Original Article

Human Rotavirus Genotypes Detection among Hospitalized Children, A study in Tehran, Iran

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Abstract

Background: Human rotavirus genotypes G1-G4, G9, P[4] and P[8] are major worldwide causes of acute gastroenteritis in children. Rotavirus genotype G1P[8] is predominant in many countries. In this study, the genotypic diversity of group A rotaviruses were detected in children <5 years of age who were treated for dehydration and diarrhea in Tehran, Iran from October 2004 to September 2008.

Methods: A total of 700 stool specimens were collected from children and assessed for the presence of rotaviruses by the dsRNA-PAGE technique. G and P typing of the positive samples were performed by semi-nested multiplex RT-PCR.

Results: Rotaviruses were isolated in 19% of samples. A total of 14 rotavirus dsRNA different electrophoretypes were detected. The predominant genotype was G1 (76.3%), followed by G4 (11.5%), G8 (0.8%), P[4] (9.2%) and P[8] (66.4%), respectively. In mixed type samples, the majority were of genotype G1P[8] (53.4%), followed by G1P[4] (9.2%) and G4P[8] (4.6%). Mixed types consisted of 3.1% of the total sample followed by G1G2/-P (1.5%), G1G4P[4] (0.8%) and G1G4P[8] (0.8%).

Conclusion: In this study, a high prevalence of the G1P[8] genotype was determined to be the cause of childhood gastroenteritis in Tehran, Iran. The sequence of G and P genotypes showed high levels of similarity to strains from other Asian countries. Our data will be useful for future vaccine formulation in Iran.

Keywords: gastroenteritis, genotype G, genotype P, human rotavirus, RT-PCR

Introduction

Acute gastroenteritis is a major cause of morbidity and mortality among children worldwide.[1] Each year rotavirus causes more than 125 million episodes of childhood gastroenteritis and over 500,000 deaths among children throughout the world.[1,2] Among the enteropathogenic viruses, *Reoviridae*, genus *Rotavirus*, species *group A Rotavirus* (ICTVdB No. 00.060.0.03.001) is recognized as the most common etiologic agent of gastroenteritis in children. Rotavirus consists of genome 11 dsRNA segments enclosed in a triple-layered capsid consisting of a core, inner capsid, and outer capsid layer.[2] Rotaviruses are divided into seven groups (A-G) on the basis of their antigenic properties, of which group A rotaviruses are the major cause of acute dehydration and gastroenteritis in children. Group A rotaviruses are further divided into the G and the P types according to the antigenic property of VP7 (glycoprotein) and VP4 (protease-susceptible protein), respectively. At least 23 G types and in excess of 31 P types have been described, of which at least 10 G types and 11 P types have been found in humans.[2,3] G1 rotavirus is the most widespread genotype that causes acute gastroenteritis in children. Globally, there are five combinations of G and P genotypes, which are most common; P[8] with G1,G3,G4 and G9,and P[4] with G2.[2–4] Determination of the prevalence and types of rotaviruses within regions is essential for vaccine preparation.[5]

In this study, we determined the incidence of group A rotavirus infection in hospitalized children with acute gastroenteritis in two pediatric hospitals in Tehran, Iran and reported the distribution of G and P genotypes of human rotavirus strains that infect children. Additionally, the phylogenetic tree on the basis of nucleotide sequences of genes VP7 and VP4 of group A rotaviruses detected was also constructed.

Materials and Methods

Study design

A total of 700 fecal specimens were collected from sporadic cases of acute gastroenteritis in two pediatric hospitals in Tehran, Iran from Oct 2004 to Sep 2008. Acute gastroenteritis was defined as the occurrence of diarrhea along with symptoms such as vomiting, fever, dehydration, and abdominal pain. The ages of the patients ranged from one month to 5 years. Fecal samples were transported on ice to the laboratory of the Virology Department of Pasteur Insti-

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tute, Iran, where they were stored at -20°C until processed. In addition, a questionnaire was filled for each case and clinical data were entered. The ethical assignment was approved by the patients' parents, who signed informed consents. The local Institutional Review Board of the Pasteur Institute of Iran also confirmed the performing of this study.

Rotavirus detection and electrophoretyping

Group A human rotaviruses were detected by dsRNA electrophoresis in polyacrylamide gel as previously described.[6,7] Briefly, a 10% suspension was made in extraction buffer, which contained sodium dodecyl-sulfate (SDS). The suspension was then mixed with an equal volume of phenol/chloroform and centrifuged. After centrifugation, dsRNA was precipitated with two volumes of ethanol and resuspended in diethyl pyrocarbonate water. For analysis, 30 μL of RNA was electrophoresed in a 10% polyacrylamide gel. The gels were stained with silver nitrate and photographed.

Extraction of human rotavirus dsRNA

Rotavirus-positive fecal suspensions were used for viral double-stranded RNA (dsRNA) extraction using the QIAamp viral RNA kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions.

G and P genotyping by semi-nested multiplex RT-PCR

Rotavirus dsRNA were used as templates for M-MuLV reverse transcriptase (Fermentase) to synthesize cDNA copies from viral strands using random primers. G and P typing of the strains were performed by semi-nested multiplex PCR assays using consensus and type specific primers (Table 1) as described previously by Gouvea et al. and Gentsch et al.[8,9] PCR amplification was carried out in a 25 μL reaction volume containing 10× PCR buffer (Fermentase), 50 mM $MgCl_2$ (Fermentase), 10 mM deoxynucleoside triphosphate mixture (dNTPs) (Fermentase), 10 μM of primers, Taq DNA polymerase (5 u/μL; Fermentase), and 3 μL of the cDNA product. Thermal-cycling was performed as indicated in Table 2, with the addition of an initial 5 min denaturation step at 94°C and a final 7 min extension step at 72°C for each assay. The PCR products were analyzed by electrophoresis on a 1.5% Ultra Pure™ Agarose gel (Invitrogen, Spain) in tris-borate buffer (TBE), which contained ethidium bromide and visualized under ultraviolet light. The 100-bp DNA ladder (Fermentase) was used as a size marker to estimate the lengths of the products. PCR amplicons were purified by a column-based purification kit (Millipore, USA, LSKS09601) using vacuum for filtering and sequenced automatically with an automated DNA sequencer (ABI 3730XL, USA).

Table 1. G and P consensus and type-specific primers

Primer	Sequence (5′- 3′)	nt Position	Amplicon (bp)	Reference
G typing 1st round VP7- F	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28	988	Gouvea et al. (1990)[8]
VP7- R	ATTTAATGATCTTGATCTTTTGGAC	988–963		Modified from Gouvea Designed in (1990),[8] present study
2nd round G1	CAAGTACTCAAATCAATGATGG	314–335	674	Gouvea et al. (1990)[8]
G2	CAATGATATTAACACATTTTCTGTG	411–435	577	Gouvea et al. (1990)[8]
G3	CGTTTGAAGAAGTTGCAACAG	689–709	299	Gouvea et al. (1990)[8]
G4	CGTTTCTGGTGAGGAGTTG	480–498	508	Gouvea et al. (1990)[8]
G8	GTCACACCATTTGTAAATTCG	178–198	810	Gouvea et al. (1990)[8]
G9	CTAGATGTAACTACAACTAC	757–776	231	Gouvea et al. (1990)[8]
P-typing 1st round Con-3	TGGCTTCGCCATTTTATAGACA	11–32	876	Gentsch et al. (1992)[9]
Con-2	ATTTCGGACCATTTATAACC	868–887		Gentsch et al. (1992)[9]
2nd round P[4]	CTATTGTTAGAGGTTAGAGTC	474–494	484	Gentsch et al. (1992)[9]
P[6]	TGTTGATTAGTTGGATTCAA	259–278	267	Gentsch et al. (1992)[9]
P[8]	TCTACTTGGATAACGTGC	339–356	345	Gentsch et al. (1992)[9]

Table 2. G and P typing PCR conditions*

	Denaturation		Annealing		Extension		No. cycles
	Temperature (°C)	Time	Temperature (°C)	Time	Temperature (°C)	Time	
G typing * 1st round	94	50″	60	50″	72	1′ ,20″	25
2nd round	94	50″	57	50″	72	1′ ,10″	20
P typing * 1st round	94	50″	50	1′, 10″	72	1′ ,30″	30
2nd round	94	50″	55	55″	72	1′	20
* This study							

Phylogenetic analysis of nucleotide and amino acid sequences

Some of the partial VP7 and VP4 nucleotides, and amino acid sequences were analyzed. As a result, two phylogenetic trees were constructed by the neighbor joining method using the Clustal W.[10,11]

Results

Rotavirus RNA detection

Group A human rotavirus was detected in 131 of 700 (19%) stool samples by the dsRNA-polyacrylamide gel electrophoresis (PAGE) method between Oct. 2004 and Sep. 2008. The incidence of rotaviral diarrhea during the four-year study period was 25.2% (Oct. 2004), 22.1% (Oct. 2005), 20.6% (Oct. 2006) and 32% (Oct. 2007), respectively (Table 3). Fourteen different rotavirus dsRNA electrophoretypes, short and long profiles, were detected in the study area (Figure 1). G2 and G8 rotaviruses exhibited short RNA patterns, while G1 strains showed long RNA profiles.

Determination of G and P genotypes

G and P genotypes were detected by semi-nested multiplex RT-PCR in 120 (91.6%) and 101 (77.1%) of samples, respectively. Overall, among the G genotypes (Figure 2, Table 3) G1 (76.3%) was the most dominant followed by G4 (11.5%), G8 (0.8%), and mixed types (3.1%). During the cooler months of 2004, two samples of mixed type G1G2 were observed. G4 genotypes were detected during 2004 – 2008 and were most prevalent type after G1 during this study period. Overall the following P types were found: P[8] (66.4%) and P[4] (9.2%). For each year of the study, P[8] was the dominant type whereas P[4] circulated during the four-year study period. During the warm and cool seasons of 2004 – 2008, G1P[8] (53.4%) was the dominant genotype. Other combinations detected were G1P[4] (9.2%) and G4P[8] (4.6%). A total of 22% of samples were identified as only G type and 8.4% of specimens were P[8] type (Table3). Four samples showed G mixed infections, G1/G4P[8] (0.8%), G1G4P[4] (0.8%), and G1G2/-P (1.5%).

Partial sequence analysis of VP7 and VP4

We determined the VP7 and VP4 nucleotide sequences for G1, G2, G4, G8, P[4], and P[8] isolates over the study period. It was determined that some G1 isolates maintained a similarity of 97% to 99% between Oct. 2004 and Sep. 2008. Over 50% and approximately 10% of the G1 strains isolated were G1P[8] and G1P[4] during this period, respectively. G4 strains analyzed during the period of this study showed a 98% similarity with the others. The genetic

Table 3. Human rotavirus genotype distribution in Tehran, Iran from 2004 – 2008

G type/P type	*n* (%)				
	Oct 2004-Sep 2005	Oct 2005-Sep 2006	Oct 2006-Sep 2007	Oct 2007-Sep 2008	Total
G1/[P4]	12 (4)	13.8 (4)	7.4 (2)	4.8 (2)	9.2 (12)
G1/[P8]	54.5 (18)	62 (18)	51.8 (14)	47.6 (20)	53.4 (70)
G1/-	15.2 (5)	3.5 (1)	14.8 (4)	19 (8)	13.7 (18)
G4/[P8]	3 (1)	10.3 (3)	0	4.8 (2)	4.6 (6)
G4/-	0	0	11 (3)	14.3 (6)	6.9 (9)
G8/-	3 (1)	0	0	0	0.8 (1)
G1G2/-	6 (2)	0	0	0	1.5 (2)
G1G4/[P8]	3 (1)	0	0	0	0.8 (1)
G1G4/[P4]	0	0	3.7 (1)	0	0.8 (1)
-/P[8]	3 (1)	10.3 (3)	11 (3)	9.5 (4)	8.4 (11)
Total	33 (25.2)	29 (22.1)	27 (20.6)	42 (32)	131

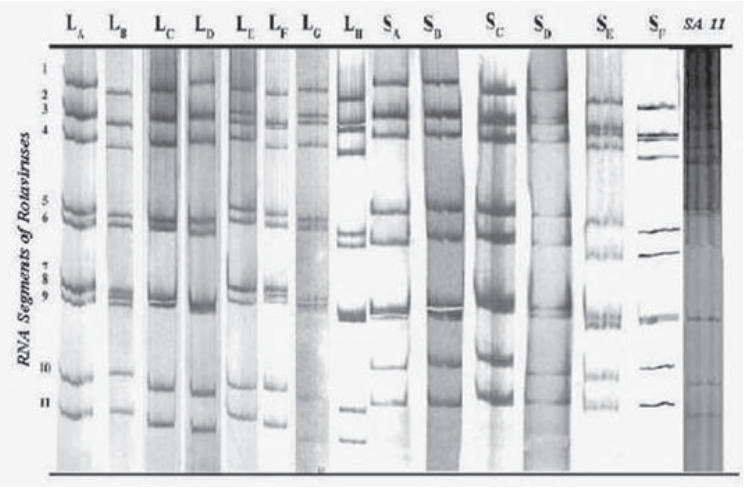

Figure 1. Electrophoretic migration pattern of RNA from the 14 rotavirus strains isolated. SA-11 is used as the marker. The viral RNAs were analyzed by electrophoresis in a 10% polyacrylamide gel and visualized by staining with silver nitrate.

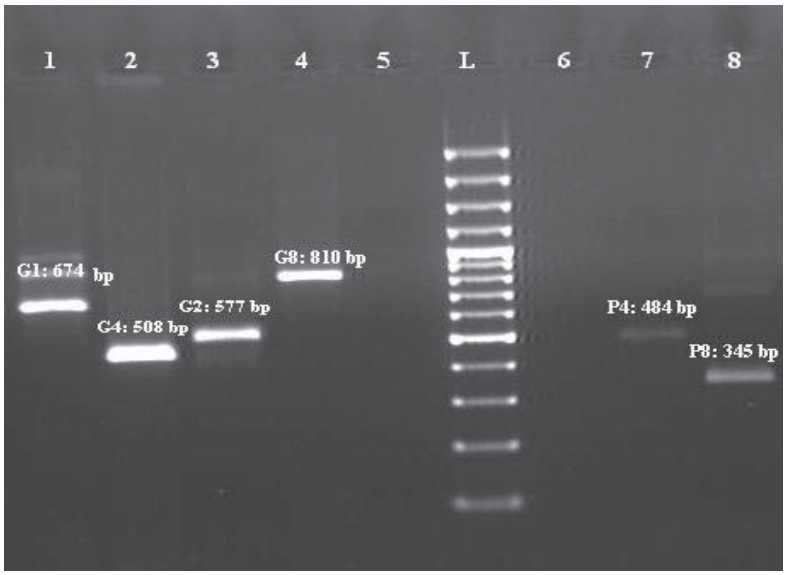

Figure 2. Amplification products of the VP7 gene and VP4 gene. Lane 1, genotype G1; lane 2, genotype G4; lane 3, genotype G2; lane 4, genotype G8; and lanes 7 and 8, genotypes P[4] and P[8], respectively. Lanes 5 and 6: Negative control. L: 100 bp ladder (gene ruler 100 bp plus DNA ladder, Fermentase, SM0323) was used as the molecular size (M) standard.

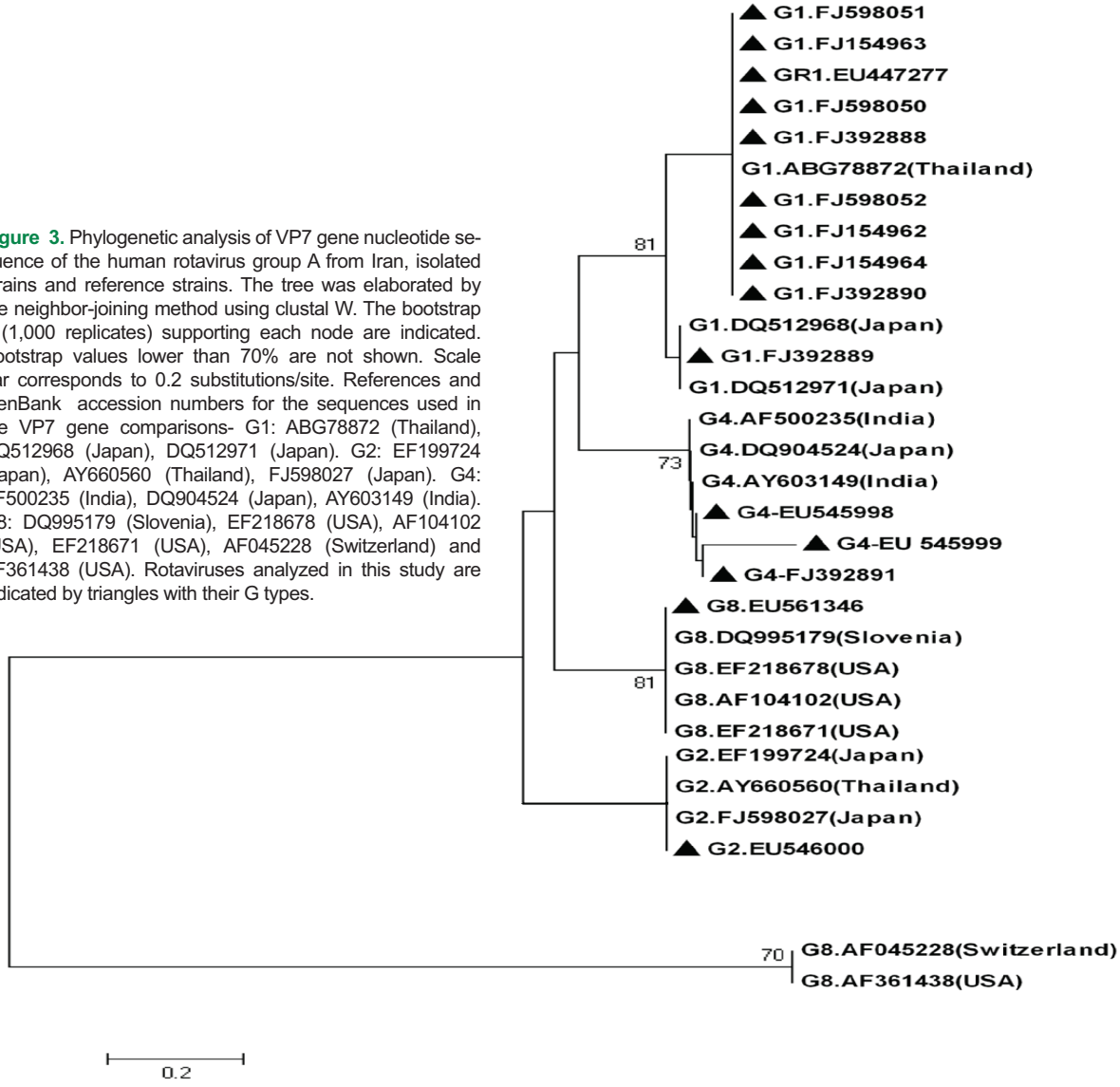

Figure 3. Phylogenetic analysis of VP7 gene nucleotide sequence of the human rotavirus group A from Iran, isolated strains and reference strains. The tree was elaborated by the neighbor-joining method using clustal W. The bootstrap a (1,000 replicates) supporting each node are indicated. Bootstrap values lower than 70% are not shown. Scale bar corresponds to 0.2 substitutions/site. References and GenBank accession numbers for the sequences used in the VP7 gene comparisons- G1: ABG78872 (Thailand), DQ512968 (Japan), DQ512971 (Japan). G2: EF199724 (Japan), AY660560 (Thailand), FJ598027 (Japan). G4: AF500235 (India), DQ904524 (Japan), AY603149 (India). G8: DQ995179 (Slovenia), EF218678 (USA), AF104102 (USA), EF218671 (USA), AF045228 (Switzerland) and AF361438 (USA). Rotaviruses analyzed in this study are indicated by triangles with their G types.

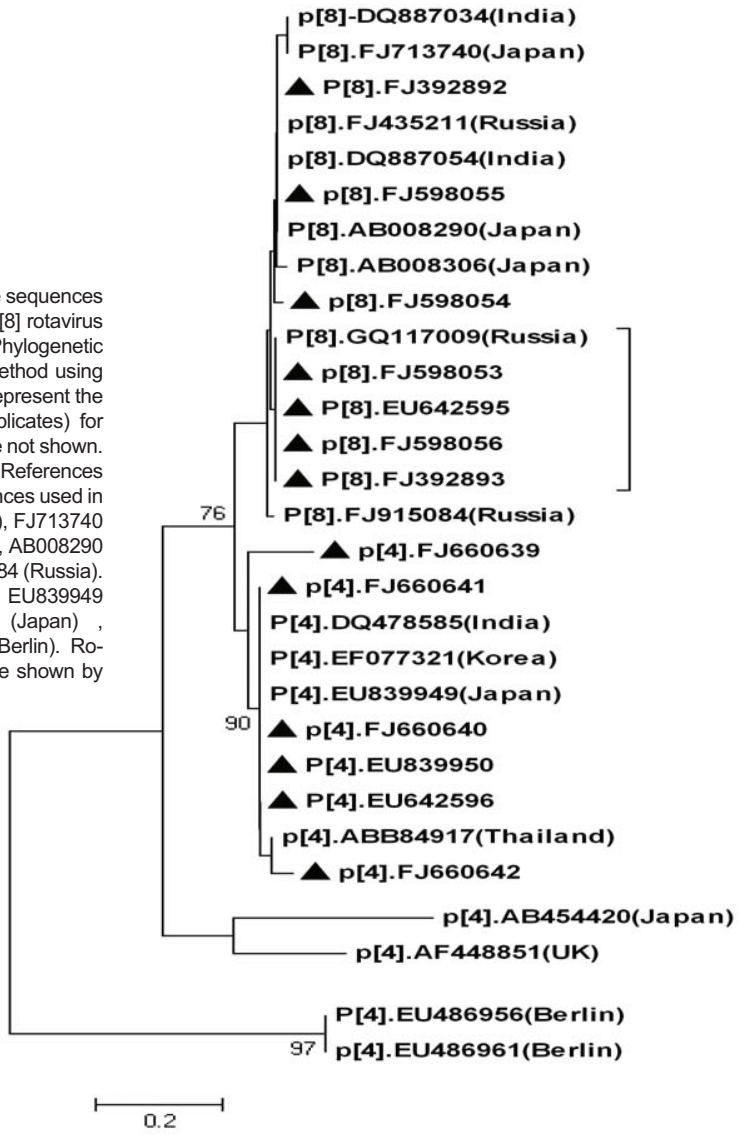

Figure 4. Phylogenetic tree based on nucleotide sequences of the VP4 gene for Group A human P[4] and P[8] rotavirus strains isolated from Iran and reference strains. Phylogenetic tree was constructed by the neighbor-joining method using clustal W. The numbers adjacent to the nodes represent the percentage of bootstrap support (of 1,000 replicates) for each node. Bootstrap values lower than 70% are not shown. Scale bar corresponds to 0.2 substitutions/site. References and Genbank accession numbers for the sequences used in VP4 gene comparisons- P[8]: DQ887034 (India), FJ713740 (Japan), FJ435211 (Russia), DQ887054 (India), AB008290 and AB008306 (Japan), GQ117009 and FJ915084 (Russia). P[4]: DQ478585 (India), EF077321 (Korea), EU839949 (Japan), ABB84917 (Thailand), AB454420 (Japan) , AF448851 (UK), EU486956 and EU486961 (Berlin). Rotavirus strains analyzed in the present study are shown by triangles with their P types.

relationships between strains, partial nucleotide sequences (nt1-988), and (nt11-887) of VP7 and VP4, respectively, and the full length of the amino acids (329aa) and (292aa) were analyzed. The nucleotide sequences obtained from this study have been submitted to the GenBank database under accession numbers: VP7 sequences of FJ154962-64, FJ392888-91, EU447277, EU545998-6000, EU561346, and FJ598051-53; VP4sequences of FJ392892-93, FJ598053-56, EU642595-96, FJ660639-42, and EU839950. G1 strains were closely related to ABG78872 (Thailand; 99%) and DQ512968 (Japan; 98%). In the case of G2, EU546000 sequence isolated in 2004 was related to AY660560 (99%), which originated in Thailand, but showed lower similarity with EF199724 from Japan (95%). G4 strains were closely related to strain AY603149 isolated in India (99%). G8 strain was related to DQ995179 (Slovenia; 100%) and EF218678 (USA; 97%) (Figure 3). Phylogenetic analysis of the nucleotide sequences revealed the existence of two different VP4 gene clusters among rotavirus P[8] strains. One cluster was formed by the three Iranian P[8] strains, Indian strain DQ887034, strain FJ713740 from Japan and the Russian strain FJ435211; the second cluster was represented by the Russian strain GQ117009. Six P[4] samples were selected and the VP4-encoding gene of each virus was sequenced and compared to other P[4] rotavirus VP4 gene sequences available in the GenBank. The best match was with the Indian strain DQ478585, which exhibited 98% nucleotide identity (Figure 4).

Discussion

Diarrhea is recognized as the second most common cause

of death among children under the age of five worldwide, and human rotaviruses are the single most cause of severe diarrhea in children.[1] Determination of the prevalence of different human rotavirus genotypes in different geographic regions and their genetic variations over time can guide any vaccination strategy.[5,12,13] In our study, rotavirus gastroenteritis was associated with 19% of pediatric inpatients under age five, and the predominant rotavirus dsRNA genomic profile detected was the long electrophoretype. In a previous report from Iran, rotavirus infection was detected in 15% of patients with acute diarrhea.[14] We found genotype G1 (76.3%) was predominant, and genotypes G4 and G8 followed at 11.5% and 0.8%, respectively. Among the G genotypes, G1 was the most common in Tehran, Iran between 2001 and 2004.[15,16] Although G9 was reported in several Asia countries, such as Iran, Japan, and Thailand, it was not detected among our samples.[16–18] In each year of the study, G1P[8] was the prevalent combination type, whereas G1P[4] and G4P[8] circulated over this study. In previous reports from Iran, G1P[8] was the most prevalent type detected in patients with acute gastroenteritis.[15,16] According to worldwide reports, in human rotaviruses the major genotypes are G1, G2, G3, G4, and G9, which are combined with P[4], P[6], and P[8].[3–5] In Turkey, G4P[8] (42%) and GIP[8] (27%) have accounted for more than two thirds of the strains.[19] In Bangladesh, the combinations G1P[8] and G2P[4] accounted for more than 50% of rotavirus strains.[20] However, in other countries such as the Netherlands, Eastern India and Brazil, G1P[8] was the prevalent strain while G1P[4], G2P[4], G4P[8], and G9P[8] were predominant genotypes in America, Korea, Argentina, and Thailand, respectively.[4,21–26] Partial sequences were obtained for their VP7 genes (G1 strains), which showed 97% to 99% similarity to each other over the study period. G1 strains analyzed in this study with long RNA electrophoretic profiles were grouped into two discrete clusters. One cluster showed a close genetic relationship with a G1 strain isolated in Thailand (99%), while the other had high homology (98%) with a Japanese strain.[27] This study revealed that there have been no considerable changes in the VP7 gene analyzed from rotavirus G1 strains. This finding is similar to studies that have been performed during the last decade in Japan, China, Thailand, and Vietnam.[27] The partial sequence of the VP7 gene (G2 strain) of rotavirus isolated with short RNA profile seemed to be closely related (99%) to strain G2P[4] from Thailand, which reemerged in the epidemic season of 2003 in this region. G4 isolates were 99% similar to Indian strains.[4] The Iranian G8 strain showed homology (100%) with G8P[8] of Slovenia, which is more closely related to the Cody 1801 bovine strain than to other human strains, and supports the theory of interspecies transmission of rotaviruses and animal-human genome reassortment.[28] P[8] strains analyzed in this study belonged to the Asian cluster and strains, which had major identities at the nucleotide sequences each other (<95%).[29–30] The VP4 partial nucleotide sequences P[4] strains of rotaviruses in our study were included in a single cluster, which was related to genotypes of Bangladesh and Korea. The best match was with the Indian strain, which exhibited 98% nucleotide identity.[20,31] Currently, prevention of rotavirus disease through immunization has been recommended and a live attenuated vaccine based on G1P[8] human strain, Rotarix™, has shown efficacy against rotavirus gastroenteritis caused by G1, G3, G4, and G9 with P[8], but lower efficacy was reported against G2P[4] in many countries.[32,33] In this study, continuous circulation of the G1P[4] strain without the obvious presence of G2P[4] was observed. This finding may be considerable in vaccination programs in Iran. The main outcome of this study is our corroboration of previous reports regarding the high prevalence of strain G1P[8] in Tehran. This study will provide useful data for epidemiological studies and future vaccine formulation in Iran. The surveillance of rotavirus gastroenteritis will be continued in Iran and other countries, therefore the preparation of the rotavirus vaccine is necessary.

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