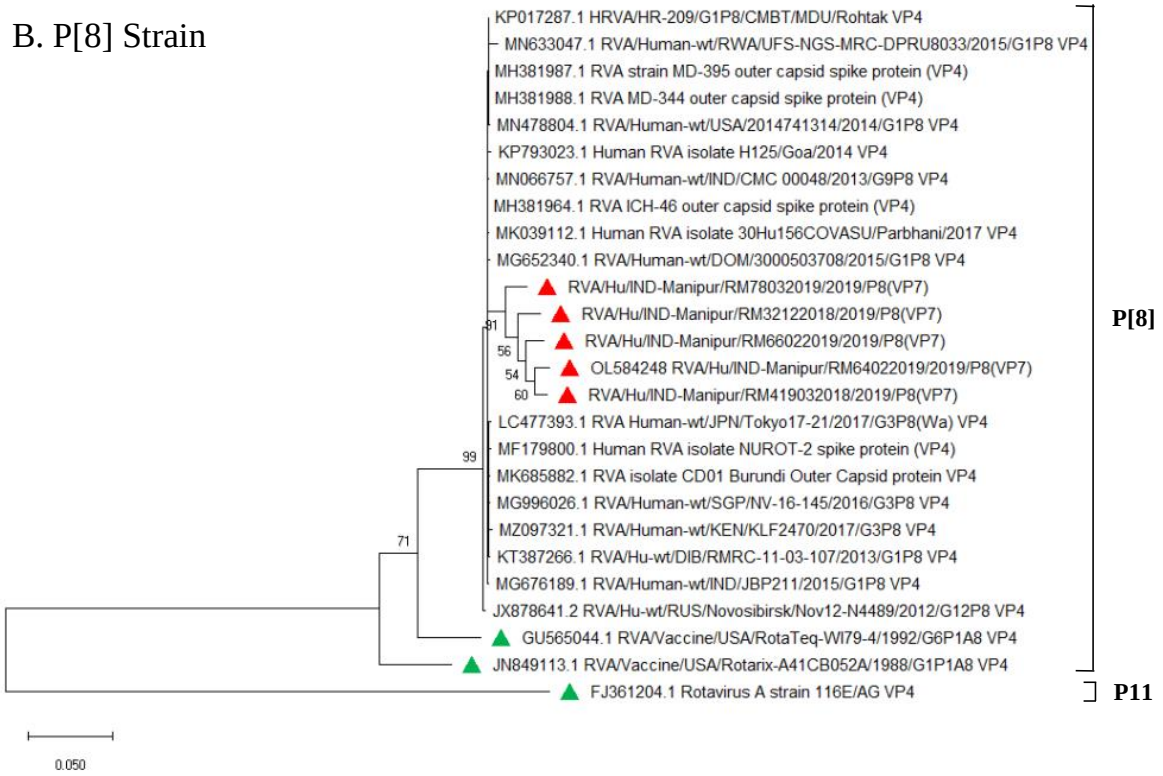
Red triangle indicates strains isolated in this study
Green triangle indicates vaccine strains

Figure 3.9. Phylogenetic tree based on the VP7 nucleotide sequence of human rotavirus A strains. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X. The strains in this study are shown in red triangles, the vaccine strains in green triangles and genotypes on the right side. A. G1 strains, B. G2 strains, C. G3 strains and D. G9 strains.

A. P[6] Strain



B. P[8] Strain



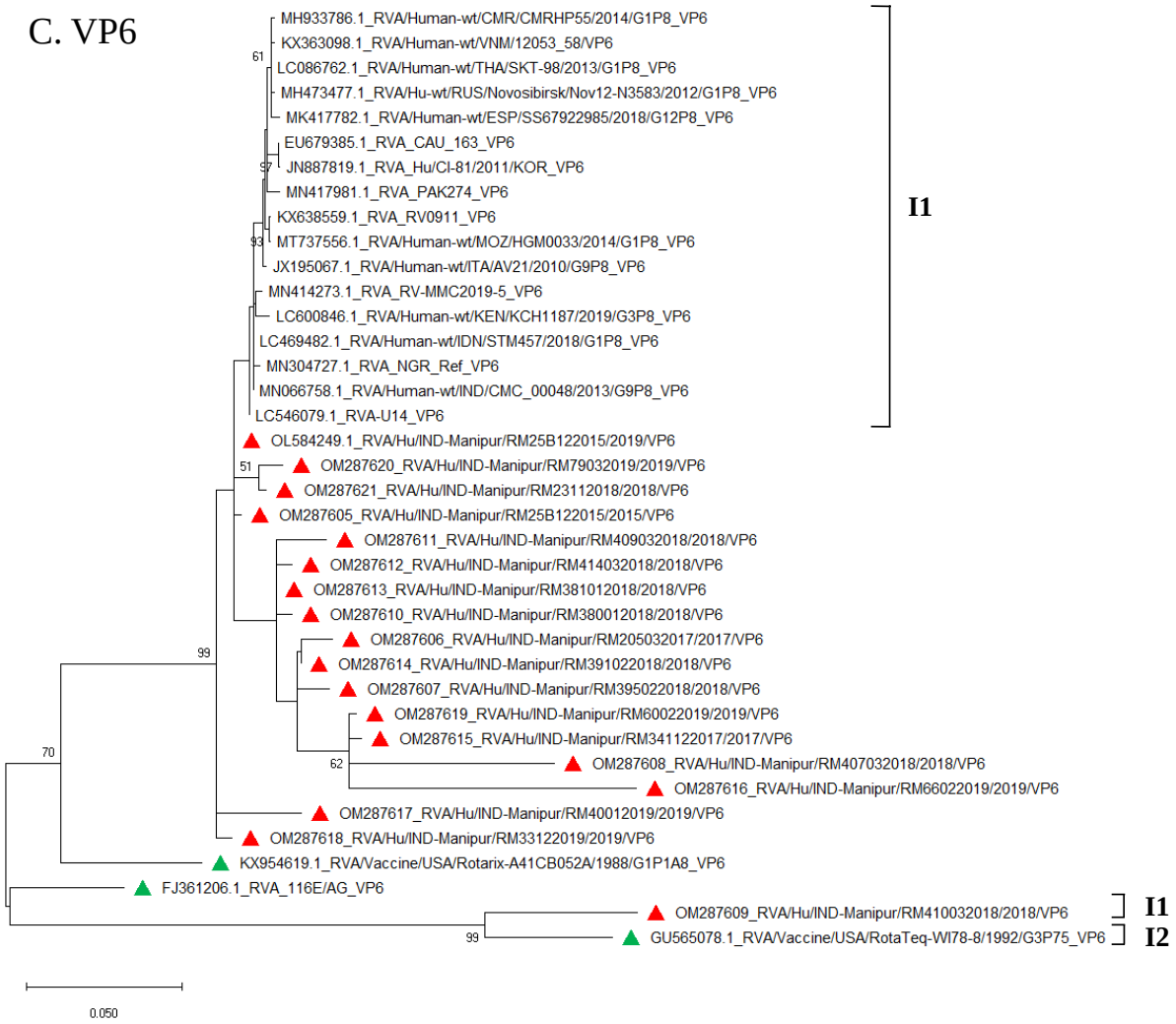


Figure 3.10. Phylogenetic tree based on the VP4 and VP6 nucleotide sequence of human rotavirus A strains. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X. The strains in this study are shown in red triangles, the vaccine strains in green triangles and genotypes on the right side. A. P[6] strains, B. P[8] strains and C. VP6.

3.3.5. Co-infection of rotavirus with other enteric viruses

All rotavirus positive and negative samples as tested by VP6-ELISA (Premier Rotaclone) have been analyzed for the presence of other common diarrhoea associated enteric viruses such as norovirus (group I and II), adenovirus and astrovirus using Rota+Adeno+Astro+Noro EIA Combo Card test (Table 3.1 and Figure 3.11). The test could be performed on each of the 527 samples. 121 healthy control samples were taken for qualitative studies during detection of co-infection of other enteric viruses. Analysis by CerTest revealed nearly 374 (70.97%) positive for rotavirus (Table 3.1) and proportion of positivity was found correlated to 356 (68.86%) by VP6-ELISA (Premier Rotaclone®). Co-infection of rotavirus with norovirus (Geno group I and II), adenovirus and astrovirus was detected in many fecal samples. To further confirm the Certest result, PCR using gene-specific primer was performed as given in Figure 3.13A-D. It was confirmed by sequencing the virus specific genes and the gene sequence have been deposited in NCBI Genbank with accession numbers (OM349536, OM349537 and OM349538). Phylogenetic analysis was made and is shown in Figure 3.14.

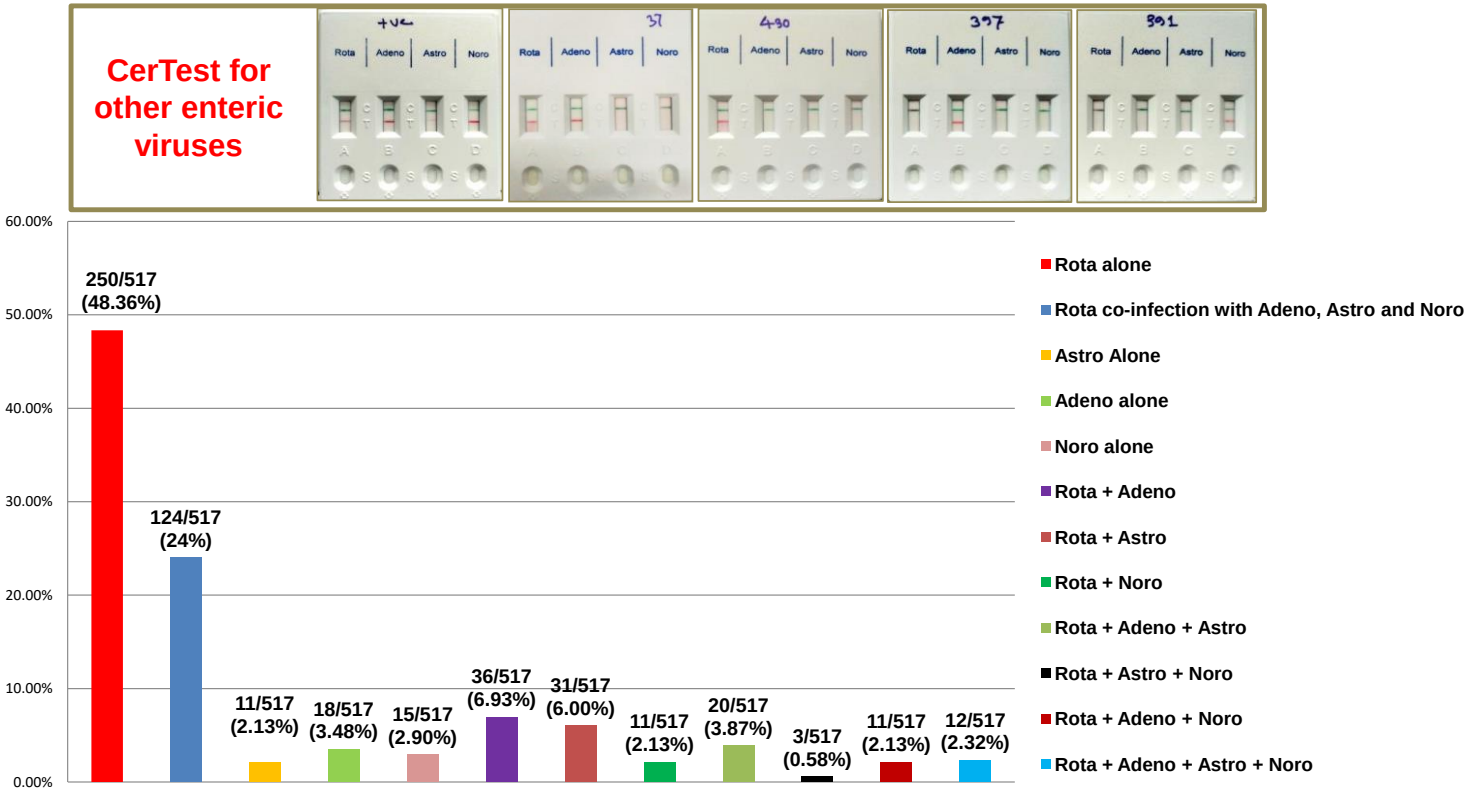


Figure 3.11. A representative photograph showing the detection of rotavirus, adenovirus, astrovirus and norovirus using CerTest Combo card test (CerTestBiotec). Top green line represents control line and bottom red line indicates presence of the virus under test. (b) Bar graph showing no. of hospitalized individuals infected with rotavirus and co-infection of rotavirus with other three viruses Adenovirus, Astrovirus and Norovirus.

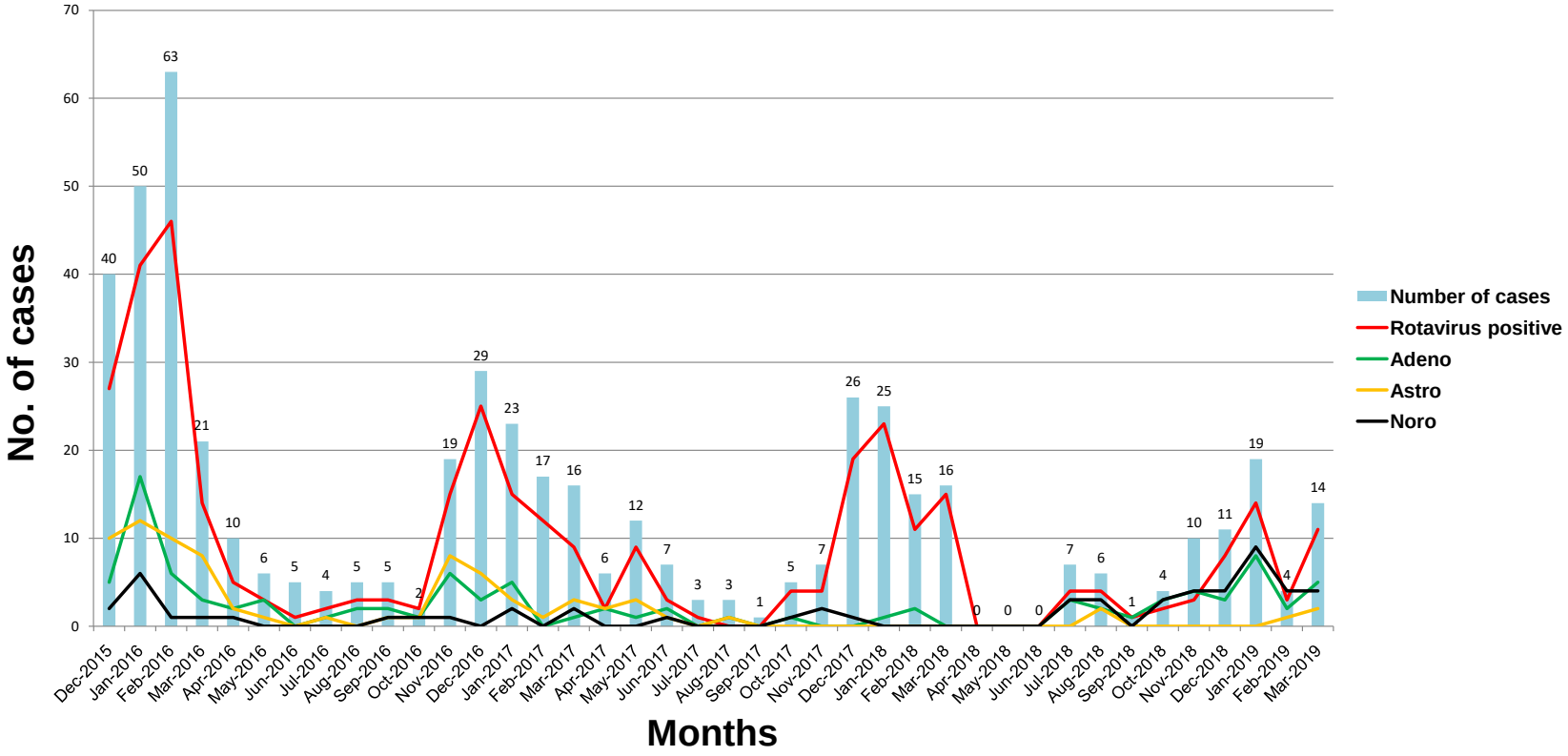
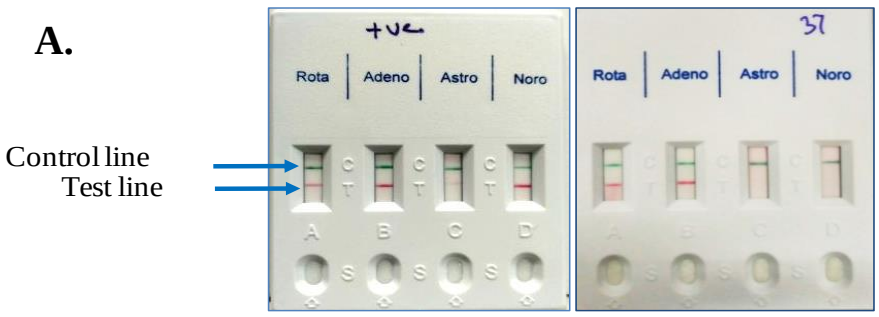


Figure 3.12. Seasonal prevalence of rotavirus and other common enteric virus (Adenovirus, astrovirus and norovirus) gastroenteritis from December 2015-March 2019.

Table 3.6. Oligonucleotide primers used for PCR amplification of other enteric viruses detected in clinical stool samples; norovirus, adenovirus and astrovirus genes.

Target virus	Primer name	Sequence 5′ to 3′	ORF /Region	Position (nt)	Fragment (bp)	Reference
Norovirus genogroup I	GI MON 431-F	TGGACIAGRGGICCYAAYCA	Capsid	5093	579	[51,52]
	G1SK R	CCAACCCARCCATTRTACT		5671		
Norovirus genogroup II	GII Cog 2F	CARGARBCNATGTTYAGR TGGATGAG	Capsid	5003	395	[52,53]
	G2SK R	CCRCCNGCATRHCCRTTRTACAT		5401		
Adenovirus	AdFfib(+)	ACTTAATGCTGACACGGGCAC	Partial shaft region of the fiber gene	1734	540	[54]
	AdFfib(−)	TAATGTTTGTGTTACTCCGCTC		2274		
Astrovirus	Ast Mon 269 (+)	CAACTCAGGAAACAGGGTGT	ORF2 (encoding capsid gene)	4526	449	[55]
	Ast Mon 270 (-)	TCAGATGCATTGTCATTGGT		4974		



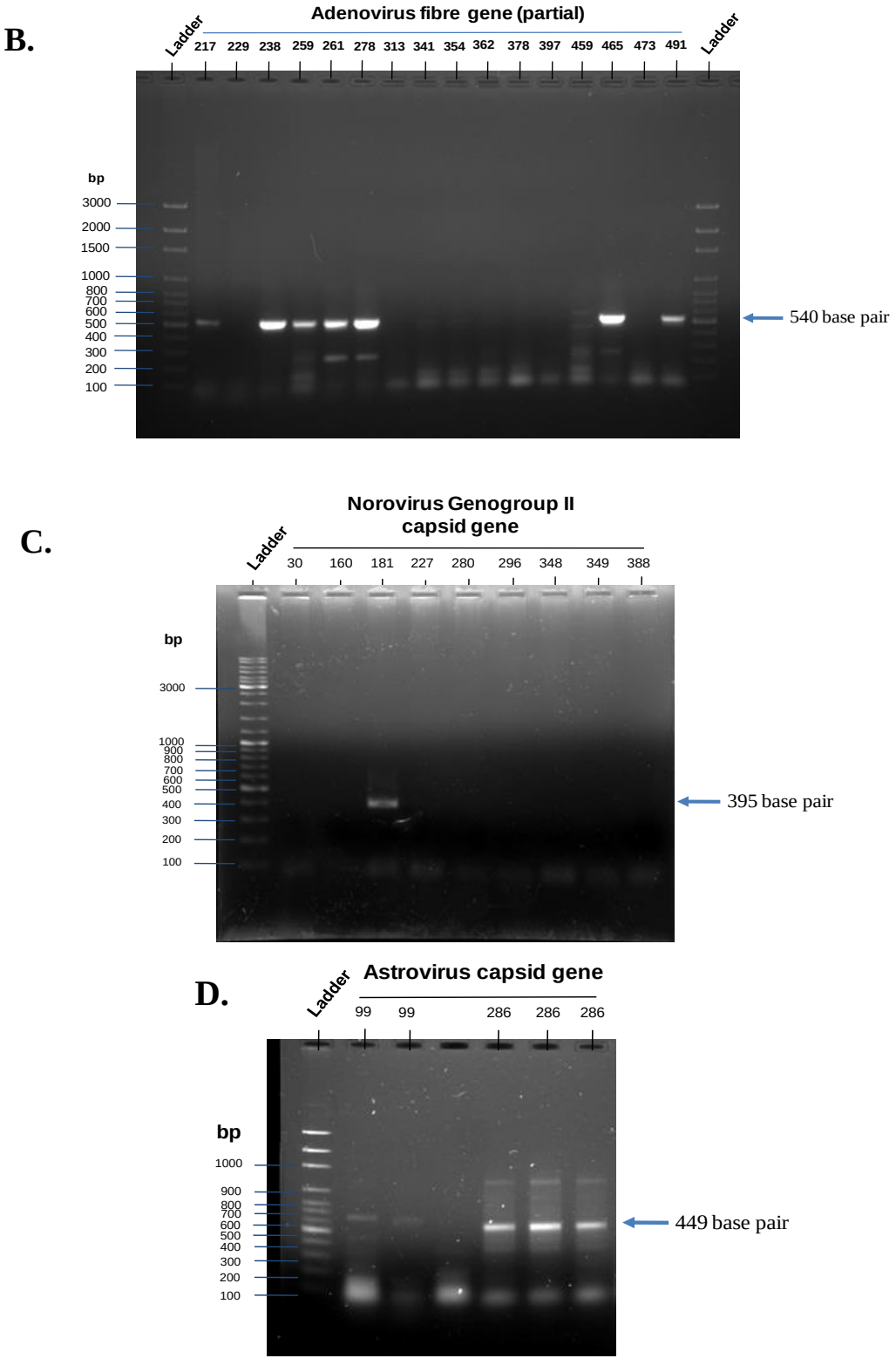


Figure 3.13. Detection of rotavirus, adenovirus, astrovirus and norovirus. A. Detection of other enteric viruses by Rota+Adeno+Astro+Noro EIA Combo Card (Cat No. RG862001V) where the green line shows control line and red line shows test line; Agarose gel image for B. adenovirus fibre partial gene (540 bp), C. norovirus genogroup II capsid gene, and D. astrovirus capsid gene PCR product (449 bp). lane 1 in all the gel images are DNA ladder (Thermo Scientific-SM0333 & Axygen® M-DNA-100BP).

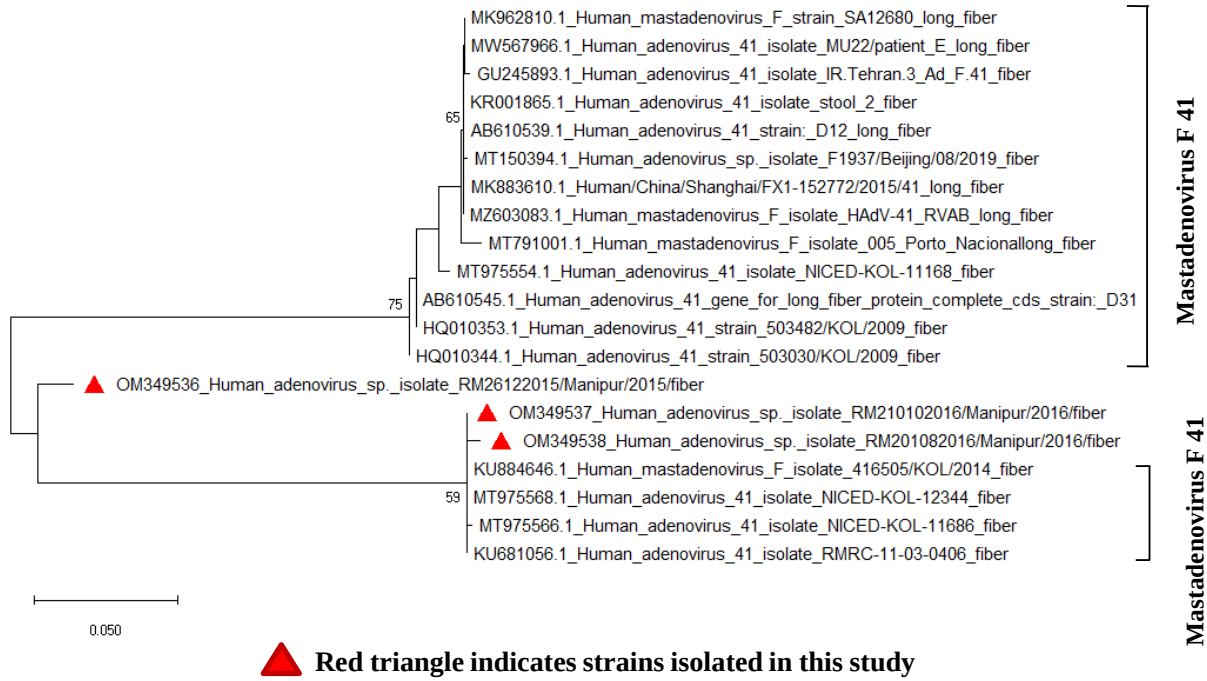


Figure 3.14. Phylogenetic tree based on the Adenovirus fiber gene sequence. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X.

3.4. Discussion

Epidemiological surveillance studies provide a better understanding about the common, unusual, rare, and emerging strains in the study region. This information is required for measuring the impact of the vaccine(s) on disease prevalence. There is lack of sufficient studies on rotavirus epidemiology and prevalent genotype strains in the North-east region of India. The present study is hospital-based surveillance conducted at RIMS, Imphal during the period from December 2015 to May 2019 prior to the introduction of rotavirus vaccine in the region. The study aimed to understand the clinical features of rotavirus diarrhoea and circulating strains in children under five-year-old.

In this study, RV was detected in 72.11% (380/527) of the total diarrheic cases in children presented with acute watery diarrhoea. A previous study has observed ~50% (244/489) rotavirus positive in Manipur [16]. A 2003 study reported a rotavirus positive rate of 23.27% (47/202) in Dibrugarh, Assam [19]. A recent study reported an RV positive rate of 28.7% [20], which is lower than the similar

studies' findings of rotavirus cases (20/50) of 40% [21]. Another investigation done in the area found a positive rate of 35.9% (51/142) [22].

In the past ten years, research from eastern Indian states have documented a significant rate of RV infection, with the highest percentage (54.75%, 357/652) recorded in Odisha [23] and the lowest percentage (6.04%, 44/728) reported in Kolkata [24]. Other studies from the eastern region show that Bhubaneshwar had a positive rate of 30.62% (98/320) in the pre-vaccination period, 36.9 percent (447/1213) [25] and 28.9% (246/850) [26] in the post-vaccination period. In the same city of Bhubaneshwar, a recent study found high RV rates of 54.6% (395/723) in pre-vaccination and 34.0% (379/1113) in post-vaccination period [27]. The present study showed highest percentage of RV positivity in the region which could be attributed to the lack of RV vaccination in the region and many other associated factors.

Children between the ages of 6 months and 2 years in this study had significant incidence of acute watery diarrhoea and rotavirus positivity. A lower number of cases and RV positivity rate in age group less than 6 months could be due to partial protection from infections by maternal antibodies to the fetus through the placenta [28]. According to published reports, passive antibody titers offer protection for the first six months of a child's life before they start to decline over time [29]. A higher positivity rate of rotavirus was also observed among children >23 months in our study. In contrast to other studies that demonstrated the protective immunity of natural infections upon follow-up reinfections [30,31], one possible explanation might be frequent reinfection in the region with high viral diversity may results in lower protection from virus [32]. Cases of diarrhoea and RV infection were observed throughout the year which is likely due to temperate climate of the region. However, rotavirus cases were more common in winter months compared to summer months as reported previously [19,22,26,33].

G and P genotype distribution overlap with other studies and some rare strains detected. G3P[6], G3P[8] and G1P[6] were the most common genotype combinations detected in this study, where G3P[8] and G1P[6] was in agreement with other studies conducted in different parts of India and other nations [34,35,36,37,26,38,39,40,41,27,42,33,43,23,44]. However, G3P[6] which was the most common strain in this study was rarely detected in northeast and other parts of India.

Further, the diverseness of electropherotype (E-types) of rotaviruses circulating in the study region was investigated. The 11-segments of RV genome has specific migration pattern. It migrates into four clusters: I, II, III and IV [45]. Group A rotaviruses can be characterized into three E-types namely, 'long', 'short' and 'supershort' based on the faster or slower migration of segment 11, respectively in polyacrylamide gels [46]. This study has identified some RV strains with atypical migration patterns that may be reassortant strains and whole genome sequencing of a few isolates have been carried out.

Various studies have reported co-infection of rotavirus and other enteric viruses in India [56-59]. Our study found co-infection doesn't increase overall severity and most of the cases were found moderate in severity. The reason for detection of more than one virus in the stool specimens could be due to prolonged virus shedding and exposure to multiple viruses. Seasonal trend of astro and nororvirus were found in agreement with other studies conducted in various parts of India [56, 60]. However, adenovirus has been reported circulating throughout the year [61] which was observed in a seasonal pattern in this study. This could be due to the fact that adenovirus is a respiratory virus and the cold and dry air favors virus survival and transmission.

Limitation of this study is that only children with diarroea admitted or visited RIMS were enrolled for the study. With the inclusion of Rotavac in universal immunization program in Manipur in June 2019, there is need for continuous surveillance to monitor the effectiveness of the indigenous vaccine in the region. Our study presents the most recent pre-vaccination data on the diverse strains and the most prevalent strains circulating in the region which will be an important contribution in public health management and research community in understanding the circulating strain diversity and effectiveness of the indigenous vaccine(s).

3.5. Conclusions

In summary, rotavirus diarrhoea disease in pre-vaccination period, clinical manifestation and circulating strains were documented. RV positivity rate {~68.86% (356/527)} was found to be high in the region. The cases of RV diarrhoea were observed mostly in cooler months. Between rotavirus-infected and -uninfected children under the age of five, there were statistically significant correlations in the frequency of diarrhoea episodes within a 24-hour

period. However, there was no significant correlation between vomiting in these two groups. The circulating strains in the region were G3P[6],G3P[8], G1P[6], G1P[8] and G9P[6]. Some unusual RV strains were observed in the region emphasizing the need for extensive t and continuous surveillance of RV in the study area.

3.6. Bibliography

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3.7. Manuscript of the chapter:

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