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RESEARCH ARTICLE

GENOTYPIC LINKAGES OF VP6 GENE OF HUMAN ROTAVIRUS ISOLATES CIRCULATING IN PEDIATRIC PATIENTS WITH ACUTE GASTROENTERITIS IN HARYANA AND COMPARISON OF ANTIGENIC EPITOPES WITH VACCINE STRAINS

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ABSTRACT

The major capsid protein, VP6 specify the sub-group specificity of Rotavirus and high titre of antibody are generated against VP6 gene. The Viral protein 6 (VP6), encoded by 6th segment of genomic double stranded RNA, is the main target for rotavirus detection both by serological methods and molecular techniques. A reverse transcription – PCR was standardized to amply the complete open reading frame (1194 bp) of human group A rotavirus VP6 gene. The genome segment 6 of local Rotavirus isolates from Haryana region were partially sequenced to analyze the variation within circulating rotaviruses and its epitopes were compared with that of vaccine strains.

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INTRODUCTION

Group A Rotaviruses (GARs) are one of the most important cause of viral gastroenteritis in infants and children below five years. It also cause diarrhea in neonates of a number of animal species as well. This rotaviral gastroenteritis occurs in approximately 114 million children and causes an estimated 4,53,000 deaths yearly (Tate *et al*., 2012). Most of these deaths occur in developing countries like India, South Asian and South African countries. In India alone, Rotavirus causes 4,57,000 to 8,84,000 hospitilization and 1,22,000 to 1,53,000 deaths yearly (Tate 2009). Since human and dairy animals share close proximity in India socio-demographic fabric, a high genetic diversity may be resulted due to continuous reassortment of segmented RNA. Rotaviruses belong to genus rotavirus within the *Reoviridae* family (Estes and Kapikian 2007). GAR is 70 nm in diameter, spherical, non-enveloped virus having triple layer of capsid coating. The 11 segments of double stranded RNA are encased in a core composed of VP1, VP2 and VP3. The major capsid protein (VP6) constitutes more than 50% of capsid protein (Estes and Kapikian, 2007) and forms the intermediate layer of capsid. A major titer of antibodies is directed against VP6 protein and this protein is targeted in most of the immunological assays (ELISA, Immunoflorescence and immunochromatography) for Rotaviral detection (Greenberg *et al*. 1983; Estes and Kapikian, 2007). On the basis of genetic data of VP6, rotaviruses have been classified into eight groups (A to H) (Matthijnssens *et al*., 2012). GARs are further subclassified into subgroups (SG) I, II, I+II and non-I, non-II based on their reactivity with VP6 specific array of monoclonal antibodies (Greenberg *et al*., 1983). Increase use of sequencing, has complemented serotyping methods and is commonly used. Rotavirus A genome segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are referred to as the Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx genotypes, respectively according to Rotavirus classification working group (Matthijnssens *et al*., 2008).

Conventional method of Rotavirus diagnosis includes ELISA and electron microscopy but sensitivity and specificity of ELISA is less as compared to molecular techniques. In addition, availability of monoclonal antibodies is inconsistent. Electron microscopy is time taking, cumbersome and expensive method. Therefore, molecular techniques offer an edge in GAR diagnosis and classification. Till date, 27 G and 37 P genotypes have been determined using nucleotide sequence based classification system (Matthijnssens *et al*., 2011; Trojnar *et al*., 2013). Nucleotide sequence of VP6 was

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utilized in designing of first molecular assay for Rotavirus detection in early 1990s (Grinde *et al*., 1995). Reverse transcription-PCR assays have been developed for determination of various VP6 genogroups of human rotavirus (Lin *et al*, 2008; Matthijnssens *et al*., 2012). Protein VP6 play an important role in organization of Rotavirus and act as a physical adapter between outermost shell and inner core. Presence of VP6 is essential for transcription process (Bican *et al*., 1982). Monoclonal antibodies generated against VP6 were shown to inhibit transcription process and to protect the mice from challenge infection (Burn *et al*., 1996).

Continuous molecular surveillance of VP6 nucleotide sequence is of prime importance in Indian subcontinent owing to diverse G and P genotypes variation reported (Jain *et al*., 2001; Ray *et al*., 2007; Kang *et al*., 2009; Ghosh *et al*., 2011). Such analysis will intimate in advance the potential emerging variations in local isolates that might eventually affect the sensitivity and specificity of VP6 based rotavirus detection and characterization methods. In this study, the genetic variations of VP6 of local rotavirus strains collected recently in Haryana, India were characterized and its epitopes compared against those of strains isolated from other part of the world and vaccine strains.

MATERIALS AND METHODS

Ethical Approval and sample collection

The research work was approved by Institutional Human Ethical Committee (PHY/HEC/13/419 dated 26/4/13). Ten children diarrheic stool samples were selected randomly from the previously screened ELISA rotavirus positive samples (Meridian Co., USA) collected from children presented with acute gastroenteritis in private clinics and laboratories during 2013-14. Stool suspensions of 10% (w/v) were prepared in Phosphate buffer saline (pH-7.4).

RNA extraction and PAGE

Total viral genomic RNA was extracted from 10% stool suspension using the RNeasy plus universal kit (Qiagen, Valencia, CA) as per the manufacturer protocol. The final elution was done in 30 µl of elution buffer. An aliquot of extracted RNA was analyzed on native PAGE after silver staining (Svensson *et al*., 1986)

VP6 RT-PCR and molecular cloning

Primers designed in our laboratory NCDV-VP6F (5'CGCGGATCCATGGATGTCCTGTAC3') and NCDV-VP6R (5'CCCAAGCTTTCATTTGACAAGCAT3') were used to amplify the complete open reading frame (1194 bp) of VP6 gene using Qiagen one step RT-PCR kit (Qiagen, Valencia, CA). Briefly, genomic RNA was denatured at 70°C for 10 minutes followed by reverse transcription at 50°C for 30 minutes. Reverse transcriptase was inactivated by heating at 95°C for 15 minutes. Thirty five cycles of 95°C for 30 sec, 58.5°C for 1 minute and 72°C for 1 minute, followed by final elongation step of 72°C for 10 minute was performed (Qiagen, Valencia, CA). The PCR products were gel purified, cloned in pGEM-T easy vector (Promega, Madison, USA) and transformed in competent DH-5α E. coli cells prepared by calcium chloride method (Sambrook and Russel, 2001)

Nucleotide sequencing and sequence analysis

Plasmid extracted from these clones were used for nucleotide sequencing by Automated DNA sequencer (ABI Prism). Genotyping on the basis of VP6 was done by RotaC [http://rotac.regatools.be/] (Maes *et al*., 2009). Nucleotide sequence of vaccine strains and other rotavirus strains was retrieved from Genbank. Nucleotide and deduced amino acid sequences were aligned using the MUSCLE program within MEGA version 6 (Tamura *et al*., 2013). Once aligned, JTT matrix based model (Jones *et al*., 1992) was used to construct maximum likelihood trees using MEGA version 6 with 500 bootstrap replicates to estimate branch support. Amino acid distance matrix was prepared using the p-distance algorithm in MEGA version 6 (Tamura *et al*., 2013). Structural analysis of VP6 (PDB 1QHD) were performed using the UCSF Chimera-Molecular Modeling System (Pettersen *et al*., 2004).

RESULTS

In the present study, three samples were taken randomly from earlier positive samples. Segmented RNA genomic segments got separated in a characteristic pattern of 4:2:3:2 which is unique to group A Rotavirus. All three strains exhibited long electropherotype (Figure 1).

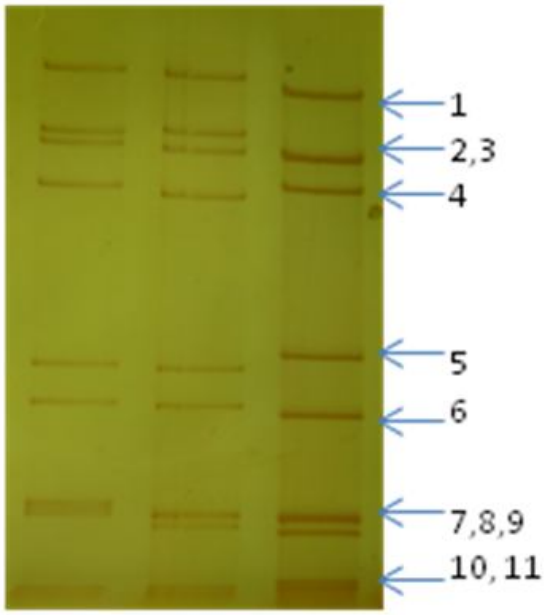


Figure 1. Electropherotype of genomic RNA of Haryana Rotavirus field strains HR-9, HR-11 & HR-48. All the three strains exhibited the typical pattern of 4:2:3:2 unique to group A Rotavirus with long electropherotype

After reverse transcription PCR full length ORF of VP6 gene was observed at 1194 bp (Figure 2). Negative control was run to rule out chances of cross contamination. Gel purified product was used for cloning in TA vector. Ampicillin was used as marker to select the recombinant clones.

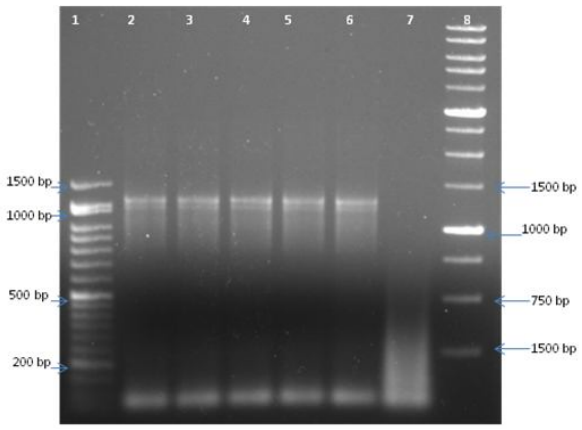


Figure 2. Agarose gel (2%) showing RT-PCR amplification of VP6 gene. Lanes 1: 50 bp Ladder; 2: HR-9; 3:HR-11; 4:HR48; 5:HR49, 6:HR-56; 7:Negative control; 8: 1 Kb ladder. DNA band of 1194 bp was observed whereas no band was observed in negative control

Colony touch PCR and plasmid profiling was done to confirm the presence of recombinant plasmid in the bacteria. The three isolates used in this study were already genotyped. Two isolates were of G9P [4] type while one was of G9P[8] type. (Using primers of Gouvea *et al.*, 1990; Iturriza-Gomara *et al.*, 2002) The partial VP6 sequences were submitted to the Genbank under accession numbers KJ804257, KJ855229 and KJ855230. Partial VP6 nucleotide and amino acid sequence of three RVA isolates (HRVA HR-9 G9P[4], HRVA HR-11 G9P[8] and HRVA HR-48 G9P[4]) were further analyzed. Genotyping of these isolates by RotaC [http://rotac.regatools.be/] indicated I2 genotype for all the three strains. Phylogenetic tree based on VP6 deduced amino acid consisting of the circulating Haryana strains as well as those of 116 E, Rotateq, Rotarix, reference human and animal strains is represented in Fig 3. All the three Haryana strains share more proximity with animal reference strain RVA/Sheep-tc/ESP/OVR762/2002/G8 [P14] (amino acid identity- 98.4-99.6%) as compared to human reference strain RVA/Human-tc/USA/DS-1/1976/G2[P4]

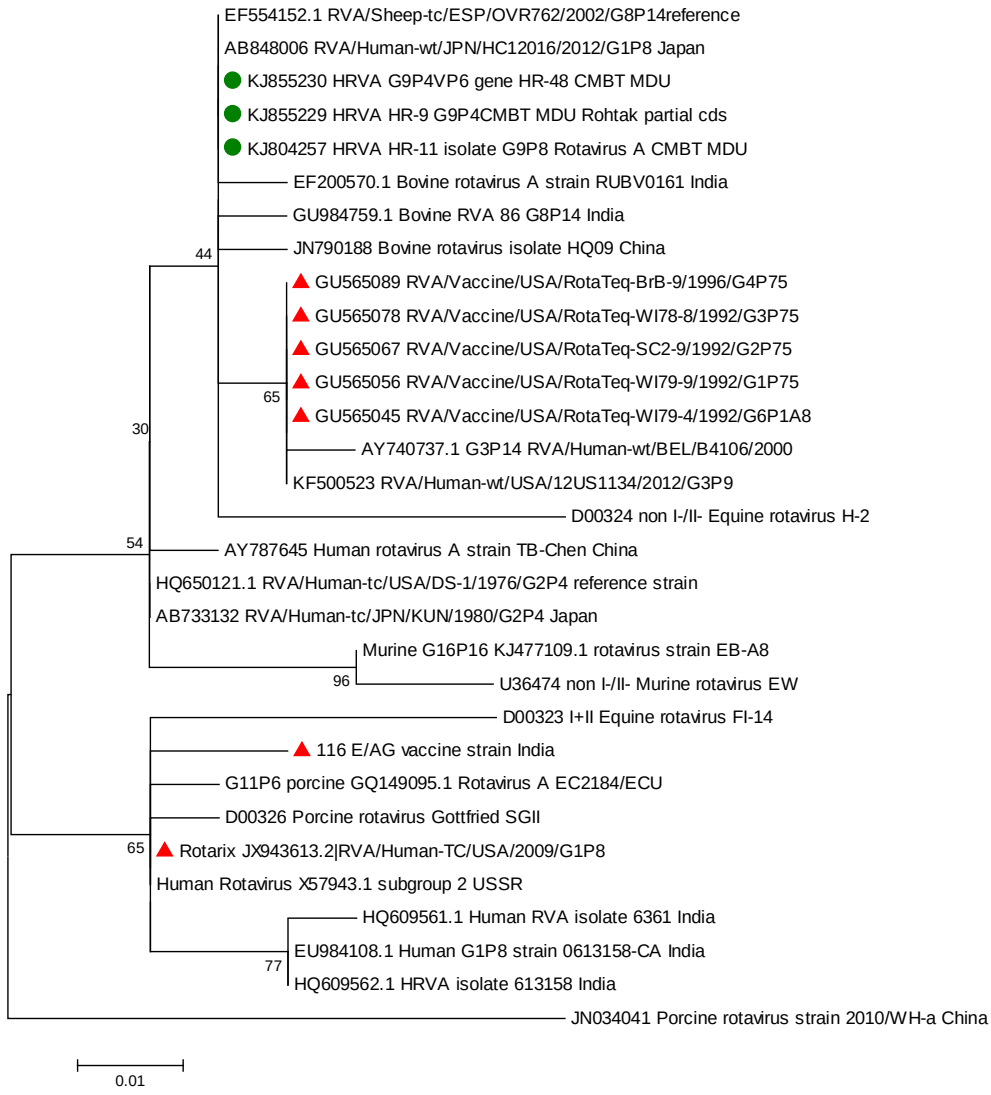


Figure 3. Maximum likelihood phylogenetic trees built in MEGA version 6.02 with a bootstrap value of 500, show the genetic relationships of amino acid sequences of VP6 of human strains from Haryana with vaccine strains and known human and animal rotavirus VP6 sequences from Genbank. Bars represent 0.01 substitutions per amino acid position. Haryana study strains are indicated by filled green circle while vaccine strains indicated by filled red triangle

(amino acid identity: 97.7-98.8%) as depicted in phylogenetic tree (Fig 3). Very interestingly, these local strains exhibited high amino acid identity (98.8-99.6%) with Bovine rotavirus A strain RUBV0161 and Bovine RVA 86 G8P[14] India as compared to Indian Human strains (91.6-93.7%) such as Human RVA isolate 6361, Human G1P[8] and HRVA isolate 613158. All the five strains of RotaTeq vaccine segment 6 share very high amino acid identity with Haryana strains (98.8-99.6%) while the percentage amino acid identity shared with Rotarix (91.6-94.9%) and 116E strain (92-94.1%) was slightly lower than RotaTeq. This respective distance with Rotarix and 116E strain is clearly depicted in phylogenetic tree (Fig 3). There are very few strains with subgroup I+II and non I-/II- reported. The Haryana strains [I2 genotype] HR-11 G9P[8] CMBT exhibited very low amino acid identity (66.9%) with a non I-/II- Equine rotavirus H-2. The I2 genotype Haryana strains shares 92.3-92.9% amino acid identity with subgroup I+II Equine rotavirus FI-14 and this distance is well depicted in phylogenetic tree.

Sequence comparison of VP6 proteins of Haryana strains, coupled with vaccine strains: RotaTeq, Rotarix and 116E, reference animal strain, reference human strain reveals several regions that are completely or highly conserved among Haryana strains. However, Haryana strains belonging to I2 genotypes were closely related to RotaTeq vaccine and reference animal strain.

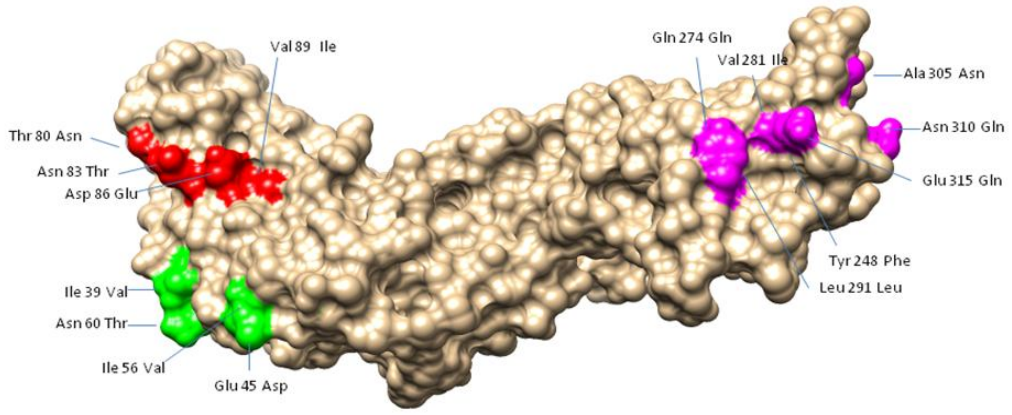
Comparison of subgroup specific antigenic regions of Haryana strains with vaccine strains shows that local strain HR-11 G9P[8] CMBT exhibits high degree of conservation in subgroup determining region with RotaTeq vaccine strains with only two amino acid substitution at positions 281 (Val>Ile) and 291 (Leu>Ser) as depicted in Fig 4-A. On the other hand, when compared with VP6 protein sequence of Rotarix vaccine (I1 genotype), four amino acid substitutions were observed at position 281 (Val>Ile), 305 (Ala>Asn), 310 (Asn>Gln) and 315 (Glu>Gln) as depicted in molecular model (Fig 4-B). Amino acid sequence from position 40 to 60 was found highly immunogenic and Haryana strains differs from Rotarix vaccine VP6 protein at three position: 45 (Glu>Asp), 56 (Ile>Val) and 60 (Asn>Thr). In variable region (aa position 80-89), four amino acid difference was observed between Haryana strains and Rotarix strain at position: 80 (Thr>Asn), 83 (Asn>Thr), 86 (Asp>Glu) and 89 (Val>Ile) as depicted in Fig 4-B.

DISCUSSION AND CONCLUSION

The VP6 protein in this study exhibited significant amount of genetic variations compared to Rotarix and 116E strain. The fact that the I2 genotype Haryana strains were more conserved with RotaTeq vaccine strains as compared to other vaccine strains available. Rotarix vaccine has been introduced in India and indigenous 116E will be available commercially very soon.

	Protective region				Variable region					Subgroup determining region						
	39	45	56	60	80	83	86	89	110	248	274	281	291	305	310	315
116 E/AG vaccine India	V	D	I	T	N	T	E	I	A	L	Q	I	S	N	Q	Q
HRVA HR-11 isolate G9P8	I	E	I	N	T	N	D	V	A	Y	Q	V	L	A	N	E
HRVA HR-9 G9P[4]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
HRVA HR-48 G9P[4]VP6	I	E	I	N	T	N	D	V	A	Y	H	I	S	A	N	E
RotaTeq-BrB-9/1996/G4P7[5]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
RotaTeq-WI78-8/1992/G3P7[5]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
RotaTeq-SC2-9/1992/G2P7[5]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
RotaTeq-WI79-9/1992/G1P7[5]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
RotaTeq-WI79-4/1992/G6P1A[8]	I	E	I	N	T	N	D	V	A	Y	Q	I	S	A	N	E
Rotarix RVA/Human-TC/USA/2009/G1P[8]	V	D	V	T	N	T	E	I	S	F	Q	I	L	N	Q	Q

[A]



[B]

Figure 4. (A) Alignment of antigenic residues in VP6 between the strains contained in RotaTeq, 116 E, Rotarix and Haryana strains. (B) Surface representation of the VP6 protein (PDB1QHD). Protective region is coloured in green, variable region in red and subgroup determining region in magenta colour

Role of antibodies against VP6 is not confirmed although some workers have reported protective role of anti-VP6 antibodies (Burn *et al*., 1996). High percentage of amino acid substitution at antigenic region in circulating strains may affect the efficacy of these vaccines. Haryana strains share high amino acid identity with bovine strains of Indian origin as compared to other Indian human strains. This may suggest that reassortant process have taken place due to close proximity of human and bovine in socioeconomic fabric of Haryana.

Subgroup I specificity was mapped to amino acid position 305 while subgroup II specificity was mapped to amino acid position 315. Substitution at these positions may affect the binding of monoclonal antibodies 255/60 (for SG-I) & 631/9 (for SG-II). Earlier reports of VP6 protein using site directed mutagenesis (Tang *et al*., 1997) or recombination (Lopez *et al*., 1994) have demonstrated that substitutions at positions 296 to 299, 305, 306, 308, and 315 could change the reactivity to the SG I- and SG II-specific MAbs. Alanine residue at position 172 also attribute to formation of SG-1 specific epitope; however point mutations at this position resulted in low reactivity in immunoprecipitation assay with SG –I specific monoclonal antibodies (Tang *et al*., 1997). Our findings are in accordance with the earlier report as amino acid variance has been reported between Haryana strains and Rotarix strain at position 305, 310, 315. The possible effects of these reported genetic changes on the host disease susceptibility demands further understanding. The VP6 genes from these circulating strains may be gradually evolving at different rates compared to other rotavirus gene segments, due to accumulation of antigenic changes caused by point mutations and reassortments. This was clearly shown by clustering of Haryana human strains with bovine strains of Indian origin. However, amino acid changes reported here were in accordance with different studies (Ghosh and Kobayashi, 2011; Matthijnssens *et al*., 2012). These amino acid variations may lead to change in VP6 epitopes. These changes could affect the potency of the Rotavirus detection assays. Although the sample size was tiny, the findings were in consistence with those reported elsewhere (Matthijnssens *et al*., 2012). The current VP6 detection methods should work expeditiously as the changes ascertained within the antigenic regions of these Haryana strains looks subltle. These results suggest that rotavirus investigators ought to continually take into account this continuous variation and update the primers needed for the detection and characterization of major capsid protein accordingly.

Acknowledgement

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Ethical Clearance

The research work was approved by Institutional Human Ethical Committee (PHY/HEC/13/419 dated 26/4/13).

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