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Genetic characterization of group-A rotaviruses among children in eastern India during 2014–2016: Phylodynamics of co-circulating genotypes

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ABSTRACT

Background: Group-A human rotaviruses (GARV) are among the major cause of childhood diarrhea worldwide. In lieu of monitoring the circulatory GARV strains and underscoring the burden of GARV related hospitalization, a systematic surveillance was conducted in three hospitals of eastern India. In this hospital-based diarrheal disease surveillance (2014–2016), GARV was the most common cause of acute infantile gastroenteritis. The strains were genotyped and characterized to understand their prevalence and phylodynamics prior to the introduction of vaccine in eastern India.

Materials and methods: A total of 3652 stool samples were screened from children (≤5 years) hospitalized with acute diarrhea during 2014–2016. Initial screening for VP6 antigen was done by ELISA. GARV positive samples were genotyped by multiplex semi-nested PCR and DNA sequencing and phylogenetic analyses were based on the capsid proteins VP4 and VP7.

Results: Of 3652 samples, 1817 (49.8%) were GARV positive. G1, G2, G3 and G9 in conjunction with P[4], P[6]and P[8]genotypes were seen to co-circulate in the population. A sharp deflection from G1 to G3 occurred since 2016; upsurge of G9 strains was seen in alternate years, whereas G2 strains had a low frequency. All the circulating genotypes depicted a low phylogenetic relatedness to the vaccine strains. Differences in antigenic epitopes of VP4 and VP7 proteins in local strains were seen when compared to the vaccine strains. A significant difference in the degree of dehydration, duration of mean hospital stay and frequency of vomiting/24 h between GARV positive and negative children was evident.

Conclusion: The study provides a relevant set of base-line data on high burden of rotaviral gastroenteritis and the varied genotypic diversity among children prior to the introduction of GARV vaccine in this endemic region. Continuous monitoring during post-vaccination era will be required to assess the impact of vaccination in this region.

1. Introduction

In contrast to the highly developed countries, human group-A rotaviruses (GARV) are a major infantile health concern in low to middle-income countries where access to oral rehydration therapy is limited [1]. An incidence of 120,000 to 215,000 annual childhood mortality due to rotaviral acute dehydrating gastroenteritis has been estimated globally [2]. Among the five countries: Democratic Republic of Congo, Ethiopia, Nigeria, India and Pakistan which are

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liable for more than 50% of rotavirus related deaths, India solely stands out for almost 22% mortality [3]. Worldwide, studies conducted on efficacy of GARV vaccines namely Rotarix (G1P[8]) and RotaTeq (G1, G2, G3, G4, P1[8]), indicate that there has been a significant reduction (median of 67%) in rate of infantile hospitalization and deaths among countries with low to high childhood mortality due to rotaviral diarrhea [4–7]. Thus, to address the high burden of childhood gastroenteritis, the government of India has introduced two indigenous vaccines, namely ROTAVAC (Parental strain 116E, G9P[11]; monovalent) (Bharat Biotech International Limited, Hyderabad, India) and Rotasiil (G1, G2, G3, G4, G9, P[8]; multivalent) (Serum Institute of India, Pune, India) in a phased manner in its expanded program on Immunization (EPI) during 2016 and 2018, respectively [8]. By the end of 2018, eleven states in India comprising of about 50% of the birth cohort have been covered under routine administration of the vaccine.

GARV genome is constituted of 11-segmented double-stranded RNA, encoding six structural proteins (VPs) and six non-structural proteins (NSPs). The antigenic variations of the two outermost capsid proteins: VP4 (protease-sensitive protein-P) and VP7 (glycoprotein-G) are the prime immune targets for vaccine development against GARV because both the antigens have a crucial role in protective immunity [9]. According to Rotavirus Classification Working Group (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg), presently 36 G (VP7 glycoprotein-G protein) and 51P (VP4 protease-cleaved protein-P protein) types have been found in human and animal species worldwide. Global GARV surveillance studies have reported various genotypic combinations, which underscores the fact that it adapts varied mechanisms to produce genomic variations in nature. Inter-species transmission between multiple hosts, recombination between human and animal GARVs upon co-infection of a single host and introduction of point mutations due to the activity of error-prone viral RdRp (RNA dependent RNA polymerase) could be few plausible causes of genetic drift leading to GARV evolution [10–13]. Common human GARV genotypes G1-G4, G9, G12, in combination with P [4], P[6] and P[8] are responsible for causing more than 90% rotaviral diarrhea worldwide. Sporadic incidence of infection with rare genotypes like G5, G6, G8, G10, G11, P[1], P[3], P[9], P[10], P[19], P[14] and P[25] have also been detected worldwide [14–18]. Geographical inclination of certain genotypes like G8, G5 has also been observed in some parts of the world [19–22].

The predominantly circulating endemic strains of GARV vary in different geographical settings within the same country [23,24]. In India, G1P[8] has been reported as the predominantly circulating strain, though periodic upsurge of G2, G9 and G12 strains has also been observed [18,25–27]. In the present study (2014–16), a dynamics in the distribution pattern of different G-types were observed. G1P[8] genotype predominated during 2014–15, followed by a shift in prevalence to G3P[8] since 2016. Upsurge of G2 and G9 strains was also observed in every alternate year. Other genotypes like G10P[6] and G12P[6], G12P[8], G12P[11] were identified at very low frequency. Evolutionary analysis of G3 strains from this region has already been reported [28]. In this study, focus has been given to the phylogenetic and molecular characterization of other major co-circulating G1, G2 and G9 strains.

The subjects included in this study belonged to both urban and rural population with poor socio-economic background in West Bengal (eastern part of India). In this area, GARV vaccines have not yet been introduced in Universal Immunization Program. Determination of prevalent genotypes and its antigenic characterization is crucial in identifying major targets for the vaccines. Prior to the introduction of vaccines in this state, adequate information regarding currently circulating GARV strains and efficacy of the vaccines in imparting heterotypic protection against them is absolutely necessary. As the degree of heterotypic protection imparted by the vaccines is comparable to the homotypic strains, it is advantageous to keep a baseline record of the circulating strains. Therefore, the purpose of this study was to estimate the proportion, seasonal incidence and genotypic variation of the GARV genotypes among children hospitalized due to gastroenteritis in rural and urban population of eastern India. Children $\leq$ 5 years of age which required hospitalization due to severe dehydrating diarrhea were enrolled in this study.

2. Materials and methods

2.1. Recruitment of subjects and collection of clinical specimens

During January'2014 through December'2016, surveillance for GARV among children ($\leq$5 years) was conducted in three hospitals, namely Infectious Disease and Beliaghata General Hospital (IDH), Institute of Child Health (ICH) in Kolkata (urban settings) and Midnapore Medical College and Hospital (MMCH) in Midnapore (rural settings). Inclusion criteria for enrolment included children ($\leq$5 years) with acute gastroenteritis having 3 or more episodes of watery stool in 24 h along with moderate to severe dehydration requiring hospitalization. The children presenting with mild dehydration, were treated in out-patient facility and thus were excluded. Immunocompromised children or those $\geq$ 5 years were also excluded from the study. In ICH and MMCH, surveillance was conducted 5 days/week; whereas in IDH every 5th child was enrolled and samples were collected two days/week. 16867, 1919, 1830 paediatric patients were admitted in IDH, ICH and MMCH, from which 5.82% (n = 983), 79.10% (n = 1518), 60.21% (n = 1102) samples were screened, respectively. Consent of the Institutional Ethical Committees, of all participating institutes was taken prior to execution of this study. As per approved study protocol, only GARV was tested in these samples.

2.2. Screening for GARV antigen in clinical samples by ELISA

Initially stool samples were screened for the detection of GARV VP6 antigen by ELISA as per kit protocol (Premier Rotaclone 48 T, Meridian Bioscience, Inc., Cincinnati, USA). The optical density (O. D.) value of the positive control provided in the kit [$\geq$0.31] was considered as the cut-off value for determining the GARV positivity.

2.3. GARV genome isolation and genotyping

GARV double-stranded RNA was extracted from the 30% w/v viral suspension prepared from the positive stool samples using Trizol reagent, followed by reverse transcription in the presence of random hexamers. One-third of the rotavirus positive samples were genotyped by multiplex semi-nested PCR using different G-P type specific primers (G1, G2, G3, G4, G9, G10, G12, P[4], P[6], P[8], P[9], P[10] and P[11]) as per National Rotavirus Surveillance Network protocol [27,29]. PCR products were analysed by agarose-gel electrophoresis and subsequently the genotypes were confirmed by Sanger sequencing for the VP7 gene and partial VP4 (VP8* region) gene, using VP7 and VP4 specific primers (VP7F, VP7R, CON3, CON2 respectively). Rest of the two-third positive samples were amplified for full length VP7 gene and partial VP4 (VP8*) gene, followed by direct genotyping through Sanger sequencing. The PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) prior to proceeding for sequencing. The primers used to amplify the VP7 and VP8* gene segments along with the amplicon size have been listed in Supplementary Table 1.

2.4. DNA sequencing and phylogenetic analysis

ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, California, USA) was used for DNA sequencing in an ABI Prism 3730 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA) [16]. Nucleotide sequence BLAST search was done in National Centre for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD) Basic Local Alignment Search Tool (BLAST) server on GenBank database release 143.0 [30]. MUSCLE was used for multiple sequence alignment. Amino acid sequences were deciphered by TRANSEQ (Transeq Nucleotide to Protein Sequence Conversion Tool, EMBL-EBI, Cambridgeshire, UK). Genotyping was determined using RotaC version 2. Phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) program, version 6. The maximum-likelihood statistical method was used to construct the phylogenetic dendrograms (at 1000 bootstrap replicates) using TN93 (Tamura Nei) model for all phylogenetic dendrograms except for the G1 tree, where T92+G (Tamura-3 + Gamma distribution) was applied. The best fit models were determined through model the testing parameter of MEGA6 software, for the VP4 and VP7 gene alignments for each of the G-P genotypes. Nucleotide sequences of VP4 and VP7 gene were submitted to the GenBank database under the accession numbers which have been mentioned against all the representative sequences in the phylogenetic dendrograms.

2.5. Statistical analysis

All statistical parameters like chi-square and p-values were calculated using the OpenEpi software. P-values < 0.05 were considered statistically significant. Statistical analysis of the clinical symptoms associated with rotaviral diarrhea was done on 2588 samples, where complete medical records were available.

3. Results

3.1. Epidemiology of GARV infection among hospitalized children (≤5 years) with severe diarrhea

During the 3 years study period (2014 through 2016), 1817 (49.8%) out of 3652 samples were found to be GARV positive. 2501 and 1102 samples were tested from urban settings (IDH and ICH) and rural settings (MMCH), respectively. Out of these, 49.34% (n = 1234/2501) and 52.9% (n = 583/1102) samples were found to be positive for GARV from urban and rural area, respectively (Table 1). Acute infantile diarrhea due to GARV infection predominated in the age group 6–12 months (55.53%), followed by 12–24 months children (55.2%) (Supplementary Fig. 1). A marked peak in increased rates of GARV diarrhea (61.7–71.1%) was observed during the cooler months of November to February in each year (Fig. 1).

3.2. Dynamics of GARV genotypes among children (≤5 years) in urban and rural settings of eastern India

Of the 1817 GARV positives, only 1/3rd samples (n = 600, 33.3%) were genotyped by multiplex semi nested PCR as per study protocol. In addition, all the GARV positive samples were G-P typed by sequencing the VP7 and VP8* gene. GARV genotype G1 in combination with P[4], P[6] and P[8] was the most prevalent strain both in

Table 1
Number of children (≤5 years) with acute gastroenteritis enrolled in the study (screened) and number of samples positive for GARV by ELISA during the study period (2014 to 2016).

Name of the hospital	2014		2015		2016		Total samples screened	Total no. of GARV positives
	Sample screened	No. of GARV positives	Sample screened	No. of GARV positives	Sample screened	No. of GARV positives		
URBAN IDH	361	183 (50.7%)	308	129 (41.8%)	314	153 (48.7%)	**983**	**465 (47.3%)**
ICH	428	223 (52.1%)	592	292 (49.3%)	498	254 (51%)	**1518**	**769 (50.6%)**
RURAL MMCH	487	266 (54.6%)	374	158 (42.2%)	290	159 (54.8%)	**1151**	**583 (50.6%)**
TOTAL	**1276**	**672 (52.6%)**	**1274**	**579 (45.4%)**	**1102**	**566 (51.3%)**	**3652**	**1817 (49.8%)**

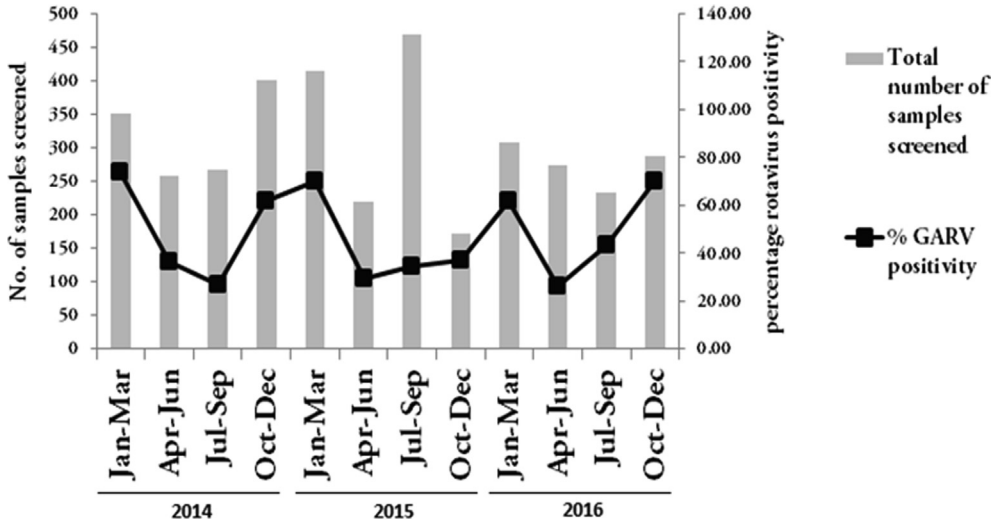

Fig. 1. Seasonal distribution of GARV diarrhea among children (≤5 years) with acute gastroenteritis during 2014–2016: upsurge of infection during winter.

urban (48.8% in IDH and ICH) as well as rural (68.01% in MMCH) settings. G9P[4], G9P[6], G9P[8] (12.93%) and G2P[4], G2P[6], G2P[8] (12.65%) strains were also seen to co-circulate among both the types of population. Rare genotypes like G10P[6], G10P[14] and G12P[11] were observed at 0.05% (n = 1/1817), 0.11% (n = 2/1817) and 0.11% (n = 2/1817) frequency, respectively. A single sporadic incidence of G4P[6] (0.14%) was seen in 2014. "Unusual combinations" such as G1P[4], G1P[6]; G2P[6], G2P[8]; G9P[4], G9P[6], etc. of commonly circulating human GARV genotypes were observed at a high frequency (n = 394/1817; 21.68%) during the study period (Table 3). Overall, there were more genetic variations in the urban area compared to the rural area (Figs. 2a and 2b).

The samples processed through multiplex semi-nested RT-PCR revealed 10.6% samples (n = 64) mixed infections (Supplementary Table 2). Sanger sequencing using respective type-specific primers however could confirm only one genotype in these samples. 5.4% (n = 99/1817) samples remained untypeable. The untypeable samples were positive for VP6 RT-PCR but had low EIA values [0.31–0.45] compared to the genotyped samples [EIA value > 1.2] (Table 2).

3.3. Statistical analysis of association between various clinical parameters and GARV positivity

A significant difference in the degree of dehydration (p-value = 0.000032), duration of mean hospital stay (p-value = 0.000212) and frequency of vomiting/24 h (p-value = 0.018316) was observed between GARV positive and negative patients. Frequency of stool passage/24 h (p-value = 0.665) and onset of fever (p-value = 0.277389) did not show any difference between GARV positive and negative children (Table 4).

3.4. Phylogenetic analysis of GARV genotypes circulating during 2014–2016

VP7 and VP4 (VP8*) gene segments of circulating GARV strains were deduced by DNA sequencing. Phylogenetic dendrograms were constructed based on the representative nucleotide sequences each of G1, G2, G9, P[4], P[6] and P[8] strains. The representative strains were selected based on the nucleotide homology of the strains. One strain from the subset of strains with

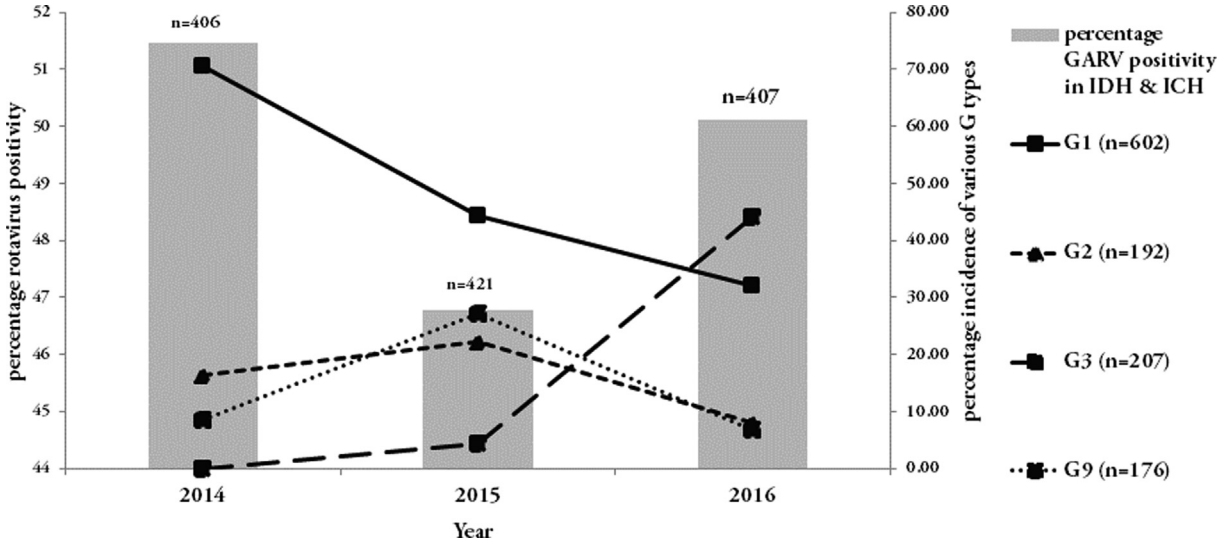

Fig. 2a. Dynamics of various GARV G-P types from 2014 through 2016 in urban settings (ICH and IDH) among children (≤5 years) hospitalized with acute gastroenteritis.

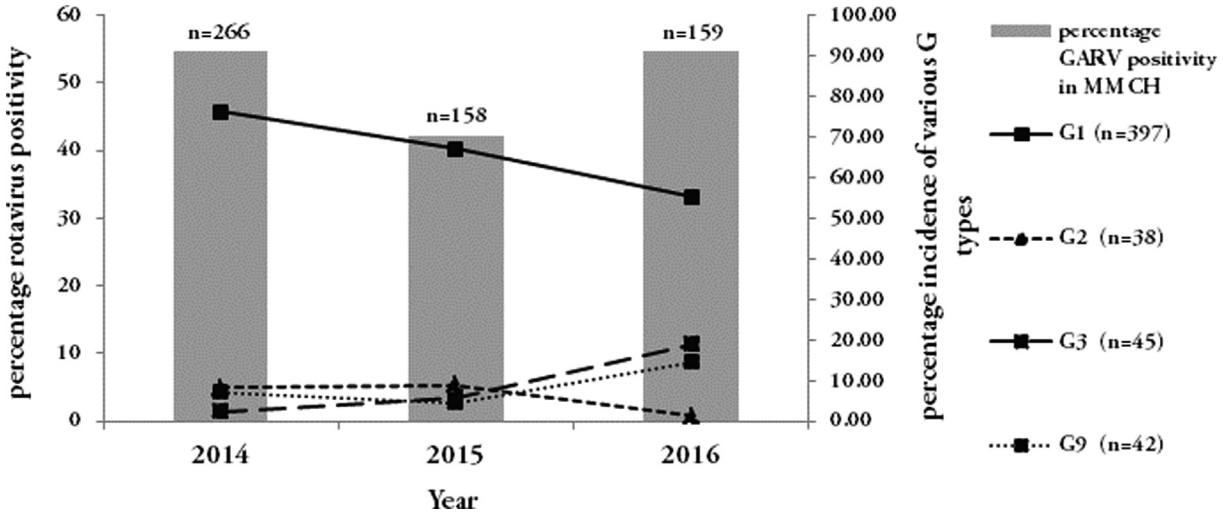

Fig. 2b. Dynamics of various GARV G-P types from 2014 through 2016 in rural settings (MMCH) among children (≤5 years) hospitalized with acute gastroenteritis.

Table 2
Frequency of distribution of various G-P genotype among GARV samples (n = 1817).

Year	Total GARV positives	G-P genotypic combinations																			
		G1			G2			G3		G4	G9			G10		G12				Gnt	
		P[4]	P[6]	P[8]	P[4]	P[6]	P[8]	P[4]	P[8]	P[6]	P[4]	P[6]	P[8]	P[6]	P[14]	P[6]	P[8]	P[11]	P[nt]		
2014	**672**	14 (2.08%)	13 (1.9%)	462 (68.7%)	33 (4.9%)	22 (3.2%)	33 (4.9%)	0 (0%)	6 (0.8%)	1 (0.1%)	13 (1.9%)	16 (2.3%)	34 (5.05%)	1 (0.1%)	2 (0.3%)	2 (0.3%)	0 (0%)	2 (0.3%)	18 (2.6%)		
2015	**579**	7 (1.2%)	19 (3.2%)	266 (45.9%)	45 (7.7%)	47 (8.1%)	16 (2.7%)	0 (0%)	27 (4.6%)	0 (0%)	111 (19.1%)	4 (0.7%)	6 (1.03%)	0 (0%)	0 (0%)	1 (0.1%)	2 (0.3%)	0 (0%)	28 (4.8%)		
2016	**566**	0 (0%)	9 (1.6%)	209 (36.9%)	19 (3.3%)	15 (2.6%)	0 (0%)	1 (0.1%)	208 (36.7%)	0 (0%)	41 (7.2%)	0 (0%)	10 (1.7%)	0 (0%)	0 (0%)	0 (0%)	1 (0.1%)	0 (0%)	53 (9.3%)		
TOTAL = 1817		**21**	**41**	**937**	**97**	**84**	**49**	**1**	**241**	**1**	**165**	**20**	**50**	**1**	**2**	**3**	**3**	**2**	**99**		
		999 (54.9%)			**230 (12.6%)**			**242 (13.3%)**		**1 (0.05%)**	**235 (12.9%)**			**3 (0.16%)**		**8 (0.4%)**			**99 (5.4%)**		

Table 3
Unusual and emerging genotypic combinations found during the surveillance period (2014–2016).

Unusual and emerging genotypic combinations	
Unusual genotypes	Proportion of the particular genotype
G1P[4]	**21**(5.33%)
G1P[6]	**41**(10.41%)
G2P[6]	**84**(21.32%)
G2P[8]	**49**(12.44%)
G4P[6]	**1**(0.06%)
G9P[4]	**165**(41.88%)
G9P[6]	**20**(5.08%)
G10P[6]	**1**(0.25%)
G10P[14]	**2**(0.51%)
G12P[6]	**3**(0.76%)
G12P[8]	**3**(0.76%)
G12P[11]	**2**(0.51%)
Total no. of unusual genotypes obtained	**394**
Total no. of GARV positives	**1817**
Percentage of unusual genotypes	**21.68%**

higher nucleotide sequence homology (≥98% DNA homology), was chosen as the representative strain of that particular lineage. Strains identified during different seasons (Winter-Spring-Summer-Monsoons) were incorporated in the phylogenetic dendrograms. The relative clustering of representative eastern Indian strains was analyzed based on previously reported human and animal strains from multiple countries along with the vaccine strains.

3.4.1. G1 strains

The representative G1 strains (n = 169) were seen to get distributed in 2 different G1 lineages: lineage-I (n = 151, 89.3%) and lineage-II (n = 18, 10.6%) (nearly 89% DNA sequence similarity among the strains of 2 lineages). Maximum strains clustered with the strains of lineage-I reported earlier from India, Bangladesh, Vietnam and Canada (DNA sequence similarity 81.4–98.3%). They were distant to the vaccine strains RotaTeq (lineage III); Rotasiil and Rotarix (lineage-II), with only 90–92.8% homology. Strains which clustered with the strains of lineage-II showed maximum identity (98–99%) with strains from India, Australia and Belgium. These strains clustered with the vaccine strain Rotasiil and Rotarix (96–97% similarity) within lineage-II, but were distant to RotaTeq (nearly 91% identity) (Fig. 3a).

3.4.2. G2 strains

All the representative G2 strains clustered within G2 lineage-IV strains, showing maximum homology with the previously reported strains from India, Bangladesh, Nepal, Thailand and Mauritius (97.5–98.5%). These strains had >97.5% identity among themselves and clustered far away from the vaccine strain Rotasiil (lineage-I) and RotaTeq (lineage-II) showing only 92–93% homology (Fig. 3b).

3.4.3. G9 strains

G9 strains (showing > 95% nucleotide homology among themselves) clustered with the strains of G9 lineage-III (major), and with the previously reported G9 strains from India and USA (>83 nucleotide identity). Previously reported Indian strain (GARV/Human-wt/IND/mani-475/2008/G9) in G9 lineage-III (minor) showed 93–95% similarity to the current G9 strains. These G9 strains clustered distantly from the vaccine strain Rotasiil (lineage-I) and 116E strain (Parental strain to ROTAVAC) (lineage-II), which exhibited only 85–88% homology with them (Fig. 3c).

Table 4
Statistical analysis of GARV positive/negative samples (n = 2588) with clinical symptoms using OpenEpi software.

	GARV-Positives (n = 1352/2588)	GARV-Negatives (n = 1236/2588)
(a) Frequency of stool passage/24 h		
≤5 (n = 31)	15	16
≥6 (n = 2557)	1337	1220
		χ^2 = 0.186, p-value = 0.665
(b) Degree of dehydration		
None (n = 879)	505	374
Some (n = 1592)	802	790
Severe (n = 117)	45	72
		χ^2 = 20.686, p-value = 0.000032
(c) Duration of stay at the hospitals		
<3 days (n = 1177)	568	609
≥3 days (n = 1411)	784	627
		χ^2 = 13.725, p-value = 0.000212
(d) Fever		
≤37 °C (n = 1621)	843	778
37.1 °C-38.9 °C (n = 947)	495	452
≥39 °C (n = 20)	14	6
		χ^2 = 2.564, p-value = 0.277389
(e) Frequency of vomiting/24 h		
0 (n = 1060)	559	501
1 (n = 46)	22	24
2–4 (n = 837)	406	431
≥5 (n = 645)	365	280
		χ^2 = 10.029, p-value = 0.018316

3.4.4. P[4] strains

The P[4] strains were seen to distribute themselves into 2 sub-lineages under P[4] lineage-V (n = 20 in lineage-Vbi and n = 7 in lineage-Vbii) bearing > 94.3% identity among themselves. Lineage-Vbi strains clustered with strains from India, Brazil and Vietnam (98–99% identity), while strains of lineage-Vbii were similar to strains reported from India (97–98% identity). Representative P[4] strains were distant to the vaccine strains Rotarix, RotaTeq, Rotasiil and 116E strain (Parental strain to ROTAVAC) (P[8], P[8], P[5] and P[11] respectively) showing less than 80% homology (Fig. 4a).

3.4.5. P[6] strains

Representative P[6] strains formed a separate cluster under human P[6] lineage-II (>96.5% identity among themselves). These strains clubbed with the human strains reported previously from India, Bangladesh and Zimbabwe showing 93.2–98.2% similarity. Eastern Indian P[6] strains exhibited only 88% homology to an Indian strain (GARV/Human-wt/IND/mcs/13-07/2007/G9P[6]; lineage-IV) which showed porcine to human interspecies transmission. Porcine P[6] strain Gottfried showed 81% homology with the P[6] strains from eastern India. The vaccine strains Rotarix, RotaTeq, Rotasiil and 116E strain (Parental strain to ROTAVAC) (P[8], P[8], P[5] and P[11] respectively) clustered away from these P[6] strains (56–70% nucleotide identity) (Fig. 4b).

3.4.6. P[8] strains

All of the representative P[8] strains from this study clustered together with the strains of P[8] lineage-III, having > 99% nucleotide identity among themselves. Strains from Italy, USA, Belgium and Vietnam had close nucleotide identity with the eastern Indian P[8] strains (96–98%). Although being in the same lineage-III, previously reported strains from India clustered at a distance from the strains identified during this period (95–96% identity). Vaccine strains Rotarix, RotaTeq, 116E strain (Parental strain to ROTAVAC) and Rotasiil clustered in different lineages having 89–92% homology with the representative P[8] strains. The OP354-like P[8] strains clustered far away in lineage-IV having only 86–87% similarity to our strains, suggesting less evolutionary relatedness to these strains (Fig. 4c).

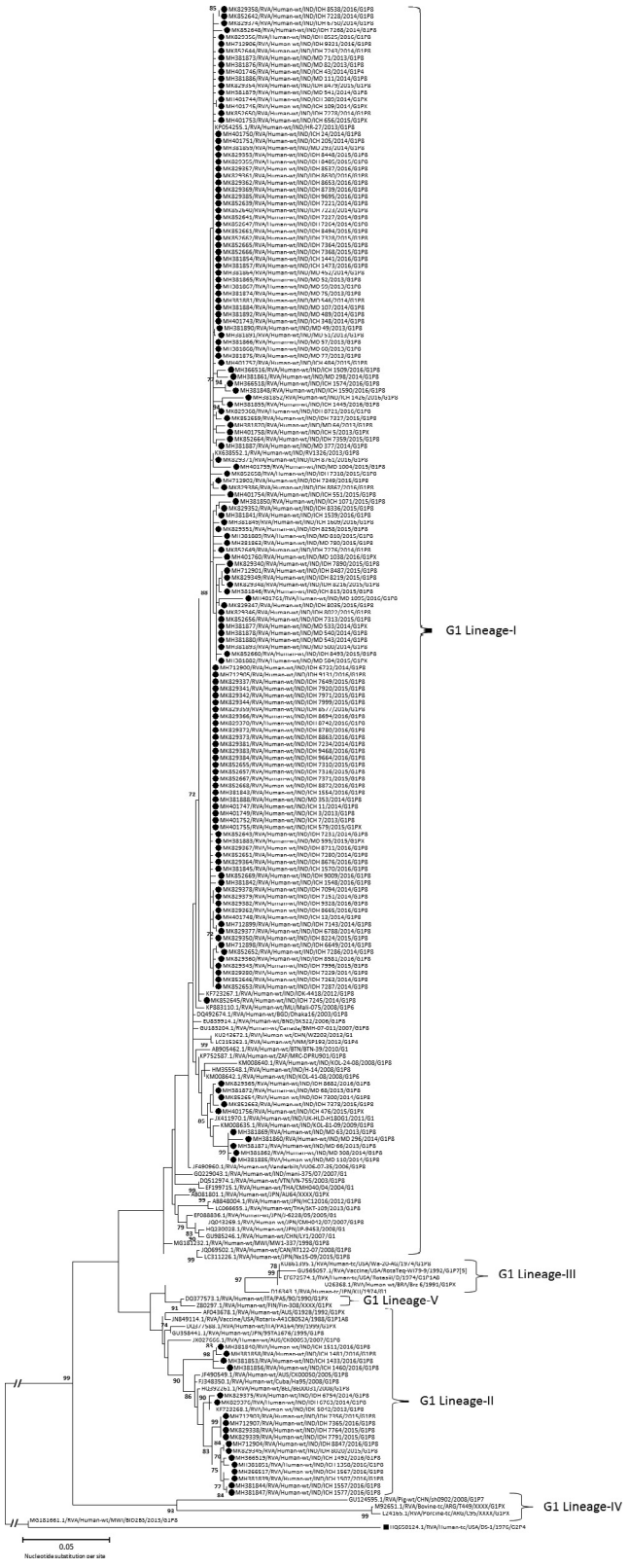

Fig. 3a. Phylogenetic dendrogram based on nucleotide sequences of VP7 gene of eastern Indian G1 strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

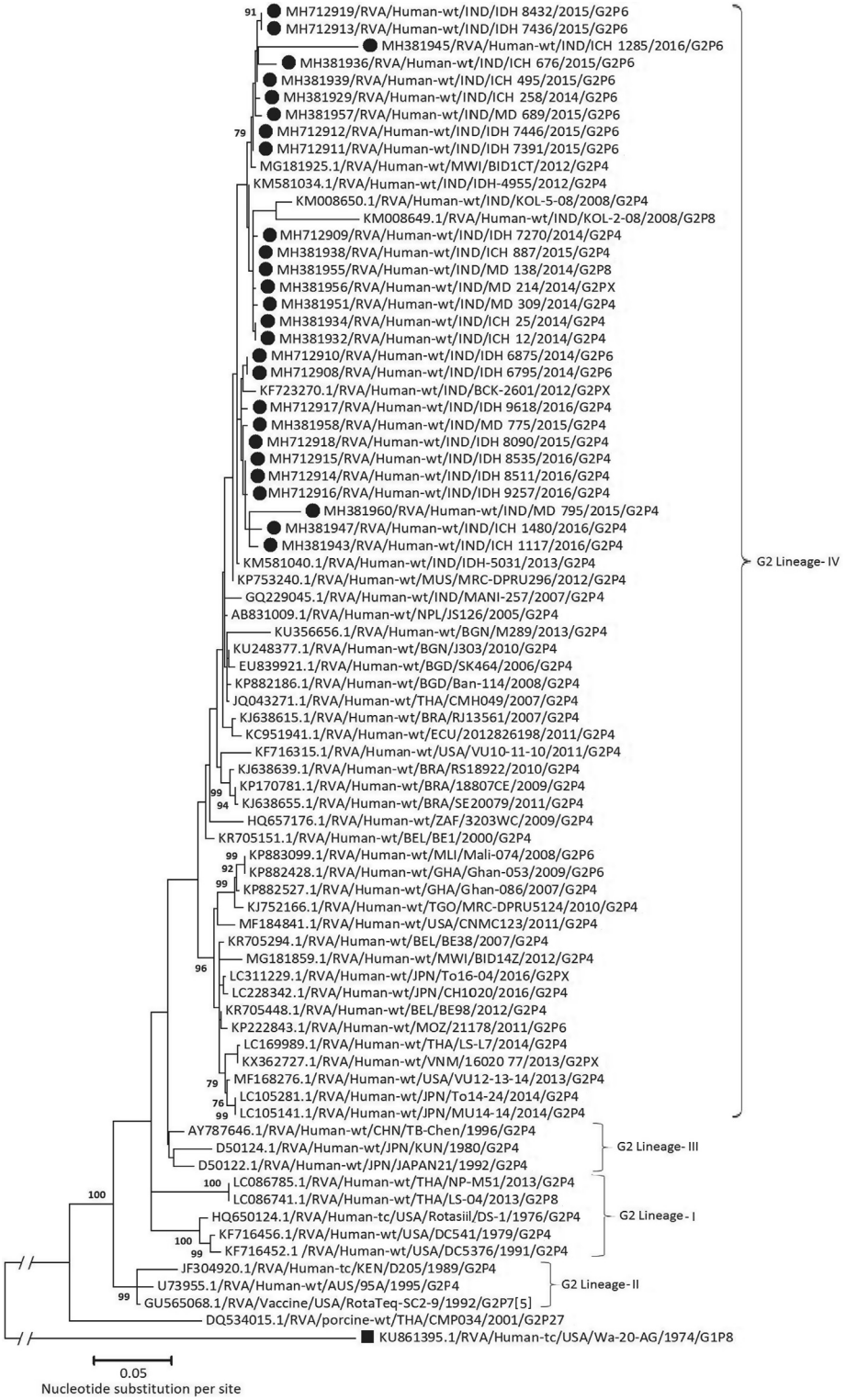

Fig. 3b. Phylogenetic dendrogram based on nucleotide sequences of VP7 gene of eastern Indian G2 strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

3.5. Analyses of the VP7 and VP4 antigenic epitopes of the various GARV genotypes with the vaccine strains

To explore any inter-genotypic variability which might exist, we compared the amino acid composition of the antigenic epitopes on the VP7 trimer and VP8* domain of VP4 protein of the identified GARV genotypes (like G1, G2, G9, P[4], P[6], P[8]) with the strains formulating vaccines like Rotarix, RotaTeq, 116E (Parental strain to ROTAVAC) and Rotasiil.

Comparison of the amino acid composition in the VP7 trimer of the G1 strains and the vaccine strains was done where 10% strains from each G1 lineage were taken (n = 100; lineage-I = 90, lineage-II = 10). These strains clustered broadly into 3 groups (A, B and C), while 3 strains from lineage-I viz., RVA/Human-wt/

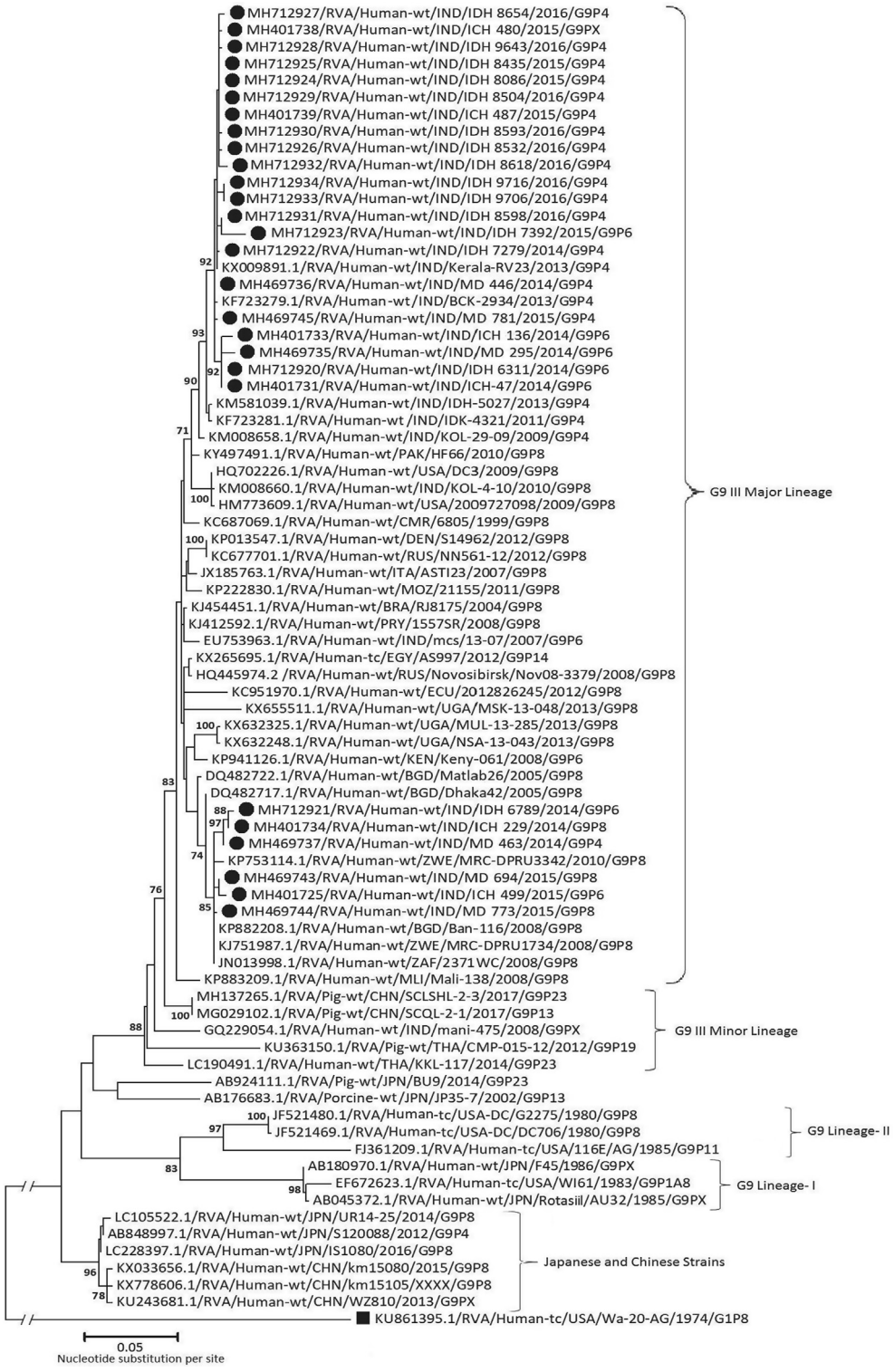

Fig. 3c. Phylogenetic dendrogram based on nucleotide sequences of VP7 gene of eastern Indian G9 strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

IND/MD 51/2014/G1P[8], RVA/Human-wt/IND/IDH 8761/2015/G1P[8] and RVA/Human-wt/IND/IDH 8538/2014/G1P[8] were unique in respect to few amino acid positions as depicted in Table 5, as they did not cluster with any of the 3 groups. The representative strains from lineage-II clustered in group B and C, while strains from lineage-I clustered in group-A. Out of the 29 amino acid residues in 7-1a, 7-1b and 7-2 epitopes, various mismatches existed with RotaTeq (G1), Rotarix (G1) and Rotasiil (G1) in respect to the G1 strains (Supplementary Table 3). An analysis of the G2 strains showed that 4 and 5 amino acid residues in the antigenic epitopes of VP7 protein differed from RotaTeq (G2) and Rotasiil (G2), respectively. The G9 strains had 4 residues that

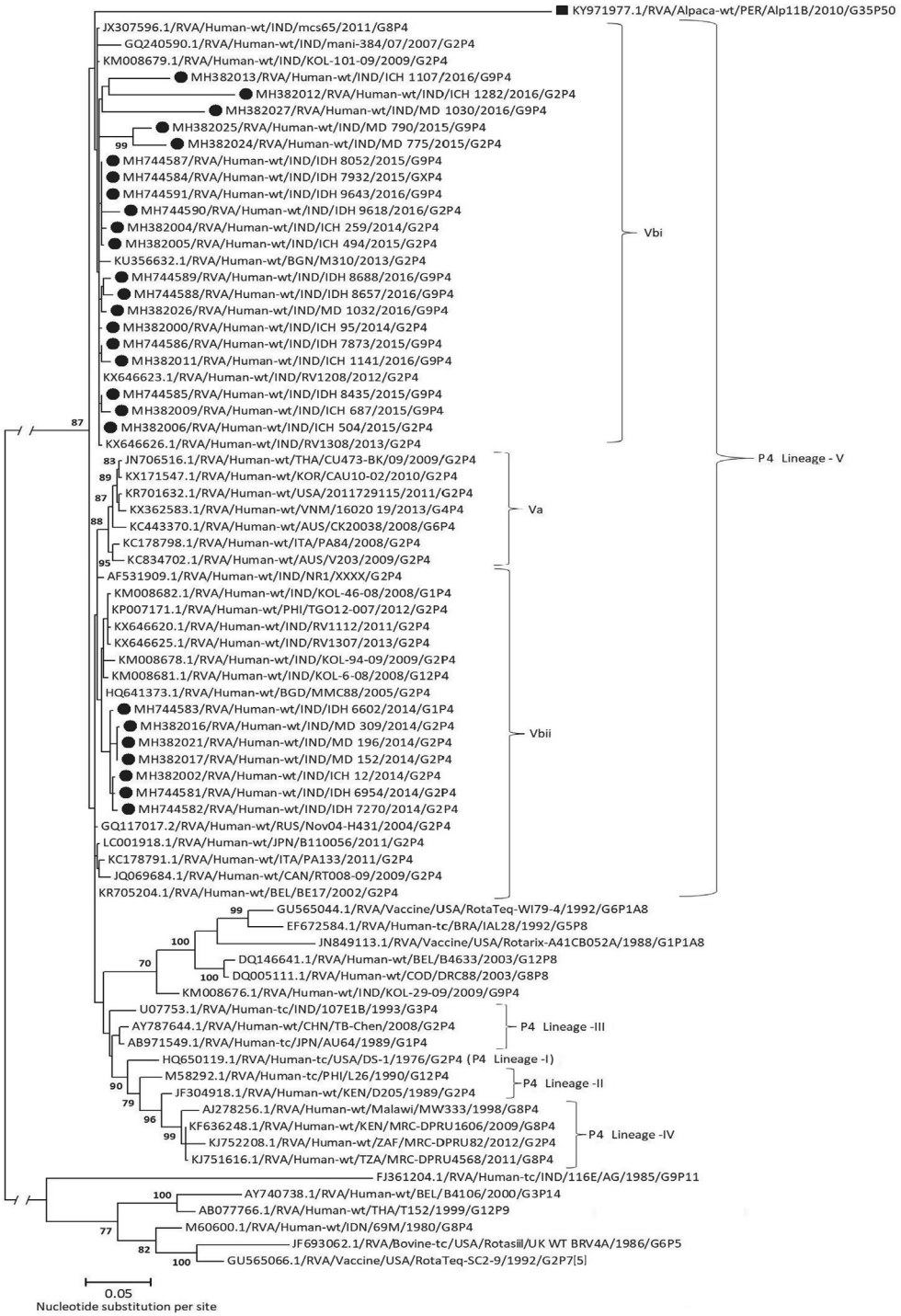

Fig. 4a. Phylogenetic dendrogram based on nucleotide sequences of VP4 gene of eastern Indian P[4] strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

were different from both 116E (G9) and Rotasiil (G9), respectively (Table 5).

Analysis of the amino acid constitution of the VP8* protein showed that of the 25 amino acid residues in 8–1 to 8–4 antigenic epitopes. 15, 16, 20 and 23 residues in all the P[6] strains were different from the vaccine strains of Rotarix (P[8]), RotaTeq (P[8]), 116E (P[11]) and Rotasiil (P[5]), respectively. 11, 10, 18 and 23 differences in residues between the P[4] strains and Rotarix (P[8]), RotaTeq (P[8]), 116E (Parental strain to ROTAVAC) (P[11]) and Rotasiil (P[5]), respectively. These differences were mostly contained in VP8* epitopes 8–1 and 8–3. The VP8* antigenic epitopes of the P[8] strains showed 5, 3, 22 and 23 amino acid residue differences with Rotarix (P[8]), RotaTeq (P[8]), ROTAVAC (P[11]) and Rotasiil (P[5]), respectively (Table 6).

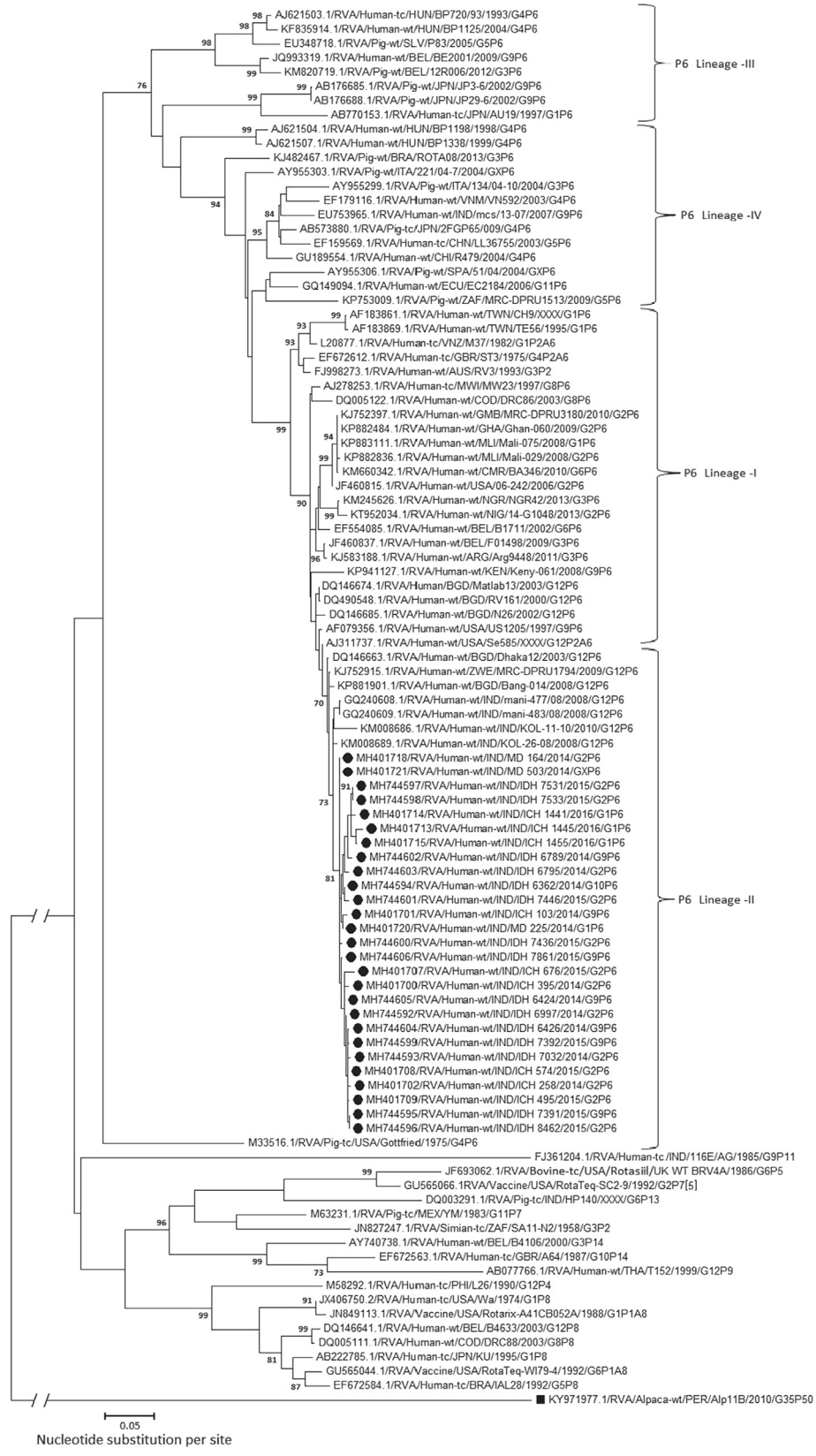

Fig. 4b. Phylogenetic dendrogram based on nucleotide sequences of VP4 gene of eastern Indian P[6] strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

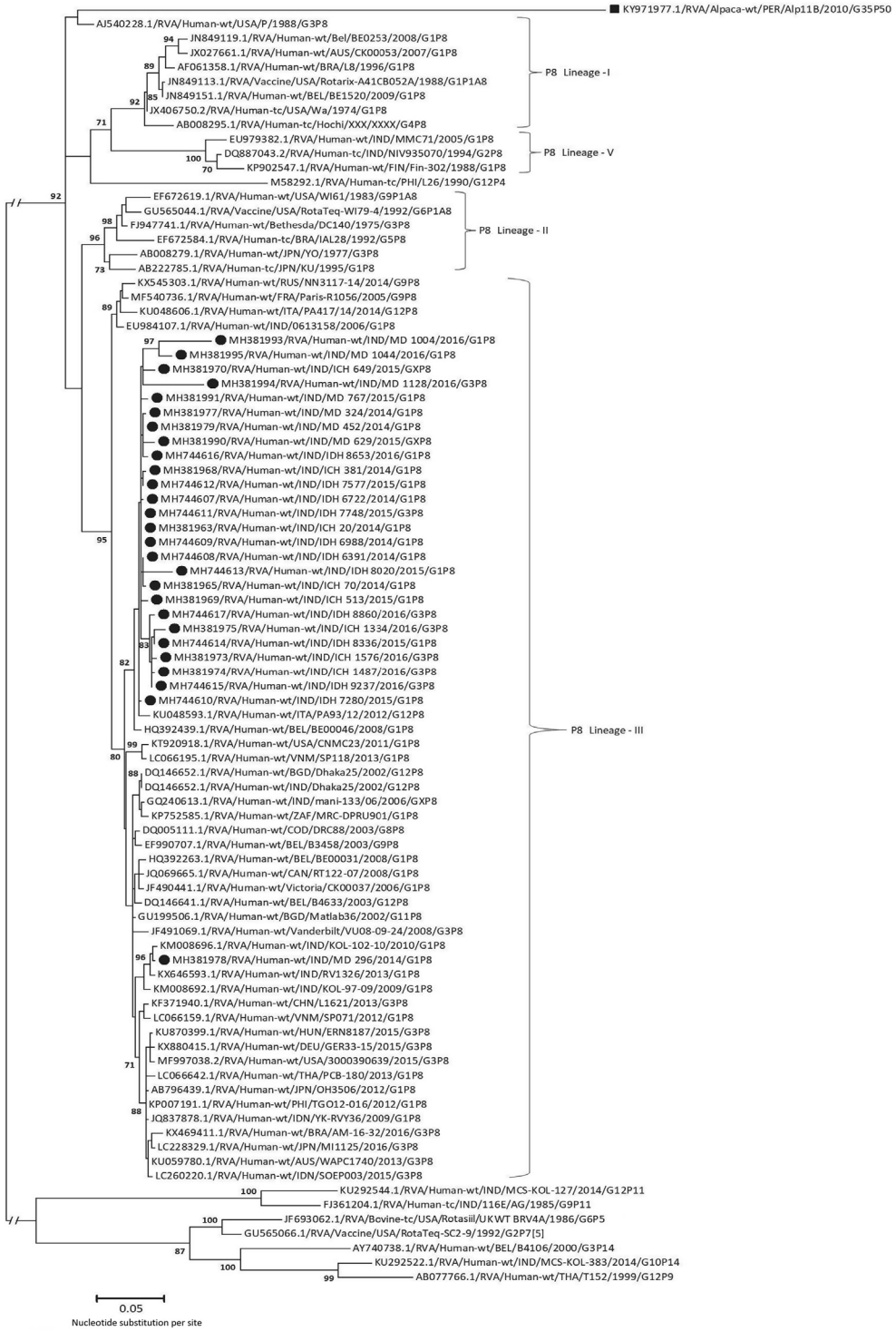

Fig. 4c. Phylogenetic dendrogram based on nucleotide sequences of VP4 gene of eastern Indian P[8] strains (marked with a solid circle ●) isolated during 2014–2016, with other known strains. The out-group has been shown with a solid square ■). Scale bar, 0.05 nucleotide substitutions per site. Bootstrap values < 70% are not shown.

4. Discussion

GARV continues to be a pervasive etiologic agent of paediatric gastroenteritis, causing high mortality in many developing countries of sub-Saharan Africa and Asia, infecting almost every child by the age of 5 years [31,32]. Estimated mortality has reduced in the latest report on rotaviral gastroenteritis in Asia; nevertheless, a high GARV disease burden (39.6% positivity) was reported from the study conducted under National Rotavirus Surveillance Network in India [33]. India suffers from huge financial burden of nearly 4.9 billion INR per year on account of paediatric hospitalizations caused by GARV diarrhea [34]. In this study, almost 50% positivity suggests a significantly high burden of GARV, in population from urban as well as rural area in eastern part of India.

Although major proportion (78%) of the circulating strains belonged to the common human GARV genotypes (G1P[8], G2P

Table 5
Alignment of antigenic amino acid residues in VP7 protein between eastern Indian strains and the vaccine strains. Single amino acid dissimilarity has been underlined and more than one dissimilarities are indicated in boldface.

G1	7-1a															7-1b						7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291		201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-tc/IND/Rotavac/116E/AG/1985/G9P[11]	I	T	G	T	E	W	K	G	Q	D	A	I	D	K		Q	N	T	A	D	N	K	N	S	T	L	S	E	N	G
RVA/Vaccine/USA/RotaTeq-W179-9/G1P7[5]	T	T	N	G	D	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
RVA/Human-tc/Rotasiil/USA/D/1974/G1P1A[8]	T	T	N	G	D	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
RVA/Human-wt/IND/MD 51/2014/G1P[8]	T	T	**N**	G	**E**	W	K	D	Q	**N**	V	V	D	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Human-wt/IND/IDH 8761/2015/G1P[8]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	**N**	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Human-wt/IND/IDH 8538/2015/G1P[8]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	**I**	D	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
A	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	D	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
B	T	**N**	N	G	**E**	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	M	N	G
C	T	T	N	G	**E**	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	**N**	L	S	M	N	G

G2	7-1a															7-1b						7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291		201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/IND/Rotavac/116E/AG/1985/G9P[11]	I	T	G	T	E	W	K	G	Q	D	A	I	D	K		Q	N	T	A	D	N	K	N	S	T	L	S	E	N	G
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]	A	N	S	D	E	W	E	N	Q	D	T	M	N	K		Q	D	V	S	N	S	R	D	N	T	S	D	I	S	G
RVA/Human-tc/Rotasiil/USA/DS-1/1976/G2P[4]	A	N	S	D	E	W	E	N	Q	D	N	V	N	K		Q	D	V	N	N	N	R	D	N	T	S	D	I	S	G
RVA/Human-wt/IND/ICH 295/2014/G2P[4]	**T**	**N**	**S**	**N**	E	W	**E**	**N**	Q	D	**T**	**M**	**N**	K		Q	**D**	V	**D**	N	**N**	**R**	D	**N**	T	**S**	**D**	**I**	**S**	G
RVA/Human-wt/IND/MD 795/2015/G2P[4]	**T**	**N**	**S**	**N**	E	W	**E**	**N**	Q	D	**T**	**M**	**N**	K		Q	**D**	V	**D**	N	**N**	**R**	D	**N**	T	**S**	**D**	**I**	**S**	G
RVA/Human-wt/IND/IDH 8511/2016/G2P[4]	**T**	**N**	**S**	**N**	E	W	**E**	**N**	Q	D	**T**	**M**	**N**	K		Q	**D**	V	**D**	N	**N**	**R**	D	**N**	T	**S**	**D**	**I**	**S**	G

G9	7-1a															7-1b						7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291		201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K		Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/IND/Rotavac/116E/AG/1985/G9P[11]	I	T	G	T	E	W	K	G	Q	D	A	I	D	K		Q	N	T	A	D	N	K	N	S	T	L	S	E	N	G
RVA/Human-wt/Rotasiil/JPN/AU32/G9P[X]	A	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	T	K	D	S	T	L	S	E	S	G
RVA/Human-wt/IND/ICH 47/2014/G9P[6]	**T**	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	**N**	K	D	S	T	L	S	E	N	G
RVA/Human-wt/IND/MD 781/2015/G9P[4]	**T**	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	**N**	K	D	S	T	L	S	E	N	G
RVA/Human-wt/IND/IDH 8532/2016/G9P[4]	**T**	T	G	T	E	W	K	D	Q	D	A	I	D	**M**		Q	N	T	A	D	**N**	K	D	S	T	L	S	E	N	G

Among G1 strains:
Cluster A represents strains with accession number with MK852639 - MK852669, MK829337, MK829340 - MK829344, MK829346 - MK829357, MK829359 -MK829370, MK829372 - MK829374, MK829377 - MK829379, MK829381 - MK829386, MH381864 - MH381867, MH381873 - MH381879, MH381881, MH381885, MH381894.
Cluster B represents strains with accession numbers MH381853, MH381856, MH381858
Cluster C represents strains with accession number with MK829338, MK829339, MK829345, MK829347, MK829351, MK829375, MK829376.

[4], G3P[8] and G9P[4]), approximately one-fifth (22%) of the isolates were "unusual/rare genotypic combinations" of usual genotypes. Out of the 33.3% samples which were genotyped through multiplex semi-nested RT-PCR, mixed infections were detected in 10.6% samples (n = 64). However, Sanger sequencing could not confirm mixed infection as only one predominant genotype was amplified. This was probably due to low sensitivity of Sanger sequencing assay to amplify and obtain sequences from low viral load samples. Mixed infection rate observed in this study is consistent with the previous report where 24% "unusual genotypic GARV combinations" and 13.8% mixed infections were reported from this geographical area, suggesting congenial environment for inter-species and intra-species reassortment in this epidemiological settings [35]. Of 1817 GARV positives, 99 samples (5.4%) remained untypeable by both multiples semi-nested RT-PCR or Sanger sequencing. These untypeable samples were VP6 antigen EIA as well as VP6 RT-PCR positive. Analysis of EIA values of these samples revealed significantly low values (O.D. = 0.31–0.45) compared to the samples which could be genotyped. This suggests that possibly a very viral load in these samples may have an impact on the amplification of VP7 and VP4 gene segments. The predominance of "unusual combination" of G9P[4] and G9P[6] over the common genotype G9P[8] which was observed in this study has also been seen in other parts of India [24,36]. G9 and G2 strains were reported to over-ride (40% and 36%, respectively) the G1 strains (16%) from this geographical area during 2011–13 among the hos-

Table 6
Alignment of antigenic amino acid residues in VP4 protein between eastern Indian strains and the vaccine strains. Single amino acid dissimilarity has been underlined and more than one amino acid dissimilarities has been indicated in boldface.

P[4]	8-1											8-2		8-3									8-4		
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RVA/Vaccine/USA/Rotarix/A41CB052A/1998/G1P1A[8]	D	S	Q	E	S	T	N	L	N	N	I	T	A	N	P	V	D	S	S	N	D	N	N	T	N
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	D	S	Q	E	S	T	N	L	N	D	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/IND /116E/AG/1985/G9P[11]	T	S	A	A	V	T	F	N	P	V	P	S	Y	S	Q	T	S	T	D	N	S	S	S	N	D
RVA/bovine-tc/USA/Rotasiil/UK WT BRV4A/1986/G6P[5]	G	T	V	G	G	F	T	N	Y	A	S	E	N	A	S	E	T	S	S	N	V	D	T	R	P
RVA/Human-wt/IND/MD 152/2014/G2P[4]	**D**	S	**Q**	**D**	**S**	T	**D**	**L**	**N**	**N**	**I**	**T**	**A**	**S**	**Q**	**T**	**N**	**N**	**E**	N	**S**	**D**	**N**	**T**	**D**
RVA/Human-wt/IND/IDH 7270/2015/G2P[4]	**D**	S	**Q**	**D**	**S**	T	**D**	**L**	**N**	**N**	**I**	**T**	**A**	**S**	**Q**	**T**	**N**	**N**	**E**	N	**S**	**D**	**N**	**T**	**D**
RVA/Human-wt/IND/ICH 1141/2016/G9P[4]	**D**	S	**Q**	**D**	**S**	T	**D**	**L**	**N**	**N**	**I**	**T**	**A**	**S**	**P**	**T**	**N**	**N**	**E**	N	**S**	**D**	**N**	**T**	**D**

P[6]	8-1											8-2		8-3									8-4		
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RVA/Vaccine/USA/Rotarix/A41CB052A/1998/G1P1A[8]	D	S	Q	E	S	T	N	L	N	N	I	T	A	N	P	V	D	S	S	N	D	N	N	T	N
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	D	S	Q	E	S	T	N	L	N	D	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/IND /116E/AG/1985/G9P[11]	T	S	A	A	V	T	F	N	P	V	P	S	Y	S	Q	T	S	T	D	N	S	S	S	N	D
RVA/bovine-tc/USA/Rotasiil/UK WT BRV4A/1986/G6P[5]	G	T	V	G	G	F	T	N	Y	A	S	E	N	A	S	E	T	S	S	N	V	D	T	R	P
RVA/Human-wt/IND/MD 164/2014/G2P[6]	**D**	**N**	**S**	**E**	**S**	T	**N**	**L**	**S**	**E**	**V**	**T**	**A**	**T**	**N**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**
RVA/Human-wt/IND/ICH 574/2015/G2P[6]	**D**	**N**	**S**	**E**	**S**	T	**N**	**L**	**S**	**E**	**V**	**T**	**A**	**T**	**N**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**
RVA/Human-wt/IND/IDH 8462/2016/G2P[6]	**D**	**N**	**S**	**E**	**S**	T	**N**	**L**	**S**	**E**	**V**	**T**	**A**	**T**	**N**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**

P[8]	8-1											8-2		8-3									8-4		
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RVA/Vaccine/USA/Rotarix/A41CB052A/1998/G1P1A[8]	D	S	Q	E	S	T	N	L	N	N	I	T	A	N	P	V	D	S	S	N	D	N	N	T	N
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	D	S	Q	E	S	T	N	L	N	D	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Human-wt/IND /116E/AG/1985/G9P[11]	T	S	A	A	V	T	F	N	P	V	P	S	Y	S	Q	T	S	T	D	N	S	S	S	N	D
RVA/bovine-tc/USA/Rotasiil/UK WT BRV4A/1986/G6P[5]	G	T	V	G	G	F	T	N	Y	A	S	E	N	A	S	E	T	S	S	N	V	D	T	R	P
RVA/Human-wt/IND/ICH 46/2014/G1P[8]	**D**	S	**Q**	**D**	**S**	T	**N**	**L**	**N**	**G**	**I**	**T**	**A**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Human-wt/IND/IDH 8860/2016/G3P[8]	**D**	S	**Q**	**E**	**S**	T	**N**	**L**	**N**	**D**	**I**	**T**	**A**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Human-wt/IND/MD 1004/2016/G1P[8]	**D**	S	**Q**	**D**	**S**	T	**N**	**L**	**N**	**G**	**I**	**T**	**A**	**N**	**P**	**V**	**D**	**T**	**R**	N	**D**	**D**	**N**	**T**	**N**

pitalized children with severe diarrhea, while no incidence of G3 was noticed [18]. On the contrary, in our study (2014–16), the frequency of G9 and G2 strains decreased significantly (12.9% and 12.6%, respectively) compared to the G1 and G3 strains (55% and 13.3%, respectively). Tracking of this continuous cycling of ubiquitously distributed GARV genotypes in subsequent epidemic seasons is essential in this region. Genotype-specific immunity is a critical parameter for vaccine-preventable disease like rotaviral diarrhea, therefore monitoring of this marked diversity of GARV strains is needed for successful vaccine implementation in this state.

GARV shows a tendency towards directional selection under persistent immunological pressure that might promote natural selection of one genotype over another in a geographical area. Temporal variation of predominant GARV genotypes has been frequently observed among populations of several countries [37–41]. Several GARV strains were seen to surpass each other as a predominant genotype temporally over the past decade in eastern India [18,28,35]. Until 2016, G1P[8] (50%) was seen as the most prevailing genotype, followed by G2P[4] (22%) all over India [24]. During 2011–13, G9 and G12 were the most prevalent genotypes [18]. Though G1 predominated during 2014–2015, but its sharp decline was evidenced in the urban population (from 70.44% to 31.94%) during the study period; while prevalence of the G3 strains increased. In 2014, there were negligible G3 strains from any of the three hospitals, but in 2015–2016 there was an upsurge in the G3 strains, especially in the urban area where it leaped from 4% to almost 44%, over-riding all other co-circulating genotypes in 2016. This is in line with previous reports where upsurge in G3 has been reported during last few years [42,43]. In the rural settings, despite the gradual decrease in the proportion of G1 during 2014–16, nevertheless it continued to pre-dominate all the other prevailing genotypes (68%). The genotype diversity was more significant among the urban conditions rather than rural, where a single genotype (G1) predominated all through the study period. This could be due to unhygienic condition, crowding and higher frequency of migratory population in urban slums compared to the more stable population in rural area. Increase in G2 and G9 strains was noticed in 2015 (18.69% and 20.9%) compared to 2014 (13.09% and 9.37%) or 2016 (6% and 9.01%), respectively. Hospitals in both the areas catered to low to middle income group. In low to middle income countries co-emergence of various genotypes and their continuous shift after short period of prevalence could exert selective immunological pressure [44].

This study focuses on the GARV genotypic dynamicity in urban and rural population of eastern India, where rotavirus vaccine will be introduced in routine immunization program by 2019. Evolutionary analyses of various G and P genotypes of co-circulating strains depicted that all the G1, G2, G9, P[4], P[6] and P[8] strains clustered with the previously reported human strains from India in the same lineage, having high nucleotide homology with them. Nevertheless, all the current strains clustered far from the vaccine

strains like Rotarix, RotaTeq, ROTAVAC (116E strain) and Rotasiil in the phylogenetic dendrograms. Multiple differences existed in the amino acid constitution spanning the potential antigenic sites of VP7 and VP4 protein of the co-circulating strains and the vaccine strains. These dissimilarities may or may not have any significant impact on the efficacy of the rotavirus vaccine in these settings. As Indian vaccines ROTAVAC and Rotasiil comprise of G9 and G1, G2, G3, G4, G9 respectively, therefore determination of amino acid substitutions in the currently circulating genotypes might provide a valuable set of data prior to the introduction of vaccines in this state. Vaccination is the key to reduce GARV related hospitalization and mortality rates among infants. Though GARV vaccine efficacy in developing countries (50–64%) is quite low compared to the developed countries (85–98%), nevertheless the infantile hospitalization rates have significantly reduced through vaccination in low-income countries after vaccine implementation [45,46]. Following the introduction of rotavirus vaccine in NIP/UIP, decrease in severe gastroenteritis among unvaccinated children has also been observed, suggesting indirect effects of vaccination such as herd immunity and herd protection [47,49].

Conclusively, our study highlights the substantial burden of GARV positivity among the rural and urban infantile population of West Bengal, together with the phylodynamics of prevalent GARV genotypes and mutational analysis of their antigenic epitopes in comparison to the prevailing vaccine strains. This data is relevant prior to the introduction of vaccine in this region expectedly in 2019. Impact of the rotavirus vaccines in these epidemiological settings of eastern India with high genotypic diversity will provide crucial data on vaccine efficacy and heterotypic protection. Thus, continuous surveillance of the proportion and genotypes of rotavirus in the post-vaccination era is needed to monitor the hospitalization rates, dynamics and lineage distribution of the circulating strains along with the upsurge of any potential reassortant strains.

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Declaration of Competing Interest

The authors declare that no conflict of interest exists.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.062.

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