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Characterisation of rotavirus strains identified in adolescents and adults with acute gastroenteritis highlights circulation of non-typeable strains: 2008–2012

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ABSTRACT

Background: Group-A Rotavirus (RV) is the main causative agent of acute gastroenteritis in children < 5 years of age. Its role as a pathogen in adults needs to be monitored. The aim of this study was to characterise the group-A RV strains that cause infections of acute gastroenteritis in adolescents and adults and determine the temporal variations in the circulating strains during 2008–2012 in continuation of an earlier study conducted in 2004–2007, in Pune, India.

Methods: A total of 371 stool samples were tested by RV antigen capture ELISA. VP4, VP6, VP7 and NSP4 genes of all of the RV strains detected in the study were analysed using reverse transcription PCR, multiplex PCR and sequencing.

Results: Group-A RV was detected in 9.4% (35/371) of the stool samples examined in the study period. The frequency of detection of RV was found to decline from 18.0% (16/90) in 2008 to 3.8% (2/52) in 2012. Of the 6 strains typed for both VP7 and VP4 genes, G2P[4], G1P[8] and G9P[4] were detected in 3, 1 and 2 samples, respectively. Sequencing and phylogenetic analysis of the VP4, VP6, VP7 and NSP4 genes revealed an infrequently reported NSP4-E6 genotype and circulation of heterogenous [G2 (lineage IIC and IID), G9 (lineage 3), P[4] (lineage P[4]-5), P[8] (lineage P[8]-3), VP6 I1 / I2 and NSP4 E2] genotypes/lineages in the RV strains. Analysis of linkage within these genes showed concordance (G2-P[4]-I2-E2) and discordance (G9-P[4]-I2-E6), equally. The sequences of amplified VP6 (n = 20) and NSP4 (n = 2) genes from G and P nontypeable RV strains (80.0%, 28/35) were most homologous to human group-A RV strains.

Conclusion: The study underscores the significant temporal variations in RV strains, identifies circulation of intergenogroup reassortants among adolescent and adult patients with acute gastroenteritis and emphasizes the need for continued surveillance and whole genome analysis of emerging rotavirus strains.

1. Introduction

Group-A Rotaviruses (RV) are the most important etiologic agents of acute gastroenteritis in infants and young children, worldwide. Globally, group-A RV infections account for 37% of all cases of diarrhoea and 4,53,000 deaths per year in children under the age of 5 years [1]. RV has been less appreciated as a pathogen of adults, although cases of rotavirus gastroenteritis have been identified in elderly and immunocompromised individuals [2–4]. In healthy adults, infection usually causes few or mild symptoms. However, in immunocompromised patients, infection can be severe and persistent, with patients presenting with vomiting, malaise, abdominal pain, diarrhoea and fever [2].

RVs belong to the family Reoviridae, and are classified in eight antigenic groups (A–H), of which, groups A, B and C are known to infect humans. The virus carries a genome of 11 segments of double-stranded RNA (dsRNA) encoding six structural (VP1–VP4, VP6 and VP7) and six non-structural (NSP1–NSP6) proteins. The two outer-layer proteins VP7 and VP4 form the basis of the current dual classification system of RVA into G and P genotypes [5]. To date, at least 27 G (G1–G27) and 37 P (P[1]–P[37]) genotypes of group-A RV have been identified globally, with various combinations of G and P genotypes [6–8]. However, only the five most common types (G1–G4, P[8]) have been targeted in the RV vaccines.

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In order to assess the impact of vaccines on circulation of wild type strains, long-term surveillance for group-A RV infections and strains have been conducted in several countries [9–11]. In India epidemiological, clinical and virological features of RV infections occurring among children (<5 years) with acute gastroenteritis have been well studied [12–14]. Such studies are also essential in higher age groups for better understanding of RV spread in the community.

In our earlier study carried out to characterise RV infections in adolescents and adults, a rise in RVA infections in 2004–2007 as compared to 1993–1996 was reported [15]. Infections with uncommon G-P and mixed infections were higher in these age groups when compared to those in children. In the present study, the surveillance of RV infections was continued in the same age groups of patients with acute gastroenteritis to understand the temporal variations in the rate of RV infections and the strains during the 5 year period, 2008–2012.

2. Material and Methods

2.1. Specimens

A total of 371 stool specimens were collected from adolescent (10–18 years) and adult (>18 years) cases of acute gastroenteritis, admitted to or visiting out-patient departments of local hospitals from Pune city during 2008–2012. The study was approved by the ethical committee of the National Institute of Virology. Epidemiologic data including age, gender, dates of diarrhoea onset and specimen collection were available from all patients.

Ten percent (w/v) stool suspension of each of the specimens was prepared in 0.01 M phosphate buffered saline (PBS), pH 7.2 containing 0.01 M $CaCl_2$. The suspensions were centrifuged at 805 g for 15 min to remove debris. The supernatants were stored in aliquots at -70 °C until tested for RVA antigen and genotypes.

2.2. RVA antigen capture ELISA

All specimens were tested for the presence of RV by using Generic Assay ELISA kit for rotavirus (Cat. No. 6001, Germany) as per manufacturer's instructions. Specimens with optical density (OD) values above the cut-off value (0.2 + mean value of OD of negative control wells) were considered positive for rotavirus antigen.

2.3. RNA extraction, RT-PCR and genotyping

RVA dsRNA was extracted from stool specimens by using TRIZOL®LS reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. The VP7 and VP4 genes were genotyped by multiplex reverse transcription (RT)-PCR using the methods described earlier [16,17] and modified thermal cycling programme [18]. The full-length NSP4 genes (751 bp) and VP6 gene subgrouping region (379 bp) were amplified using the NSP4-F and NSP4-R primers [19] and forward (F) VP6 and reverse (R) VP6 primers [20], respectively, with the one step RT-PCR kit (Qiagen, Hilden, Germany). The PCR conditions involved initial reverse transcription step of 30 min at 45 °C and 95 °C for 15 min followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 70 °C for 2.5 min with a final extension at 70 °C for 7 min.

All PCR products, including those from the first-round and multiplex PCRs, were analysed by electrophoresis using Tris acetate EDTA (TAE) buffer, pH 8.3 on 2% agarose gels, containing ethidium bromide (0.5 ug/ml) and visualised under UV illumination.

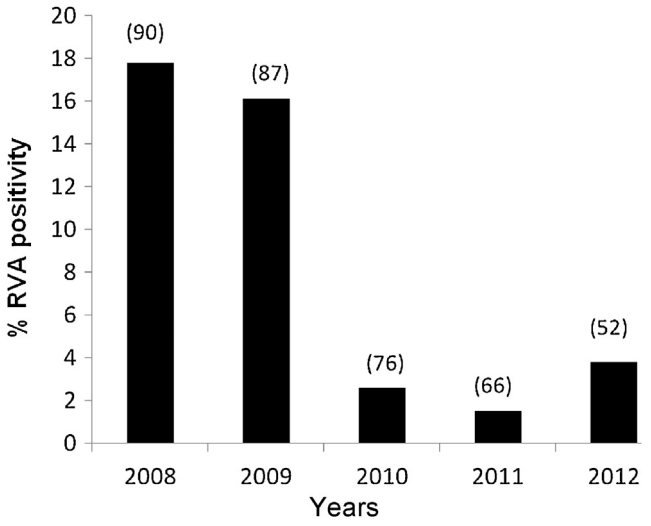

Fig. 1. Yearwise positivity for group-A rotavirus among adolescent and adult cases of acute gastroenteritis in 2008–2012. The figures in parentheses indicate total number of specimens tested. Note: Percent RVA positivity reported in the adolescents and adults of the previous study [13]. 2004: 7.1%, 2005: 5.7%, 2006: 7.2%, 2007: 43.1%.

2.4. Nucleotide sequencing and phylogenetic analysis

The first round PCR products obtained in multiplex PCR for VP7 (876 bp) and VP4 (881 bp) genes and the PCR products obtained in RT-PCR for VP6 gene (379 bp) and NSP4 gene (751 bp) were purified on minicolumns (QIAquick; Qiagen, Valencia, CA) and sequencing was carried out using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster city, CA) and a ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

All sequences obtained for VP4(P), VP7(G), VP6(I) and NSP4(E) genes were aligned with the corresponding gene sequences of RVA strains available in the GenBank by using Clustal W [21]. The phylogenetic analysis was carried out in MEGA 5 by using Kimura –2 parameter and neighbour-joining method [22]. The reliability of different phylogenetic groupings was confirmed by using the bootstrap test (1000 bootstrap replications).

2.5. Accession numbers

The RV NSP4, VP4, VP6 and VP7 gene sequences from this study have been deposited in GenBank under the accession numbers KF951361-KF951404.

3. Results

3.1. Detection of RV antigen

Group-A RV antigen was detected in 9.4% (35/371) of the specimens collected from adolescent and adult cases of acute gastroenteritis. The distribution showed a decline in the RV positivity over time (Fig. 1).

3.2. Genotyping, sequencing and phylogenetic analysis of VP7 and VP4 genes

Genotyping of VP7 and VP4 genes was conducted for all 35 strains detected in adolescent and adult cases of acute gastroenteritis. The VP7 and VP4 genes were both successfully genotyped in 6 cases and one additional VP7 was typed. For the remaining 28 samples, VP7 and VP4 genes could not be amplified despite the use of specific primers. The number of strains non-typeable for both

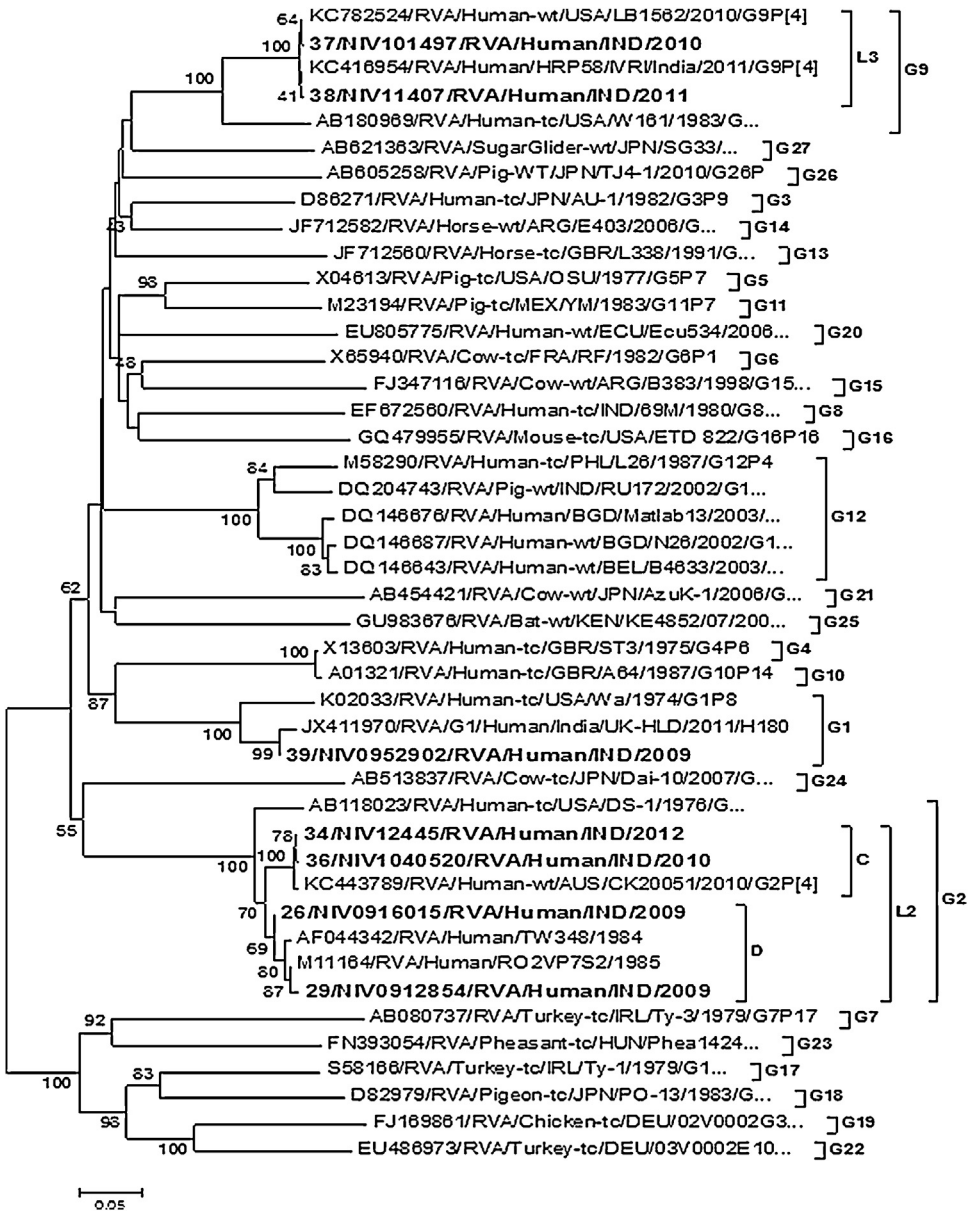

Fig. 2. Phylogenetic tree based on the partial nucleotide sequences of the VP7 gene (nt 87–897) of rotavirus strains recovered from adolescents and adults in 2008–2012. Strains of the present study are highlighted in bold. The reference strains are indicated by accession numbers followed by the strain names. Scale indicates genetic distance.

genes (n = 28) was significantly high as compared with the typeable strains ($p < 0.01$). Among the strains (n = 6) typeable for both VP7 and VP4 genes, G2P[4] (n = 3; 2 in 2009 and 1 in 2012), G9P[4] (n = 2; 1 each in 2010 and 2011) and G1P[8] (n = 1 in 2009) genotypes were detected. All 6 and 1 additional typed VP7 sequences clustered with their respective genotypes (Fig. 2). G2 strains were placed in lineage II sublineages C and D. G9 and G1 strains were classified in lineages L3 and L1, respectively. Analysis of VP4 gene sequences showed clustering of all of the P[4] strains (n = 5) in the P[4]- 5 lineage and that of the P[8] strain (n = 1) in the P[8]-3 lineage. Two of the P[4] strains did not amplify sufficiently in the first round of PCR and hence were not included in the phylogeny (Fig. 3).

3.3. RT-PCR amplification, sequencing and phylogenetic analysis of the VP6 and NSP4 genes

Twenty seven of the 35 strains which typed or did not type for VP7 and VP4 genes were amplified in the VP6 PCR and sequenced. Analysis of VP6 gene sequences showed clustering of the majority (24/27; 89%) in the I2 genotype, in two clusters with the remaining 3 strains (3/27, 11%) clustering in the I1 genotype (Fig. 4).

Six of the 35 strains were amplified by NSP4 PCR and sequenced, 4 of 6 amplified genes clustered in the two different groups of E2 genotype and the remaining two clustered with the E6 genotype (Fig. 5).

The VP6 and NSP4 genes amplified from 20 and 2 strains, respectively, which were non-typeable for VP7 and VP4 genes were most homologous to human RV strains.

3.4. Analysis of linkage between VP4(P), VP6(I), VP7(G) and NSP4(E) genotypes

Of the 4 strains that were typed for all four genes, the common combination of G2-P[4]-I2-E2 was detected in 2 of the 4 strains. The unusual genotype combination G9-P[4]-I2-E6 was noted in the remaining 2 strains.

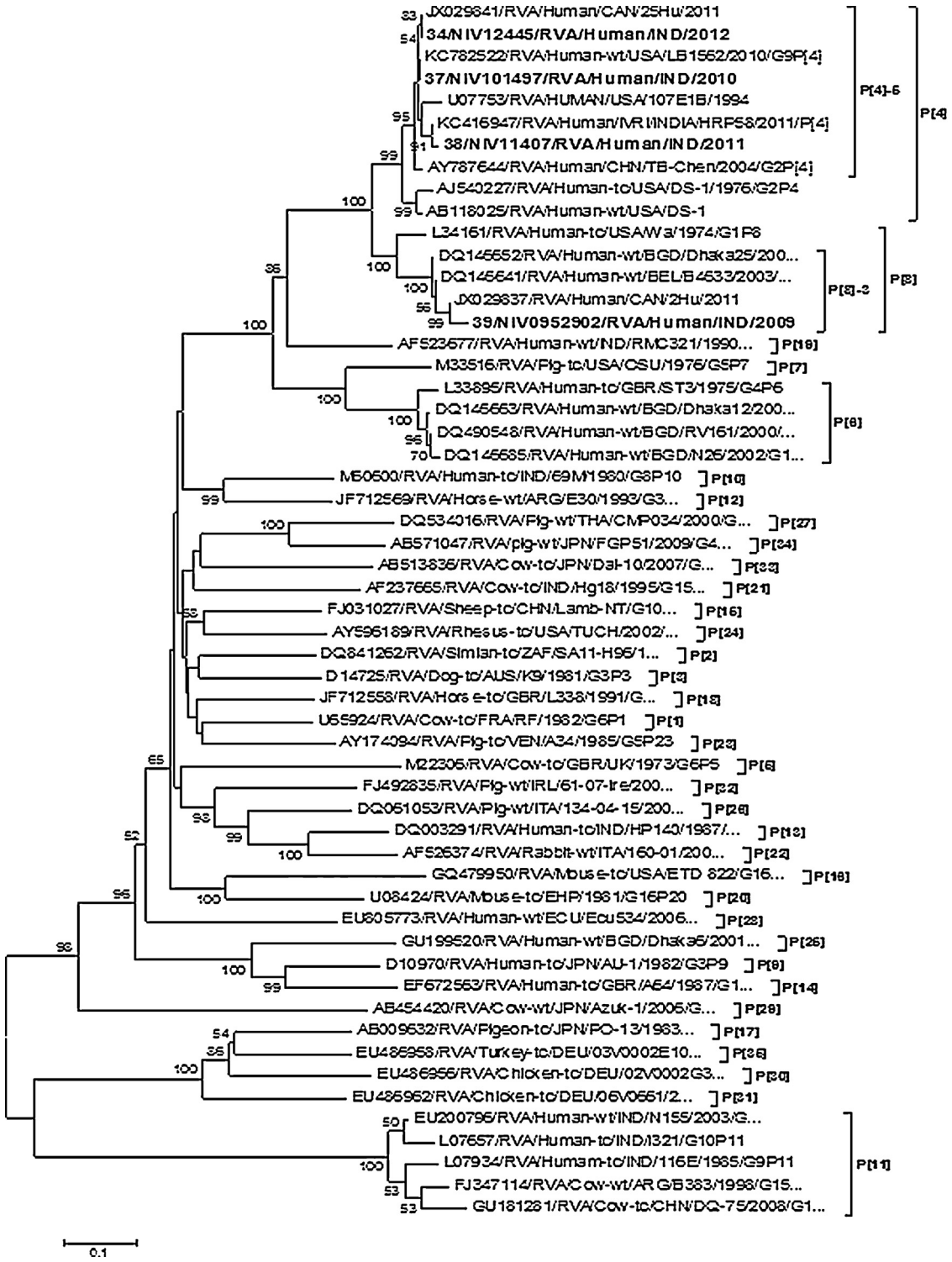

Fig. 3. Phylogenetic tree based on the partial nucleotide sequences of the VP4 gene (nt 122–842) of rotavirus strains recovered from adolescents and adults in 2008–2012. Strains of the present study are highlighted in bold. The reference strains are indicated by accession numbers followed by the strain names. Scale indicates genetic distance.

4. Discussion

The key to develop targeted care or prevention strategies is to recognise the pathogens causing disease in different age groups. Based on surveillance for RV disease and strains, RV vaccines have been recommended in national immunisation programmes, worldwide [23]. A few studies have reported indirect protection of adults by vaccination in the paediatric population [10]. However, more studies are required to compare the RV strains circulating in children and adults, and to understand the effects on infections in adults as a result of herd immunity due to vaccine introduction in children.

The study, although conducted over 5 years, on a relatively limited number of cases each year, showed an overall decline in the frequency of RV infections in adolescents and adults during 2008–2012 (9.4%) as compared to an earlier report (16.9%) in a

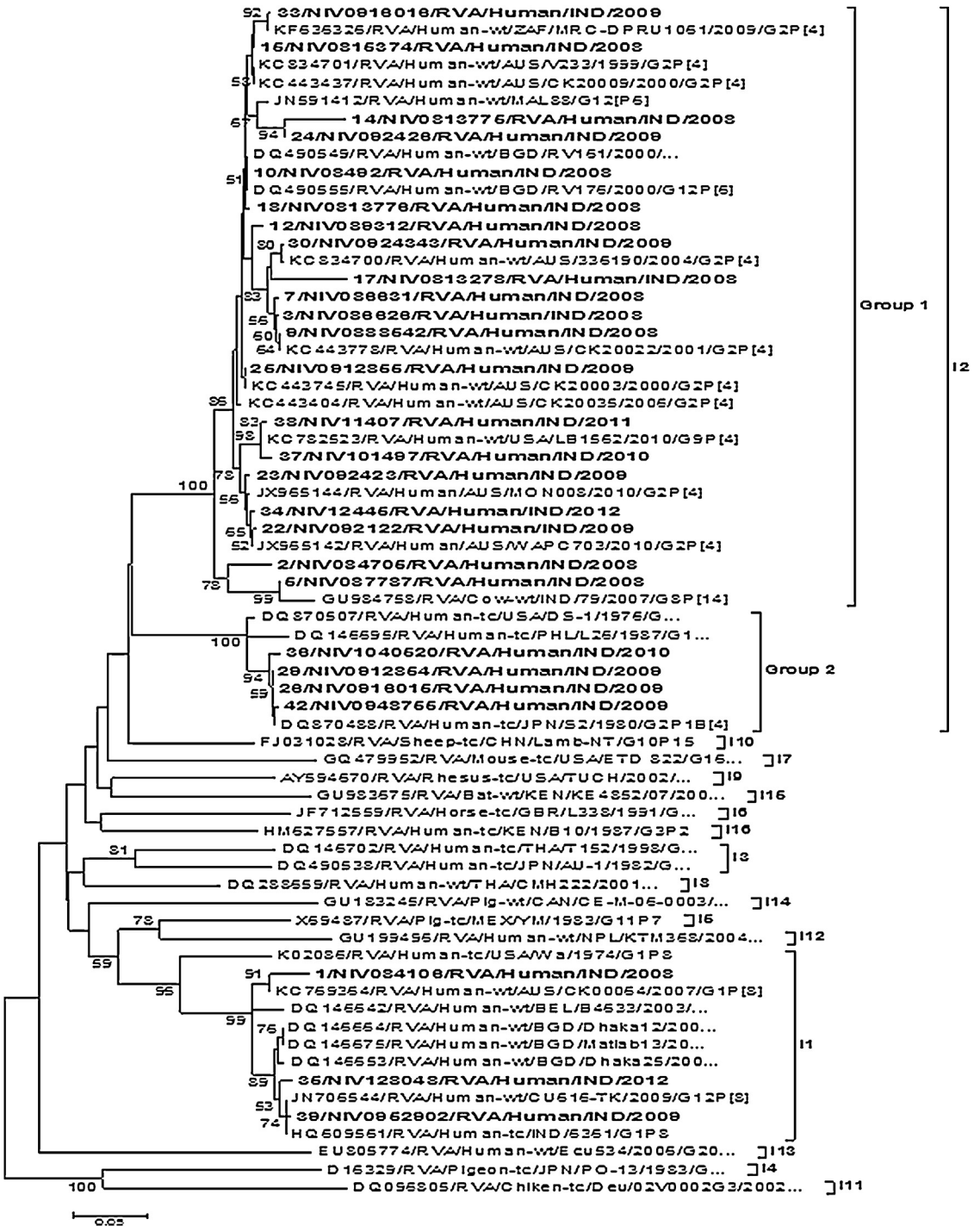

Fig. 4. Phylogenetic tree based on the partial nucleotide sequences of the VP6 gene (nt 761–1112) of rotavirus strains recovered from adolescents and adults in 2008–2012. Strains of the present study are highlighted in bold. The reference strains are indicated by accession numbers followed by the strain names. Scale indicates genetic distance.

similar group of patients [15]. It may be noted that the prevalence of RV among adults declined from 4.4% in 2006–2007 to 2.3% in 2008–2010 in USA, suggesting an indirect protection of adults by paediatric rotavirus vaccination [10]. It may not be possible to explain the decline in the RV infections observed in the present study on the similar basis as only 9.7% of the paediatricians in India have reported routine administration of RV vaccines [24] and the vaccines are not in the public vaccination programme.

Similar to the studies reported in the 2000s in Brazil, Ireland, India and US [4,11,15,25–27], G2P[4] strains were found to be the common strains in adolescent and adult patients in the present study. These results, however, differed from those found in children from the same region and period (2009–2012) from India describing G1P[8], G2P[4] and G9P[8] strains as the most common types and the emergence of G9P[4] and G12 P[6]/P[8] strains (under communication) and worldwide [28,29]. Interestingly, an uncommon genotype combination G9P[4] was detected in the years 2010 and 2011, a finding similar to that described recently in children from Latin America [30], Africa [28], Bangladesh [29], Kerala [31] and also from Pune, India (under communication). Among the other commonly circulating RV strains, G1P[8] was detected only in 2009.

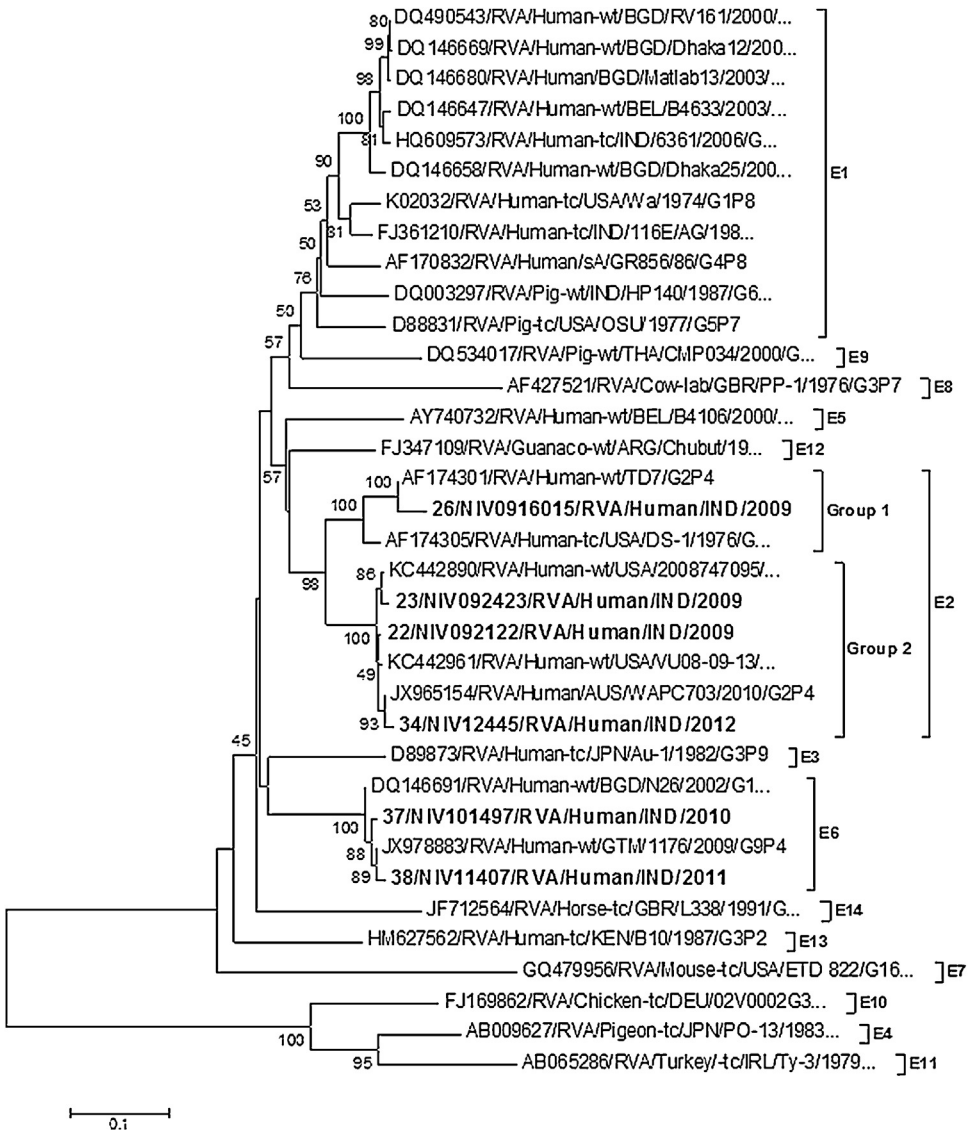

Fig. 5. Phylogenetic tree based on the partial nucleotide sequences of the NSP4 gene (nt 42–569) of rotavirus strains recovered from adolescents and adults. Strains of the present study are highlighted in bold. The reference strains are indicated by accession numbers followed by the strain names. Scale indicates genetic distance.

Our earlier RV surveillance study [15] conducted for the period from 2004–2007 in adolescents and adults from the same region has documented almost equal similar contribution of nontypeable (11.6%) and mixed (13.9%) RV strains in causing gastroenteritis. Surprisingly, none of the patients with gastroenteritis in the present study were detected to have mixed rotavirus infection. This may be attributed to the decline in the rate of RV infection as well as diversity in rotavirus strains noted in the present study as compared to that reported earlier [15]. The finding of a large proportion of nontypeable strains (77.1%) in the present study highlight their dominance in causing gastroenteritis infections in adults. It may be noted that in this study false ELISA positivity of nontypeable rotavirus strains was ruled out in 77% of the strains by RT-PCR and sequencing of the VP6 gene. The remaining 23% of the samples (strains) may have contained empty particles or virus at such low levels that there was insufficient template for amplification. The possibility of the presence of PCR inhibitors that may cause interference in the assay also needs to be considered.

The co-circulation of lineages IIC and IID of the G2 strains differed from an earlier report of I and IIB from India [15] and IIC from Ireland [27]. All of the G9 strains clustered in the L3 lineage commonly circulating worldwide [27,32]. Likewise, all of the P[4] strains clustered in the widely detected P[4]-5 lineage [15,27]. The proportion of circulating VP6 I1 and I2 genotypes was similar to that reported earlier from India [33]. The presence of the rare NSP4 E6 genotype is reported for the first time in adolescents and adults in this study, although this genotype was detected earlier in children from Bangladesh [29].

Occurrence of intergenogroup reassortments has been considered as random events that contribute to the emergence of new combinations of serotypes and genotypes within the human population [34]. In the present study, sequence analysis of VP4, VP6, VP7 and NSP4 genes revealed intergenogroup reassortment, however, analysis limited to these genes may not be adequate to obtain definite data on the overall genetic diversity or origin of the strains. Complete genome sequencing of strains will be of importance to determine the genotype constellation in common and reassortant human group-A rotaviruses.

In conclusion, group-A RV infections have been detected to be a notable cause of acute gastroenteritis in adolescents and adults from Pune, India. The pattern of their transmission between paediatric and adult populations is not clearly understood. The

finding of occurrence of new genotype combinations in the adolescents/adults indicates that understanding genomic diversity and evolution of rotaviruses requires characterisation of strains from all age strata.

Conflict of Interest

The authors have no conflict of interest.

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