

Full Length Research paper

# Intragenotypic diversity in the VP4 encoding genes of rotavirus strains circulating in adolescent and adult cases of acute gastroenteritis in Pune, Western India: 1993 to 1996 and 2004 to 2007

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Genetic variability of rotaviruses circulating in Pune, India at the two time points was determined by characterizing VP4 genes in 131 rotavirus strains detected in adolescent and adult cases of acute gastroenteritis. The multiplex RT-PCR classified the VP4 genes in 73 P[4] (43.2%), 69P[8] (40.8%) and 27 P[6] (16.0%) genotypes. Sequencing and phylogenetic analysis revealed increase in the prevalence of P[4]-5 and P[8]-2 and decline of P[8]-3 lineages in 2000s as compared to those identified in 1990s (92.8% Vs 100%, 4.2% Vs 33.3% and 93.7% Vs 66.7%, respectively). The P[4]-1 and P[8]-4 lineages circulated at low levels (7.1% / 2.1%) while presence of only P[6]-1 (100%) lineage was detected at both time. The strains with different VP4 genotype specificities displayed 0.2 to 2.3% amino acid divergence. A significant difference ( $P < 0.01$ ) in their association with common and nontypeable G strains was noted between the two time points studied. This is the first report to describe the intragenotypic diversity in the rotavirus VP4 genes from adolescent and adult patients of acute gastroenteritis from India. VP4 being one of the major protective antigens, monitoring the mutations in this protein would be crucial to understand the evolutionary changes in rotaviruses and devise more effective vaccine strategies in developing countries.

**Key words:** Group A rotavirus, VP4 genotypes, G and P typing, genetic diversity.

## INTRODUCTION

Rotaviruses are well-recognized causative agents of severe gastroenteritis among infants and children, worldwide (Parashar et al., 2006). Taxonomically, these viruses belong to the family Reoviridae whereas in morphological analysis they appear as icosahedral particles consisting of 11 segments of double stranded (ds) RNA encased within a triple layered capsid composed of VP4, VP6, VP7 and VP2 proteins. The outermost layer has two proteins: the spike protein VP4 (P-protease sensitive) and the coat protein VP7 (G-glycoprotein) (Estes, 2001). Based on these proteins 27 G and 35 P types have been identified globally (Matthijnssens et al., 2008; Matthijnssens et al.,

2011).

More than sixty different G-P combinations have been found in the rotavirus strains circulating worldwide. Of these 12G and 15P genotypes have been detected in humans (Patel et al., 2011). The most commonly found G and P types are G1, G2, G3, G4, G9, P[4] and P[8], respectively (Santos and Hoshino, 2005; Matthijnssens et al., 2008). Of these most of the VP7 genotypes but only one VP4 genotype have been included in currently licensed rotavirus vaccines. It is expected that the implementation of the vaccination programs reduce significantly the hospitalizations and mortality related to rotavirus infection. However, existence of vast variations in the rotavirus strains and genotypes causing gastrointestinal infections is evident from the rotavirus surveillance studies conducted worldwide including India (Santos and Hoshino, 2005; Kang et al., 2009; Tatte et al., 2010a; Pietsch et al., 2011). Intragenotypic diversity

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in VP7, NSP4 and VP6 encoding genes of such strains has been also reported from India (Arora et al., 2009; Tatte et al., 2010b). However, variations within VP4 genotypes circulating in the Indian population have been rarely reported (Samajdar et al., 2008).

A recent molecular epidemiology study carried out in adolescent and adults has reported an increase of mixed infections and increasing trend of genetic reassortment among rotavirus strains (Tatte et al., 2010a). Since adults frequently infected with rotavirus could act as reservoir of infection in early ages, more studies on the diversity within the rotavirus genotypes are necessary. The present study describes the assessment of temporal variations in the VP4 encoding genes of rotavirus strains recovered from adolescents and adults.

## MATERIALS AND METHODS

### Specimens

In earlier studies conducted at the two time points (1993 to 1996 and 2004 to 2007) in Pune, India for rotavirus surveillance in adolescent (9 to 18 years) and adult (>18 years) cases of acute gastroenteritis, a total of 131 fecal specimens were detected positive for group A rotavirus by ELISA (n = 118) (Tatte et al., 2010a) and RNA PAGE (n = 13). These consisted 26 (18/8) and 105(66/39) specimens respectively from adolescents and adults from the years, 1993 to 1996 (n = 84) and 2004 to 2007 (n = 47). Typing of rotavirus VP7 genes by multiplex PCR showed multiple G genotypes in the rotavirus strains. In the present study VP4 genes of these strains were scrutinized to classify them into lineages and sublineages. These also included VP4 genes from rotavirus strains (n = 31) detected in mixed infections reported earlier (Tatte et al., 2010a).

### RNA extraction, RT-PCR and genotyping

Rotavirus ds RNA was extracted from 10% stool suspension prepared in phosphate buffered saline containing calcium chloride (PBS-CaCl<sub>2</sub>) of pH 7.2, using Trizol® LS reagent (Invitrogen, Carlsbad, CA, USA), as per the manufacturer's protocol. The VP4 genes of rotavirus strains were subjected to RT-PCR followed by multiplex PCR for genotyping according to the method described earlier (Gouvea et al., 1990; Gentsch et al., 1992; Ituriza-Gomara et al., 2004) using a modified thermal cycling program (Chitambar et al., 2008). All of the PCR products, were analyzed by electrophoresis using 1X Tris Acetate EDTA (TAE) buffer, pH 8.3 on 2% agarose gels, containing ethidium bromide (0.5 ug/ml) and visualized under UV transilluminator.

### Nucleotide sequencing and phylogenetic analysis

The multiplex PCR products obtained for different VP4 genotypes (P[8] – 345 bp, P[4] – 483 bp and P[6] – 267 bp) were purified on minicolumns (QIAquick, Qiagen, Hilden, Germany) and sequencing was carried out using ABI-PRISM Big Dye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster city, CA) and a ABI-PRISM 310 Genetic Analyzer (Applied Biosystems Foster city, CA). The sequences were aligned with the corresponding sequences of rotavirus strains available in the GenBank by using Clustal W (Thompson et al., 1994). The phylogenetic analysis was carried out in MEGA 4 by using Kimura –2 parameter and

neighbour-joining method (Tamura et al., 2007). The reliability of different phylogenetic groupings was confirmed by using bootstrap test (1000 bootstrap replications) available in MEGA 4.

### Accession numbers

Nucleotide sequences of the VP4 genes of this study have been deposited in the GenBank sequence under the accession numbers HQ260459-HQ260577 and FJ6323188-FJ623237.

### Statistical analysis

The proportions across two different periods were compared using the Chi-square test with Yates's correction. P-values less than 0.05 were considered statistically significant.

## RESULTS

### P-typing of rotavirus strains

In a multiplex RT-PCR performed for amplification of VP4 genes of 131 rotavirus strains, a total of 169 amplicons were obtained indicating 74 single and 95 mixed rotavirus infections. Based on the electrophoretic mobilities of the PCR products obtained in multiplex PCR, VP4 genes were classified into 73 P[4] (43.2%), 69 P[8] (40.8%) and 27 P[6] (16.0%) genotypes. All of these VP4 genotypes were found to be in combination with different G genotypes (Table 1).

### Nucleotide sequencing

All of the multiplex PCR products were sequenced to confirm the presence of P[4] / P[8] / P[6] genotypes of VP4 in single or mixed infections. The sequences were compared with those available in GenBank.

### Phylogenetic analysis of P[4] strains

Phylogenetic analysis of the 73 P[4] strains recovered from 10 adolescents and 63 adults at two time points 1993 to 1996 (n = 41) and 2004 to 2007 (n = 32) showed clustering of the strains in two distinct lineages P[4]-5 and P[4]-1 of the five lineages known to date for this genotype (Figure 1).

All of the 70 strains placed in P[4]-5 lineage showed 95.3 to 99.5% / 94.8 to 100% and 95.9 to 100% / 96.1 to 100% nucleotide / amino acid identities respectively with the reference strains-TB-Chen, RMC/G66, 107EIB, Py04ASR42, Py05ASR60, KO-2, VN594, SC-185 and TW569 and within themselves (Figure 1). These strains were predominantly associated with mixed infections (n = 24, 34.3%) followed by G2 strains (n = 21, 30.0%), G non-typeable strains (n = 18, 25.7%) and unusual G types [G1 (n = 3, 4.3%), G3 (n = 1, 1.4%), G4 (n = 1, 1.4%) and G9 (n = 2, 2.9%)]. All three strains classified

**Table 1.** Distribution of rotavirus VP4 genotype combinations in different G types identified in 1993 to 1996 and 2004 to 2007.

G-P Types (%)	P[4]		P[8]		P[6]	
	No. positive (%)					
	1993-1996	2004-2007	1993-1996	2004-2007	1993-1996	2004-2007
Common n = 47 (27.8)	n = 17(40.5)	n = 4 (12.9.0)	n = 25(52.1)	n = 1(4.8)	n = 0(0.0)	n = 0(0.0)
G1 (38.3)	0(0.0)	0(0.0)	17(35.4)	1(4.8)	0(0.0)	0(0.0)
G2 (44.7)	17(40.5)	4(12.9)	0(0.0)	0(0)	0(0.0)	0(0.0)
G3 (10.6)	0(0.0)	0(0.0)	5(10.4)	0(0)	0(0.0)	0(0.0)
G4 (6.4)	0(0.0)	0(0.0)	3(6.3)	0(0)	0(0.0)	0(0.0)
Unusual n = 12(7.1)	n = 0 (0.0)	n = 7(22.6)	n = 1(2.1)	n = 0(0.0)	n = 4(16.7)	n = 0(0)
G1 (25.0)	0(0.0)	3(9.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
G2 (8.3)	0(0.0)	0(0.0)	1(2.1)	0(0.0)	0(0.0)	0(0.0)
G3 (8.3)	0(0.0)	1(3.2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
G4 (8.3)	0(0.0)	1(3.2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
G9 (50.0)	0(0.0)	2(6.5)	0(0.0)	0(0.0)	4(16.7)	0(0.0)
Mixed n = 67(39.6)	n = 21(50.0)	n = 6(19.4)	n = 17(35.4)	n = 5(23.8)	n = 17(70.8)	n = 1(33.3)
G1 (31.3)	6(14.3)	1(3.2)	7(14.6)	1(4.8)	6(25.0)	0(0.0)
G1, G2 (13.4)	3(7.1)	1(3.2)	2(4.2)	1(4.8)	2(8.3)	0(0.0)
G1 G4 (1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(4.2)	0(0.0)
G2 (5.9)	1(2.4)	1(3.2)	0(0.0)	1(4.8)	1(4.2)	0(0.0)
G2, G4 (4.4)	1(2.4)	1(3.2)	0(0.0)	1 4.8)	0(0.0)	0(0.0)
G2, G3 (4.4)	1(2.4)	0(0.0)	1(2.1)	0(0.0)	1(4.2)	0(0.0)
G3 (4.4)	1(2.4)	0(0.0)	1(2.1)	0(0.0)	1(4.2)	0(0.0)
G4 (14.9)	4(9.5)	0(0.0)	4(8.3)	0(0.0)	2(8.3)	0(0.0)
G9 (12.0)	2(4.8)	2(6.4)	0(0.0)	1(4.8)	2(8.3)	1(33.3)
G1, G2, G4 (7.5)	2(4.8)	0(0.0)	2(4.2)	0(0.0)	1(4.2)	0(0.0)
Nontypeable n = 43 (25.4)	n = 4(9.5)	n = 14(45.1)	n = 5(10.4)	n = 15(71.4)	n = 3(12.5)	n = 2(66.7)
GNT (100)	4(9.5)	14(45.1)	5(10.4)	15(71.4)	3(12.5)	2(66.7)
Total	42	31	48	21	24	3

in the lineage P[4]-1 were closer to each other and with the prototype strain, DS-1 in nucleotide (96.6 to 100%) and amino acid (97.7 to 100%) sequences.

Differences within all P[4] strains by 5.4 to 6.4% / 3.7 to 6.0% and 2.5 / 2.3% in their nucleotide / amino acid sequences from the prototype strain, DS-1 of lineage P[4]-1 were observed during the 1990s and 2000s. The alignment of the partial deduced amino acid sequences (11-153 aa) showed variations at positions 32(S→N), 89(N→D), 99(N→S), and 120(I→V) as compared to the prototype strain, DS-1. Multiple strain specific changes were also shared by these strains at positions 35(V→I), 49(N→D), 113(T→A), 130(V→I) and 133(N→S).

### Phylogenetic analysis of P[8] strains

The nucleotide sequence analysis of the 69 P[8] strains recovered from adolescents (n = 14) and adults (n = 55)

at two time points, 1993 to 1996 (n = 48) and 2004 to 2007 (n = 21) showed clustering of the strains in three distinct evolutionary lineages P[8]-2, P[8]-3 and P[8]-4 of a total of four lineages described to date for this genotype (Figure 2).

All of the 59 strains classified in P[8]-3 lineage showed 97.5 to 100% / 96.1 to 100% and 96.0 to 100% / 94.7 to 100% nucleotide / amino acid identities respectively with the reference strains- Kagawa/90-544, Kagawa/90-513, Hun 9, OP351, 27B3, Dhaka 25-02, ISO22, PA 25/03, 90-551 and CAU and within themselves. All these strains were predominantly associated with commonly detected rotavirus G genotypes [(25/59, 42.4 %); G1 (17/25, 68.0%), G3 (5/25, 20.0%) and G4 (3/25, 12.0%)] followed by mixed infections (20/59, 33.9%) and G-nontypeable strains (13/59, 22.0%). Only one of the strains (939961) showed unusual combination with rotavirus genotype G2 (1/59, 1.7%).

Four of the 9 strains grouped in lineage P[8]-2 were



**Figure 1.** Phylogenetic tree based on partial nucleotide sequences of the VP4 (P[4]) gene (nt 43 to 456) of rotavirus strains recovered from adolescents and adults in 1993 to 1996 and 2004 to 2007. The strains of the present study are identified by the specimen number, followed by the genotypes identified in multiplex PCR. The reference strains are indicated by accession numbers followed by the strain names.

closer to the prototype strain, F45 with 95.7 to 96.8% and 96.7 to 98.9%, nucleotide and amino acid identities respectively. Within these strains nucleotide and amino acid identities were noted to be 96.4 to 100% and 97.8 to

100% respectively. Remaining five strains showed 100% identity with the KU strain at nucleotide and amino acid level. Majority of the strains (7/9) from this lineage were associated with non-typeable G genotypes.



1996 and 2004 to 2007 differed respectively by 6.6 to 8.2% / 4.7 to 6.4% and 3.0% / 2.2% in their nucleotide / amino acid sequences from the prototype strain, Wa of lineage P[8]-1 and from each other. The alignment of the partial deduced amino acid sequences (11-103aa) showed variations at positions 19(H→Y), 35(I →V), 38(S→G), 64(M→I) and 78(N→T) as compared to the prototype strain, Wa. These strains also showed strain specific changes at 20(T→I), 71(P→S), 75(T→K) and 77(P→L) positions.

### **Phylogenetic analysis of P[6] strains**

Phylogenetic analysis of 27 P[6] strains recovered from adolescents (n = 3) and adults (n = 24) at two time points 1993 to 1996 (n = 24) and 2004 to 2007 (n = 3), showed clustering of all of the strains in lineage P[6]-I (Figure 3). All strains showed 94.1 to 100% and 93.9 to 100% nucleotide and amino acid identities respectively with the reference strains, M37, ST-3, SC2, MtA5, RV176, US1205 and Se585. Within these strains 96.1 to 100% nucleotide and 95.5 to 100% amino acid identity was noted. Nucleotide and amino acid divergence respectively of 0.4 to 2.3% and 0.2 to 1.6% was detected between the strains from 1993 to 1996 and 2007. The alignment of the partial deduced amino acid sequences (11-80 aa) showed variations at positions 30(T→S), 73(S→N) and 78(S→N) as compared to the prototype strain, M37.

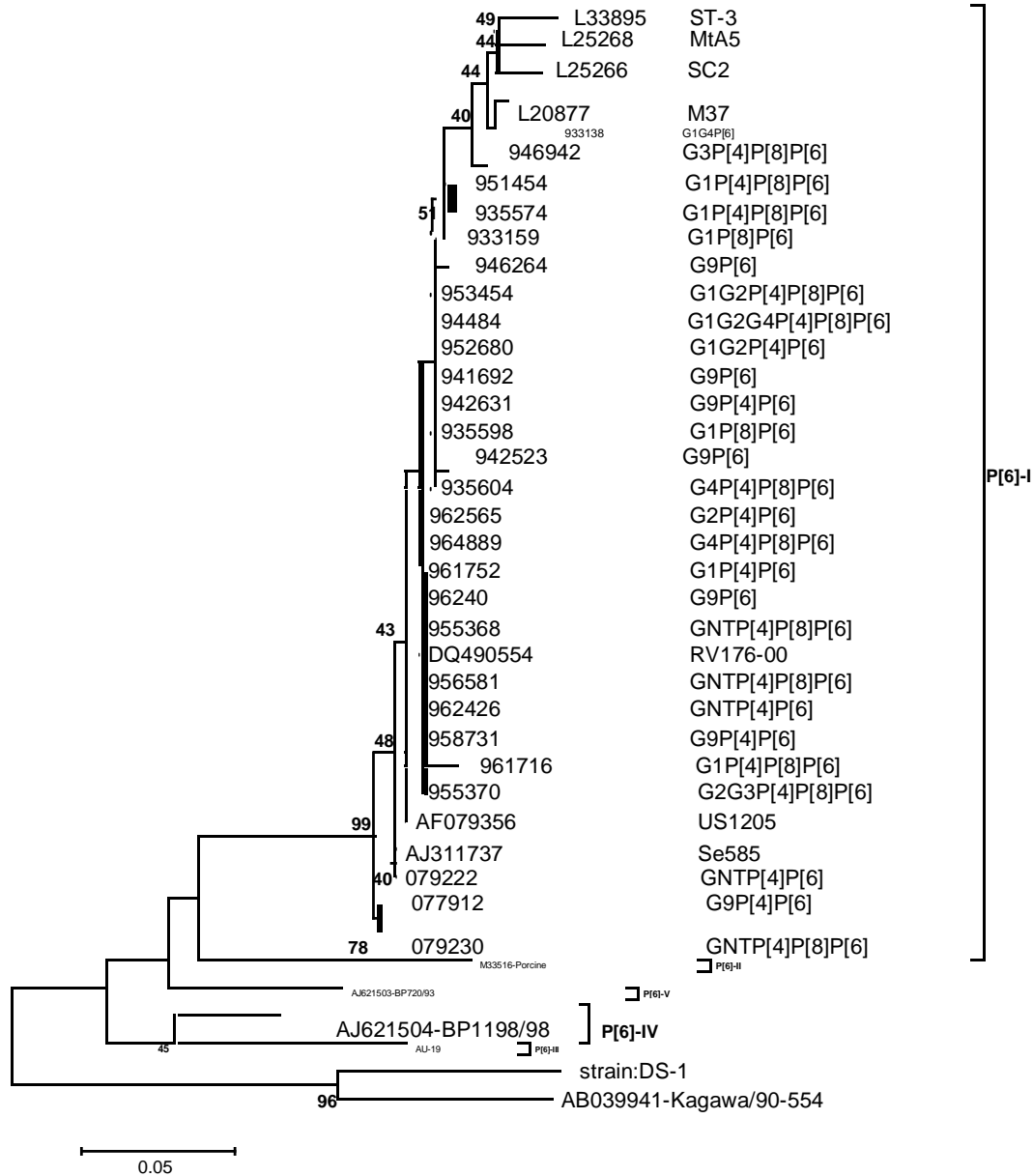
## **DISCUSSION**

Epidemiological surveys carried out in the late 1970s for the rotavirus strain surveillance used monoclonal antibodies. Though these studies established circulation of G1 to G4 serotypes in Europe, North America and Australia they also reported predominance of nontypeable rotavirus strains in South America, Africa and Asia (Desselberger et al., 2001). A complete assessment highlighting complex picture of the virus emerged with the availability of typing methods for both VP7 (G) and VP4 (P) genes (Gouvea et al., 1990; Gentsch et al., 1992). Rotaviruses identified to have different G and / or P types have indicated that a diverse gene pool contributes to human infections (Desselberger et al., 2001). Further, studies on genetic and antigenic variations in the rotavirus strains of the same genotypes have revealed intragenotypic diversity and rotavirus gene classification on the basis of lineages and sub lineages (Gouvea et al., 1999; Araujo et al., 2007; Espinola et al., 2008). Circulation of different lineages within the P[4], P[8] and P[6] genotypes of rotavirus VP4 genes isolated from UK, Hungary, Brazil and Paraguay (Itturiza-Gomara et al., 2001; Banyai et al., 2004b; Araujo et al., 2007; Espinola et al., 2008) has been reported. However, such

data from India is limited to rotavirus strains recovered from children (Samajdar et al., 2008; Zade et al., 2009). This study analyzes partial sequences of VP4 genes of rotavirus strains recovered from adolescents and adults at two different time points (1993 to 1996 and 2004 to 2007).

Varying frequencies of P[4] genotypes have been reported during rotavirus surveillance conducted in Brazil (21 to 26%), Sao Paulo (1%), India (33%), Korea (15.4%), Tunisia (13%) and Bangladesh (43%) (Araujo et al., 2002; Mascarenhas et al., 2002; Carmona et al., 2006; Volotao et al., 2006; Samajdar et al., 2006; Araujo et al., 2007; Chouikha et al., 2007; Le VP et al., 2008; Paul et al., 2010). Occurrence of five (P[4]-1 to P[4]-5) different lineages of P[4] genotype has been described on the basis of analysis of >100 strains circulating worldwide (Espinola et al., 2008). Three lineages P[4]-1, P[4]-4 and P[4]-5 have been described to combine with either of the G2, G3 and G9 types of VP7 gene. The remaining two lineages, P[4]-2 and P[4]-3 were found in association with G8 and G12 genotypes, respectively (Espinola et al., 2008). As against this, in the present study, P[4]-5 lineage clustered almost in equal proportion with the strains of G2 specificity and mixed / unusual infections / nontypeable G types. These results differed from the recent findings on clustering of G2P[4] strains in the P[4]-2 lineage from Thailand (Khamrin et al., 2010). The P[4] - 1 lineage described in the present study clustered only mixed G and / or P infections (3/73). Interestingly, a comparison between the two time points, 1993 to 1996 and 2004 to 2007 showed a significant difference (P<0.01) in the frequencies of association of P[4] with common and nontypeable G strains (Table 1).

Genotype P[8] has been found to have undergone genetic and antigenic drift (Jin et al., 1996) with cocirculation of four distinct P[8] lineages, globally (Gouvea et al., 1999; Cunliffe et al., 2001; Arista et al., 2005, 2006; Ansaldi et al., 2007; Espinola et al., 2008). Several studies have revealed cocirculation of mainly P[8]-1 and P[8]-2 lineages in the late 80's and early 90's and predominant circulation of P[8]-3 lineage in the 2000s (Cunliffe et al., 2001; Itturiza-Gomara et al., 2001; Arista et al., 2005; Araujo et al., 2007; Espinola et al., 2008; Le VP et al., 2010; Tort et al., 2010). In the present study P[8]-3 predominated at both time points (93.7% / 66.7%), thus indicating circulation of this sublineage in Pune, India since 1990s. Cocirculation of P[8]-2 and / or P[8]-4 was also noted at both time points with increase in the circulation of P[8]-2 lineage from 4.2% in 1990s to 33.3% in 2000s. Although P[8]-3 lineage grouped majority of the strains of present study, its association with G genotypes differed with time. In the 1990s it was predominantly associated with common G types (G1, G3 and G4) (25/45, 55.6%) followed by unusual and mixed infections with G and / or P types (16/45, 35.5%) and G nontypeable strains (4/45, 8.9%). On the contrary, strains (n=14) from 2000s associated predominantly with



**Figure 3.** Phylogenetic tree based on partial nucleotide sequences of the VP4 (P[6]) gene (nt 40 to 249) of rotavirus strains recovered from adolescents and adults in 1993 to 1996 and 2004 to 2007. The strains of the present study are identified by the specimen number, followed by the genotypes identified in multiplex PCR. The reference strains are indicated by accession numbers followed by the strain names.

nontypeable G types (9/14, 64.3%) followed by mixed G and / or P types (5/14, 35.7%). These results differed from the findings documenting association of P[8]-3 lineage predominantly with common strains (Min et al., 2004; Araujo et al., 2007; Espinola et al., 2008; Samajdar et al., 2008; Le et al., 2010). It was also interesting to note that the common strains (G1, G3 and G4) from the 1990s of this study clustered in the P[8]-3 lineage and that they differed from the contemporary strains of the same types that clustered in P[8]-1 or P[8]-2 lineages (Iturriza-Gomara et al., 2001; Arista et al., 2005; Espinola

et al., 2008). The existence of constraints on the G9 strains circulating worldwide to belong to the P[8]-3 lineage (Iturriza-Gomara et al., 2001; Banyai et al., 2004a) was also noted in this study.

Circulation of P[8]-2 lineage reported at different time points has displayed clustering of rotavirus strains with different G types (G1-G4, G5, G8 and G9) (Iturriza-Gomara et al., 2001; Araujo et al., 2007; Espinola et al., 2008, Samajdar et al., 2008). The rotavirus strains detected in children from Vietnam, Brazil and Bangladesh have shown association of P[8]-2 lineage with common



(G1, G2, and G9) and mixed G strains (Araujo et al., 2007; Nguyen et al., 2008; Dey et al., 2009). In the present study this lineage clustered G non-typeable strains predominantly. However, it is possible that the nontypeable strains could be commonly circulating strains that remained undetected due to less viral load or genomic variations.

OP354 like lineage designated as P[8]-4 has been described to group G1, G3, G4, G9, G1G9, G1G4 and G3G9 strains from different countries (Cunliffe et al., 2001; Banyai et al., 2004a; Nguyen et al., 2008; Samajdar et al., 2008; Nagashima et al., 2010). The association of this lineage with triple P (VP4) type rotavirus infection (G1, P4, P8, P6) was noted for the first time in this study. A significant difference ( $P < 0.01$ ) in the frequencies of association of P[8] with common and nontypeable G strains was evident between the two time points examined in this study (Table 1).

The P[6] genotype previously thought to be restricted to asymptomatic infections has also been identified in combination with G1-G4, G5, G8, G9, G11 and G12 genotypes detected in neonates and children with diarrhea (Adah et al., 1997; Pager et al., 2000; Cunliffe et al., 2002; Mascarenhas et al., 2002; Steele and Ivanoff, 2003; Page and Steele, 2004; Mascarenhas et al., 2006; 2007; Ahmed et al., 2007; Nguyen et al., 2007; Le et al., 2008; Banyai et al., 2009; Mukherjee et al., 2009; Shim et al., 2010). However, no data is available from adolescents and adults. Interestingly, majority of the strains with P[6] genotype in the present study were detected in the mixed infections and placed in lineage P[6]-I (M37 like).

In summary, the findings of this study highlight the intragenotypic diversity and temporal variations in rotavirus VP4 genes. Although it is known that homotypic and heterotypic immune response could be elicited by VP4, antigenic differences in various P[6] lineages identified in human and porcine species have been reported to generate differential neutralizing antibody response (Gorziglia et al., 1990; Li and Gorziglia, 1993; Nakagomi et al., 1999). In this context, it will be important to monitor alterations in the epitopes of P[8] and P[4] rotavirus strains grouped in different lineages and evaluate corresponding antibody responses as VP4 is a major protective antigen. Currently available rotavirus vaccines introduced mainly for the pediatric population constitute VP4 of P[8] genotype. In view of this, vigilance on the intragenotypic diversity in VP4 genes would provide a key to understand the evolution and genetic / antigenic differences in the rotavirus genotypes and assess the need for improvement in rotavirus vaccines.

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