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Sequence analysis of VP4 genes of wild type and culture adapted human rotavirus G1P[8] strains

Ritu Arora, Ganesh S Dhale, Pooja R Patil, Shobha D Chitambar*

Enteric Viruses Group, National Institute of Virology, Pune India

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ABSTRACT

Objective: To conduct a comparative analysis of the VP4 gene sequences of Indian wild type (06361, 0613158, 061060 and 0715880) and cell culture adapted (06361-CA, 0613158-CA, 061060-CA and 0715880-CA) G1P[8] rotavirus strains. **Methods:** Full-length VP4 genes of each of the four wild type G1P[8] rotavirus strains and their cell culture adapted counterparts displaying consistent cytopathic effect were subjected to RT-PCR amplification and nucleotide sequencing. **Results:** All four cell culture adapted G1P[8] rotavirus strains showed nucleotide and amino acid substitutions in the VP4 gene as compared to their wild type strains. The number of substitutions however, varied from 1-64 and 1-13 respectively. The substitutions were distributed in both VP5* and VP8* subunits of VP4 gene respectively of permeabilization and hemagglutinating activity. The presence of unique amino acid substitutions was identified in two of the four wild type (V377G, S387N in 061060 and I644L in 0715880) and all four cell culture adapted (A46V in 0613158-CA, T60R in 06361-CA, L237V, G389V and Q480H in 061060-CA and S615G and T625P in 0715880-CA) strains for the first time in the VP4 gene of P[8] specificity. Amino acid substitutions generated increase in the hydrophilicity in the cell culture adapted rotavirus strains as compared to their corresponding wild type strains. **Conclusions:** Amino acid substitutions detected in the VP4 genes of G1P[8] rotavirus strains from this study together with those from other studies highlight occurrence of only strain and/or host specific substitutions during cell culture adaptation. Further evaluation of such substitutions for their role in attenuation, immunogenicity and conformation is needed for the development of newer rotavirus vaccines.

1. Introduction

Among the diverse pathogens causing diarrhea, rotavirus has been established as the most important cause of severe diarrhea in children, accounting for an average of 39% of severe diarrheal cases [1]. Globally, rotavirus is responsible for an estimated 611 000 deaths, 2 million hospitalizations, 25 million medical visits and a total of 140 million cases among children under 5 years of age annually [1]. Mortality caused by rotaviruses occurs especially in the countries from South Asian region [2]. India alone accounts for approximately 122 000-153 000 infant deaths per annum due to rotavirus disease [3]. To reduce the burden of rotavirus disease, two orally administrable rotavirus vaccines, Rotarix (Monovalent, G1P[8]) and RotaTeq (Pentavalent, G1,G2,G3, G4 and P[8]), have been developed and licensed in more than 100 countries, and were introduced into the routine childhood immunization program in some countries in Latin America, Europe, Australia, and the United States [4].

Rotavirus belongs to the family *Reoviridae*, and is characterized by a genome of 11 segments of double-stranded RNA which encodes six structural (VP1-4, VP6, VP7) and six nonstructural viral proteins (NSP1-6) [5]. The two outerlayer proteins, VP7 (G, glycoprotein) and VP4 (P, protease sensitive) form the basis of the current dual classification system of group A rotaviruses into G and P genotypes, respectively[5]. This classification system has enabled identification of rotaviruses into 24 G and 33 P types [6,7] revealing >45 distinct G-P combinations [8]. Five combinations of G and P genotypes, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] comprise more than 90% of the human rotavirus infections, among which G1P[8] combination

*Corresponding author: Dr. Shobha D. Chitambar, Deputy Director & Group Leader, Enteric Viruses Group, National Institute of Virology, 20-A, Dr. Ambedkar Road, Pune - 411 001, India.
Tel: 91-020-26127301
Fax: 91-020-26122669
E-mail: drshobha.niv@gmail.com

prevails, worldwide [9].

Rotavirus replicates in the mature enterocytes of the small intestine, an environment rich in proteases. The rotavirus VP4 is a trimeric spike protein involved in the cell attachment and membrane penetration[5]. For efficient entry into the cell a virion needs to be activated by trypsin. Proteolytic cleavage of VP4 primes the virus for efficient entry into the cells [5]. During proteolysis, VP4 (88 kDa) is cleaved into VP8* (28 kDa) and VP5* (60 kDa), and the cleavage products remain associated with the virion. The VP8* fragment (247 amino acid, 1-247) is the viral hemagglutinin [5], while the VP5* fragment (529 amino acid, 248-776) is involved in the permeabilization of membranes [5]. The X-ray crystallographic structures of VP8* and VP5* have provided strong evidence of the distal globular domain of the VP4 spike to be composed of VP8*, with the remaining body of the spike consisting of VP5* [10,11].

The VP8* region has been described to contain four putative neutralization domains (8-1, 8-2, 8-3, and 8-4) defined by amino acid alignments and mapping of monoclonal antibody escape mutants [10,11], while VP5* fragment involved in binding to neutralizing antibody has not been well characterized.

Rotaviruses have been adapted in cell culture, however genomic changes during cell culture adaptation have been described occasionally [12,13]. The aim of the present study was to compare the VP4 gene sequences of cell culture adapted G1P[8] rotavirus strains with those of the corresponding wild type strains detected in Indian children.

2. Materials and methods

2.1. Viruses and cell culture

The stool specimens collected from children hospitalized for acute gastroenteritis in 2005-2007 were tested for the presence of rotavirus antigen by enzyme linked immuno sorbent assay (ELISA) and characterized by multiplex PCR for identification of G and P types [14]. Rotavirus strains with G1P[8] specificity were detected predominantly. Thirteen specimens indicating high optical density values (>0.8) in ELISA and found in sufficient quantity were selected for the inoculation in cell culture. The rhesus monkey kidney epithelial cell line, MA104 was procured from ATCC, USA and grown in rolling tubes containing Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum and antibiotics (Penicillin 200 U/mL and Streptomycin 20 μg/mL). The tubes were maintained at 37 ℃ and inoculated with the fecal specimens containing human rotavirus G1P[8] strains, as described earlier [15]. Prior to inoculation in the cell line, virus was activated by 4 μg per mL of trypsin (TPCK Trypsin). At the end of 1h adsorption period of virus, inoculum was removed and maintenance medium containing trypsin (1 μg/mL) was added, and incubation was continued at 37 ℃ for 7 days on the roller apparatus. The virus was passaged 10 times in the cell culture. The cell cultures indicating cytopathic effect (CPE) were subjected to two cycles of freezing and thawing for the recovery of virus. The culture supernatants were stored at -70 ℃ until tested.

2.2. ELISA

Antigen capture ELISA was performed on the supernatants of infected cell cultures at each passage level using a Group A Rotavirus detection kit (IDEIA™ Rotavirus, DAKO, USA) according to the manufacturer's protocol. To visualize the rotavirus particles under electron microscope, samples were examined by negative staining as described earlier [16].

2.3 RNA extraction and reverse transcription polymerase chain reaction (RT- PCR)

Viral RNA was extracted from the unpassaged virus (30% stool suspension) and supernatants of the infected cell cultures at passage level 10 using Trizol, LS reagent (Invitrogen, USA) according to the manufacturer's instructions. The presence of rotavirus RNA was determined by RT-PCR based amplification of the VP6 gene as described previously [17]. All samples indicating amplification in VP6 RT-PCR were subjected to full length amplification of VP4 genes using a Qiagen One step RT-PCR kit (Qiagen, Germany) and primers as described previously [18]. The PCR conditions employed in the study were similar to those reported earlier [19]. All of the PCR products were electrophoresed in 2% agarose gels containing ethidium bromide (0.5 μg/mL) and visualized under UV transilluminator.

2.4. Nucleotide sequencing

The PCR amplicons were purified using QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions and sequences in both directions were determined by the dideoxy nucleotide chain terminator method using a Bigdye Terminator cycle sequencing reaction kit V3.1 (Applied Biosystems, USA). The sequence data were collected from an automated sequencer ABI 3130 XL (Applied Biosystems, USA).

2.5. Sequence and phylogenetic analysis

The nucleotide and deduced amino acid sequences of the VP4 genes of wild type and cell culture adapted rotavirus strains were compared with each other and with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (www.ncbi.nlm.nih.gov/blast) server. Multiple alignments were carried out using CLUSTAL W. The phylogenetic tree was generated using MEGA 4.0 with neighbor-joining algorithm and Kimura 2-parameter distance model. The reliability of the analysis was tested by applying bootstrap test with 1 000 bootstrap replications [20].

2.6. Sequence submission

The nucleotide sequences of VP4 genes of the present study were submitted to GenBank under the accession numbers – EU984107 and HM467806–HM467808 for wild type strains and EU984106 and HM467803– HM467805 for cell culture adapted G1P[8] strains.

2.7. Hydropathy Index

Hopp–Woods scale (a hydrophilic index, with nonpolar residues assigned negative values) was used to compare the hydrophilic indices of the wild type and cell culture adapted rotavirus strains. At each position, the mean hydrophilic index of the amino acids within the window was calculated and that value was plotted as the midpoint of the window. A window size of 9 was used to determine the regions of maximal hydrophilicity.

3. Results

3.1. Virological analysis

Four (06361, 0613158, 061060 and 0715880) of thirteen G1P[8] rotavirus strains propogated in MA104 cell culture showed CPE of increasing strength comprising of increased granularity and rounding of cells passage 3 onwards. All (4/4) strains showed the presence of rotavirus RNA in RT–PCR of VP6 gene, however, only three showed positivity in ELISA at passage 10. The presence of virus like particles of ~70 nm diameter was confirmed by electron microscopy (data not shown). One specimen (0715880– CA) found to be negative in ELISA and electron microscopy probably carried low viral load at passage 10. The cell culture adapted strains corresponding to their wild type strains were designated as 06361–CA, 0613158–CA, 061060–CA and 0715880–CA, respectively.

3.2. Phylogenetic analysis

Phylogenetically, VP4 genes of all of the wild type and cell culture adapted strains of the present study clustered with rotavirus strains of P[8]–3 lineage, (Figure 1) with 93.6%–98.5% nucleotide and 93.8%–98.9% amino acid identity. Highest (98.5%) nucleotide identity was displayed with the strain, DH396 from Bangladesh. With other P–genotypes the nucleotide and amino acid identities shown by these strains were 59.8%– 87.4 % and 68.3%–91.2%, respectively.

3.3. Nucleotide and deduced amino acid sequence analysis

The potential cleavage site, arginine at positions 231, 241, and 247, the highly conserved proline residues at positions 68, 71, 225 and 226 and the conserved cysteine residues at positions 216, 318, 380 and 774 described earlier (19,12) were identified in all of the VP4 genes of wild type and cell culture adapted strains.

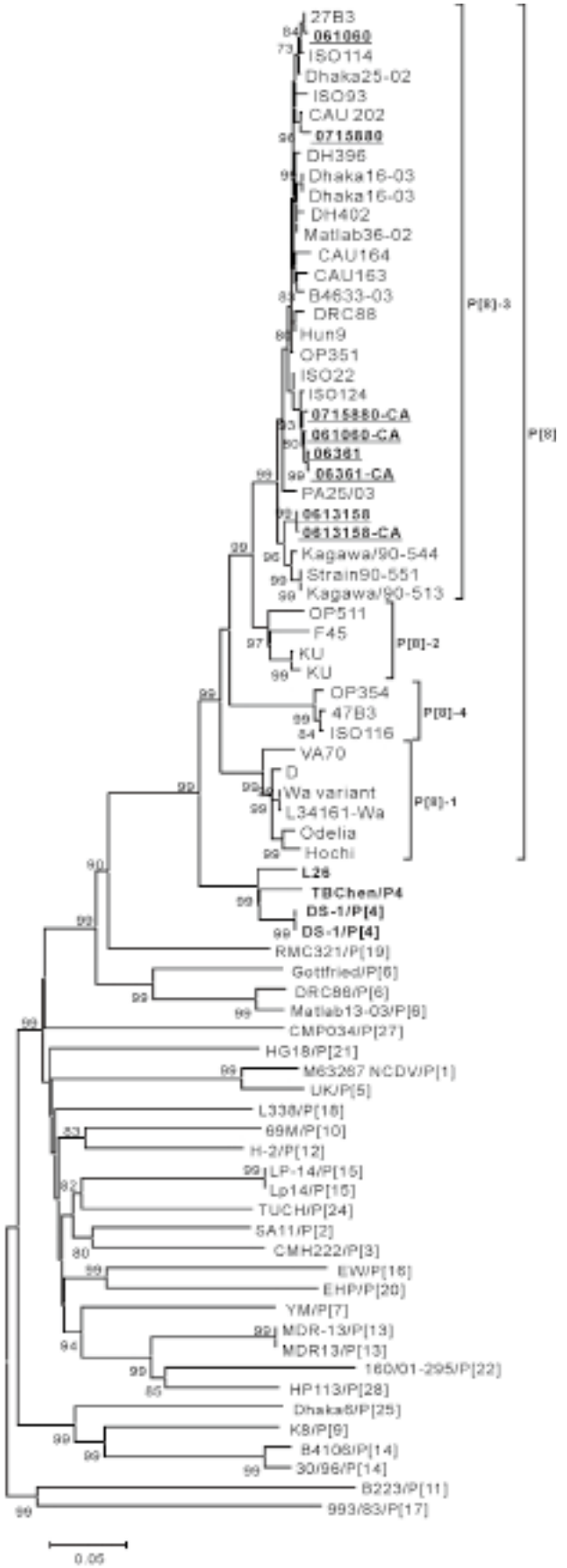

Figure 1. Phylogenetic tree constructed from nucleotide sequences of VP4 gene of study strains and other representative strains.
The strains of the present study are underlined. For each strain, the following data are given: strain name/ genotype.

3.4. Nucleotide and amino acid alignment

The VP4 gene of cell culture adapted strain, 0613158-CA showed 99.9% nucleotide and 99.9% amino acid identity with its wild type strain, 0613158. One nucleotide mismatch (C-T) was observed at position 146 which led to the substitution of alanine (A) by valine (V) at position 46 (Table 1).

The cell culture adapted strain, 06361-CA showed 99.8% nucleotide and 99.9% amino acid identity with counterpart wild type strain, 06361. However, it revealed three nucleotide mismatches which included two silent mutations (G-A and C-T) at positions 651 and 783 and one (C-G) at position 188 generating substitution of amino acid (T-R) at position 60 (Table 1).

The cell culture adapted strain 061060-CA showed 97.3% nucleotide and 98.5% amino acid identity with its wild type strain, 061060. In contrast to 0613158-CA and 06361-CA strains, 061060-CA strain revealed numerous nucleotide mismatches (n=64) in the VP4 gene. Most of them were silent mutations except 12 which replaced amino acids (R-K), (S-N), (L-V), (G-V), (N-S), (P-S), (Q-H), (A-T), (Y-V), (V-L) (K-R) and (F-Y) at positions 162, 189, 237, 377, 387, 389, 480, 586, 587, 600, 616 and 672, respectively (Table 1).

The cell culture adapted strain 0715880-CA showed 97.2% nucleotide and 98.5% amino acid identity with its wild type strain, 0715880. Similar to 061060-CA strain, this strain also displayed multiple (n=63) nucleotide mismatches in the VP4 gene sequence with silent mutations except 13 which replaced amino acids at positions 33 (I-V), 104 (V-A), 113 (D-N), 162 (R-K), 183 (D-A), 586 (A-T), 587 (F-V), 600 (V-L), 615 (S-G), 616 (K-R), 621 (R-K), 625 (T-P), and 644 (L-I) (Table 1).

3.5. Unique amino acid substitutions in the VP4 genes of Indian rotavirus strains

Unique amino acids defined as the presence of specific amino acid residues at specific positions not reported in the sequences available in the GenBank were identified by using tblastn algorithm available at NCBI website. The wild type strains, 061060 and 0715880 showed respectively two (V377G and S387N) and one (I644L) unique amino acid substitutions. The presence of unique amino acids- valine in 0613158-CA, arginine in 06361-CA, valine, serine and histidine in 061060-CA and glycine and proline in 0715880-CA were identified at positions 46, 60, 237, 389, 480, 615 and 625, respectively (Table 1).

3.6. Hydrophilicity Index

As compared to the wild type strains, cell culture adapted 06361-CA, 061060-CA, and 0715880-CA strains showed amino acid substitutions at positions 60, 237/586/587 and 586/587/615/625 respectively in VP4 protein at pH 7.0 indicating increase in the hydrophilicity of the protein while substitution of A-V at position 60 in 0613158-CA strain reduced its hydrophilicity (Figure 2 A-D).

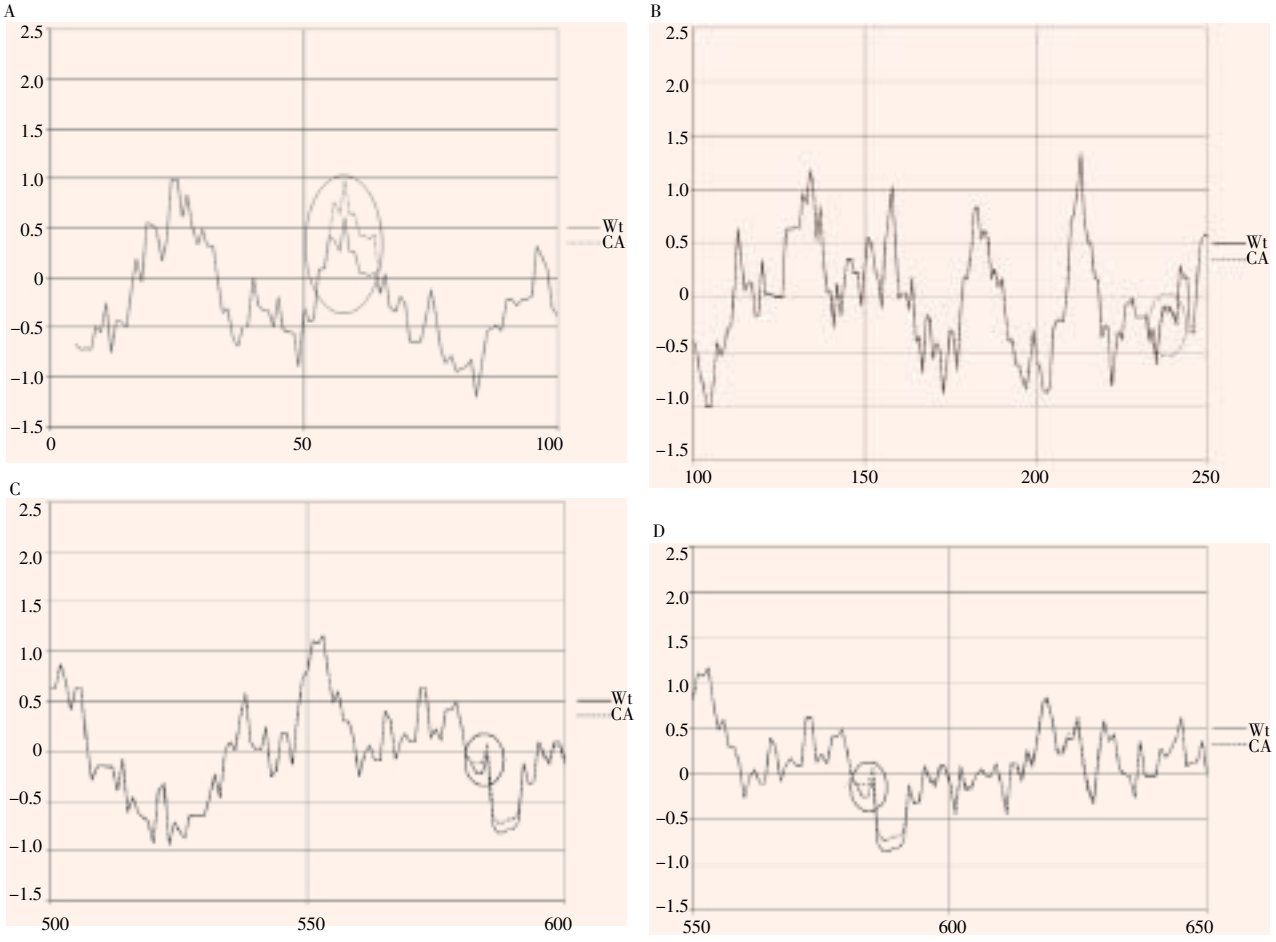

Figure 2. Hydrophilic indices of VP4 protein of (A) 06361 and 06361-CA; (B) and (C) 061060 and 061060-CA and (D) 0715880 and 0715880-CA strains. The circle indicates increases in hydrophilicity of the strains.

Table 1
Amino acid substitutions in the VP4 protein.

Strain	Genotype	Amino acid positions																						
		33	46	60	104	113	162	183	189	237	377	387	389	480	586	587	600	615	616	621	625	644	672
	Consensus aa	V	A	T	A	N	K	A	N	L	V	S	P	Q	T	I	V	S	K	K	T	I	Y
KU	G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Wa	G1P[8]	.	.	.	.	.	R	.	S	P	.	.	.	.	A	.	.	N	.	.	.	.	.
DRC88	G8P[8]	.	.	.	.	D	R	.	.	.	.	.	.	.	A	F	.	N	.	R	.	.	.
DH396	G9P[8]		.	.		.	R	.	.	.	.	.	.	.	A	F		N	.		.	.	.
06361	G1P[8]	.	.	.	.	.	R	.	.	.	.	.	.	.	.	V	L	N	R	.	.	.	.
06361-CA	G1P[8]	.	.	R*	.	.	R	.	.	.	.	.	.	.	.	V	L	N	R	.	.	.	.
0613158	G1P[8]	.	.	.	.	.	R	.	.	.	.	.	.	.	.	V	.	N	.	.	.	.	.
0613158-CA	G1P[8]	.	V*	.	.	.	R	.	.	.	.	.	.	.	A	V	.	N	.	.	.	.	.
061060	G1P[8]	.	.	.	.	.	R	.	S	.	G	N	.	.	A	Y	.	N	.	.	.	.	F
61060-CA	G1P[8]	.	.	.	.	.	.	.	.	V*	.	.	S*	H*	.	V	L	N	R	.	.	.	.
0715880	G1P[8]	I	.	.	V	D	R	D	.	.	.	.	.	.	A	F	.	.	.	R	.	L	.
0715880-CA	G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	L	G*	R	.	P*	.	.

* Unique amino acid substitutions detected in the present study, aa: amino acid.

4. Discussion

The present study reports comparative analysis of the full-length nucleotide (2354) and deduced amino acid (775) sequences of VP4 genes of wild type and cell culture adapted G1P[8] rotavirus strains. VP4 gene is known to be associated with the cell attachment, penetration, neutralization and virulence of rotaviruses [5]. The changes in this gene have been described in *in vitro* studies [13,21]. The amino acid substitutions at positions 38 (G-S), 120 (M-T), 421 (S-N), 525 (D-G) and 618 (L-F) during cell culture adaptation have been found to be supportive for cultivation of human rotavirus strain, Wa in HepG2 liver cells [21]. The same number of amino acid substitutions, however, at different positions [51 (G-D),167 (L-F),331 (S-F),385 (D-T) and 695 (N-I)] have been identified in a study carried out on African green monkey kidney cell line unpassaged and 33 times passaged 89-12 rotavirus strain[21].

Recently, a G1P[8] rotavirus strain, MMC71 isolated in MA104 cells has shown 100% identity in VP4 gene with its wild type counterpart [22]. A rotavirus vaccine candidate, CDC-9 after passaging in Vero cells has revealed five amino acid substitutions at positions 51, 331, 364, 385 and 388 [13]. The strain, CDC-9 shared two sites (51 and 385) with the strain 89-12, however, due to unavailability of sequence data of this strain (CDC-9), amino acid substitutions remain to be identified. In the present study, the positions (33, 46, 60, 104, 113, 162, 183, 189, 237, 377, 387, 389, 480, 586, 587, 600, 615, 616, 621, 625, 644 and 672) of amino acid substitutions in the VP4 gene of cell culture adapted strains were different from those reported earlier. These data suggest the strain specific/host specific substitutions in the G1P[8] strains during cell culture adaptation. Similar observations have been made for the hepatitis A, dengue and porcine reproductive and respiratory syndrome viruses adapted to cell lines of different origins [23-28].

Amino acid substitution identified at position 260 (P-L), during *in vivo* passaging of porcine rotavirus strain, 4F has been described to be essential for its replication in piglets [29]. Unique amino acid substitutions- A46V, T60R, L237V/G389V/Q480H and S615G/ T625P identified in the present study may be important respectively for the growth of 0613158, 06361, 061060 and 0715880 strains in MA104 cell culture.

All of the G1P[8] study strains showed nucleotide substitutions spread over the VP4 gene without revealing any specific susceptible site during cell culture adaptation. Very recently, it has been demonstrated that a mutation, V391D in VP5* region of VP4 protein caused failure in the permeabilization process during infection of MA104 cells with rotavirus [30]. As a result mutated rotavirus particles carried reduced hydrophobicity and found 10 000 fold less infectious than those of parent type. In view of this observation more substitutions detected in the VP5* region of VP4 genes of cell culture adapted G1P[8] rotavirus strains of the present study need to be analyzed further for conformation and immunogenicity of the VP4 protein and finally for the comparative infectivity of the cell culture adapted and wild type rotavirus strains.

Amino acid substitutions associated with the neutralization resistant mutants selected by cross-reactive monoclonal antibodies have been described in the VP4 gene at positions 88,148,305,385,388,392,428,433 and 439 [5]. Such substitutions, however, were not identified in wild type and cell culture adapted study strains. This data may indicate the potential of these strains to elicit the neutralizing antibody response against VP4 protein. A point mutation at position 469 (Q-L) indicating increased pathogenicity of porcine rotavirus strain, 4F in piglets was absent in the study strains [26]. Similar observation has been made for asymptomatic and symptomatic human rotavirus strains and wild type and cell culture adapted CDC-9 strains [13].

Currently licensed rotavirus vaccines, Rotarix and RotaTeq are developed through serial passaging of the 89-12 and WC-3 strains, respectively. G1 and P[8] genotypes are important components of both vaccines. The study presented here reports nucleotide / amino acid substitutions in the VP4 genes of serially passaged G1P[8] rotavirus strains. Such strains need further evaluation for attenuation

and immunogenicity as they may have implications in the development of newer rotavirus vaccines.

Conflict of interest statement

We declare that we have no conflict of interest.

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