Rotavirus genotypes in children with gastroenteritis in Erzurum: first detection of G12P{6} and G12P{8} genotypes in Turkey

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Abstract

Introduction: Rotavirus is one of the leading pathogens which cause acute gastroenteritis in children and is responsible for a substantial proportion of childhood deaths worldwide.

Aim: To determine the group A rotavirus (RVA) prevalence and genotypes of circulating RVA strains in 0–5-year-old children with complaints of vomiting and diarrhoea in Eastern Anatolia in Turkey.

Material and methods: RNA extracted from stool specimens of 329 children aged 0–5 years with acute diarrhoea was subjected to reverse transcription polymerase reaction (RT-PCR) and multiplex-nested PCR. The genotypes were identified based on the expected size of the amplicon, which was amplified with a genotype-specific primer.

Results: Out of 329 stool samples analyzed, 109 (33.1%) were positive for RVA. G1P[8] was the dominant genotype combination (42.2%), followed by G9P[8] (21.1%) and G12P[6] (11.0%). Mixed infections were identified in 5 cases: G3,9 in 2 cases, G1,9 in 1 case, P[4,8] in 1 case, and P[6,8] in 1 case. The P genotype could not be typed in two patients.

Conclusions: In the study, we detected six different rotavirus G genotypes, 3 different P genotypes, 11 different G-P combinations and 5 different mixed genotypes combinations. G1, G9, G12 and P[8] were found to be the predominant genotypes. G12P[6] and G12P[8] genotypes, showing an increase as new rotavirus genotypes in the world, are reported for the first time for our regions. We determined the dominant genotypes, mixed genotypes and unconventional genotypes of rotavirus in our region.

Introduction

Rotavirus is one of the leading causes of acute gastroenteritis in children and is responsible for a substantial proportion of childhood deaths worldwide. Each year, about two million people especially children < 5 years of age are affected rotavirus-related diarrhea [1].

The first rotavirus was detected in Australia in 1973 by Bishop [2] in duodenal biopsy sections taken from children with acute gastroenteritis, via examination with electron microscopy (EM) in the cytoplasm of the enterocytes. Because of the whell like apperance of virus under electron microscopy, the name 'rotavirus'was given in 1974 [3]. Not only humans, but almost all mammal and bird species can have rotavirus infections in newborns; although rotavirus was not reported in people until 1973, it has been reported earlier in animals [4].

Rotavirus is classified in the family *Reoviridae* and is a non-enveloped virus with a genome consisting of 11 segments of double stranded RNA (dsRNA). The 11 genome segments of dsRNA code for six structural viral proteins (VP1–4, VP6 and VP7) and six non-structural proteins (NSP1-6) consisting of 12 viral proteins [5]. It is structurally composed of three layers inside the virus: VP2, VP6 middle, and outer-shape VP4 and VP7 proteins. The VP6 groups and sub-groups are the original antigen, and rotaviruses up to H separate into 8 groups [6]. Viral VP4 and VP7, making up the outer capsid proteins, are antigens that trigger the production of neutralizing antibodies and determine the serotype of rotavirus P and G, and also determine the P and G genotypes of the gene encoding the protein [7]. So far,

at least 27 distinct G genotypes (G1-G27) and 37 distinct P genotypes (P[1]–P[37]) have been identified [8]. Molecular epidemiological studies in humans have been performed on the common rotavirus GP genotypes (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8]) and in the last 20 years throughout the world, G9P[8] and G12P[8] have been studied, emphasising the increasing genotypes in humans [9].

During replication, multiple rotavirus types infecting the host's intestinal cells, through the segmented genome, can develop point mutations, reassortment and new genetic regulation. As a result of these genetic conditions many genotypic variations of rotavirus have emerged. Rearrangement of the virus's genes usually occurs in the non-structural protein NSP5 and NSP6 encoding segment 11, and to a lesser extent, segments 5–10 [10]. Another factor that plays a role in the emergence of new phenotypes is mixed infections. Rotavirus has a wide genetic variation. G8 genotype is responsible for the outbreak in Africa, G5 in Latin America and G10 in India [11]. Rotavirus molecular structure is a suitable structure for genetic reorganisation which can be explained by antigenic drift and shift mechanisms. This structure leads to the emergence of reassortant types.

A reassortment opportunity can be won against the virus today with the segmented genome, G1P[8] monovalent, containing the strains of rotavirus vaccine RV1 (Rotarix, GlaxoSmithKline), and G1, G2, G3, G4 and P[8] pentavalent, containing the human-bovine reassortment strains of the human-bovine RV5 reassortment vaccine (RotaTeq, Merck), which are widely used [12].

In infected persons without diarrhoea, the virus is shed in the faeces for about a week. Rotavirus is highly contagious and can be spread human-to-human with close contact, or transmitted by the direct or indirect faecal-oral route. Rotavirus is a virus which is well adapted and rapidly spread in human and animal populations. Therefore, regular investigations on genotyping diversity of this virus is necessary.

Aim

The aim of study was to determine the RVA prevalence and genotypes of circulating RVA strains in 0–5-year-old children with complaints of vomiting and diarrhoea in Eastern Anatolia. The genotypic diversity of rotavirus in this area is explored for the first time with molecular-based methods. Genomic amplification and characterization of the rotaviruses will provide valuable data about their prevalence, transmission, dominant types, virulence and evolutionary development.

Material and methods

Collection of samples and RNA isolation

The study was carried out on stool samples sent to the medical microbiology laboratories of Erzurum Ataturk University Research Hospital and Regional Training and Research Hospital, from 329 children aged 0–5 years with diarrhoea and vomiting, during a 2-year period (January 2012 to November 2013) in Erzurum, Turkey.

10% faecal suspension was prepared in phosphate buffered saline (PBS) containing 25,000 U/ml penicillin and 20 mg/ml streptomycin, after being centrifuged at 3000 rpm for 20 min at +4°C 1,250 µl of supernatant of the precipitate was transferred to sterile test tubes and kept at –80°C until used. Isolation of nucleic acids from samples kept in test tubes was performed using the Qiasymphony DSP Virus/Pathogen Midi Kit (Qiagen, Germany) devices and supplies in accordance with the manufacturer's instructions. The obtained nucleic acid suspensions were stored in 85 µl volumes at –80°C until used.

Rotavirus positive control

We found 20 samples positive for rotavirus antigen by the rotavirus immunochromatographic test (CerTest, Biotec SL, Spain) and all samples were confirmed with RT-PCR with generic primers (Table I), which were used as controls in all polymerase chain reaction (PCR) reactions.

Reverse transcription

The synthesis of cDNA from RNA samples was accomplished with two steps. In the first step, a mixture of 1 µl of random hexamer (0.2 µg/l), 6 µl of nuclease-free water and 6 µl of the RNA sample was incubated at 65°C for 5 min in a thermal cycler, then the samples were put on ice packs.

In the second step, a mixture of 4 µl 5X reaction buffer, 2 µl (10 mM) of dNTP and 1 µl (200 U/µl) of RevertAid reverse transcriptase enzyme (Thermo Fisher Scientific Inc., ABD) was chilled on ice and cDNAs were synthesised by incubation for 10 min at 25°C, 60 min at 37°C, 5 min at 70°C and 5 min at 4°C in the thermal cycler.

Determination of P and G genotypes by multiplex-nested PCR

Every cDNA amplicon from each sample was evaluated with VP6 primers in Table I for confirmation of rotavirus presence and 379 bp of PCR amplicon presence was evaluated as positive for rotavirus [13–17]. Multiplex-nested PCR was completed on positive samples by amplification in the previously reported reaction conditions, with the reaction mixture including primers of different gene regions of VP4 and VP7. VP4 F, P[4],

Table I. Primer sequences, gene regions and product sizes

Gene region	Target and primer	Primer sequences F/R (5′-3′)	Length [bp]	Annealing [°C]	References
VP6	Jenerik VP6F-VP6R	F: GACGGVGCRACTACATGGT R: GTCCAATTCATNCCTGGTGG	379	50	[14]
VP7	Jenerik Beg9-End9 Nested VP7F-VP7R	F: GGCTTTAAAAGAGAGAATTTCCGTCTGG R: GGTCACATCATACAATTCTAATCTAAG F: ATGTATGGTATTGAATATACCAC R: AACTTGCCACCATTTTTTCC	1062 881	42 52	[15] [16]
VP4	Jenerik Con3-Con2 Nested VP4F-VP4R	F: TGGCTTCGCTCATTTATAGACA R: ATTTCGGACCATTTATAACC F: TATGCTCCAGTNAATTGG R: ATTGCATTTCTTTCCATAATG	876 663	50 50	[17]
G genotype	VP7R G1 (aBT1) G2 (aCT2) G3 or mG3 G4 (aDT4) G9 or mG9 G10 or mG10 G12	R: AACTTGCCACCATTTTTTCC F: CAAGTACTCAAATCAATGATGG F: CAATGATATTAACACATTTTCTGTG F: ACGAACTCAACACGAGAGG F: CGTTTCTGGTGAGGAGTTG F: CTTGATGTGACTAYAAATAC F: ATGTCAGACTACARATACTGG F: GGTTATGTAATCCGATGGACG	 618 521 682 452 179 266 396	42	[13]
P genotype	VP4F P[4] 2T-1 P[6] 3T-1 P[8] 1T-1D P[9] 4T-1 P[10] 5T-1 P[11] mP[11]	F: TATGCTCCAGTNAATTGG R: CTATTGTTAGAGGTTAGAGTC R: TGTTGATTAGTTGGATTCAA R: TCTACTGGRTTRACNTGC R: TGAGACATGCAATTGGAC R: ATCATAGTTAGTAGTCGG R: GTAAACATCCAGAATGTG	 483 267 345 391 583 312	45	[13]

P[6], P[8], P[9], P[10] and P[11] VP4 gene regions for P genotypes and VP7 R, G1, G2, G3, G4, G9, G10 and G12 for G genotypes were used in this process [13]. The reaction was completed and genotype-specific PCR products were introduced according to product size and separated by electrophoresis in 1% agarose gel under UV light for rotavirus P and G genotypes. Finally, P and G genotypes were identified in the 1% agarose gel by electrophoresis under ultraviolet light according to the size of the PCR product.

Ethical considerations

Ethical approval of this study was obtained from the Ataturk University Medical Faculty Institutional Review Board (Approval No. B.30.2.ATA.0.01.00/48).

Table II. Comparison of rotavirus gastroenteritis by age group

Age group [months]	Positive *n* (%)	Negative *n* (%)	Total *n* (%)
0–24	84 (25.5)	149 (45.3)	233 (70.8)
25–36	15 (4.6)	30 (9.1)	45 (13.7)
37–48	4 (1.2)	22 (6.7)	26 (7.9)
49–60	6 (1.8)	19 (5.8)	25 (7.6)
Total	109 (33.1)	220 (66.9)	329 (100.0)

Results

Table II illustrates the comparison of rotavirus gastroenteritis by age group. Rotavirus was identified by RT-PCR in 109 (33.1%) of the 329 children with gastroenteritis. A large majority of rotavirus positivity was obtained from 0–24-month-old babies.

Table III shows the seasonal distribution of rotavirus infections in the children. Two years' (2012 and 2013) worth of spring, summer, autumn and winter months were included for the relevant seasons in the table. Meteorological data are taken into consideration; hospital admission rates peak in the spring in Erzurum, when average rainfall is 51.3 kg/m^2 and there is a relatively low temperature (4.6°C), followed by the summer, fall and winter. Rotavirus positivity showed a higher rate in stool specimens examined during spring and autumn than those collected winter and summer.

In the study, six different G genotypes (G1 at a rate of 45%, G9 at 32.1%, G12 at 15.6%, G2 at 1.8% , G3 at 1.8% and G4 at 1.8%) and two mixed G genotypes (G3,9 at a rate of 1.8% and G1,9 at 0.9%) were determined in 109 positive samples. Three different P genotypes (P[8] at a rate of 72.5%, P[6] at 16.5% and P[4] at 7.4%) and two mixed P genotypes (P[4]+P[8] and P[6]+P[8]) were identified, and P genotypes in two (1.8%) cases could not be typed. The most common G and P genotype com-

Table III. Distribution of infections compared to Erzurum province's average seasonal temperature and precipitation with a breakdown of differences

Variable	*N*	Spring	Summer	Autumn	Winter
Rotavirus-positive	109	65 (59.6)	9 (8.3)	20 (18.3)	15 (13.8)
Rotavirus-negative	220	105 (47.7)	93 (42.3)	14 (6.4)	8 (3.6)
Total	329	170 (51.7)	102 (31.0)	34 (10.3)	23 (7.0)
*Seasonal temperature average [°C]		4.6	17.8	7.7	–7.9
*Seasonal rainfall average [kg/m^2]		51.3	29.5	31.9	21.2

**These data were taken from the official web site of the Republic of Turkey, Ministry of Forestry and Water Affairs (http://www.mgm.gov.tr/veridegerlendirme/il-ve-ilceler-istatistik.aspx?m=ERZURUM), and seasonal averages for Erzurum were calculated by us.*

bination was G1P[8] (42.2%), followed by G9P[8] (21.0%) and G12P[6] (11.0%), respectively. Other combined genotypes (G9P[4], G12P[8], G9P[6], G2P[4] and G4P[8]) were isolated at low rates. The mixed genotype combinations were identified as G1,9P[8], G3,9P[8], G3,9P[6], G9P[4,8] and G1P[6,8] (4.6%). The distribution of rotavirus G-P genotype combinations is given in Table IV.

Table IV. Combinations of rotavirus G-P genotypes

G-P combination	*N*	%
G1P[8]	46	42.2
G9P[8]	23	21.1
G12P[6]	12	11.0
G9P[4]	5	4.6
G12P[8]	5	4.6
G9P[6]	4	3.7
G2P[4]	2	1.8
G4P[8]	2	1.8
G1P[6]	1	0.9
G1P[4]	1	0.9
G3P[8]	1	0.9
G1P[6,8]	1	0.9
G9P[4,8]	1	0.9
G1,9P[8]	1	0.9
G3,9P[8]	1	0.9
G3,9P[6]	1	0.9
G9P[negative]	2	1.8

Discussion

It is reported that the incidence of rotavirus infections is similar in developing and developed countries, but 82% of the deaths of children in poor countries are due to factors such as poverty and malnutrition [18, 19]. Malek *et al.* reported that the rate of hospitalisations for rotavirus infections increased with income (the median for low-income countries was 8%, for low-middle-income countries was 21%, for high-middle-income countries was 31% and for high-income countries was 42%) [20].

Rotavirus infection in almost every child under the age of 5 years causes severe diarrhoea and dehydration. While that may play a role in viral gastroenteritis as well as other agents, such as bacterial, parasitic and other viral pathogens, the 33.1% rotavirus positivity rate in our study reveals the severity of this viral infection in our region.

Reassortant strains are common all over the world, and new virus types are added to the strain every day. Homotypic and heterotypic immunity obtained from the vaccine carries universal importance. While cross-immunity in dominant genotypes is acquired by the application of the present vaccines, in reassortant types there can be observed a decrease in immunity. This situation raises the need to control the circulating genotypes of human rotavirus with a surveillance study using certain intervals. In addition, it is important to follow the prevalence of new rotavirus types (especially those of animal origin), because they can cause low neutralizing antibody activity even in the event of vaccination [21].

Revealing the genetic character of rotavirus and its dominant genotypes is important in order to direct the vaccination protocols and to combat the rotavirus epidemic. The development of vaccines is the most important weapon against rotavirus, and depends on identification and isolation of rotavirus genotypes as regional, continental and global. So far, it has been reported that the most common rotavirus genotypes affecting humans are G1, G2, G3, G4 and G9, associated with genotypes P[4] and P[8]; the genotypes G1, G2, G3 and G4 are observed in 90–95% of cases in Europe, North America and Australia, 68% in South America and 50% in Africa [22, 23]. In our study, the dominant G

genotypes were identified as the G1 and G9 genotypes. These were also reported to be dominant in previous studies conducted in Turkey and around the world [24, 25]. G1 is common especially in North America, Australia and Europe (70% of infections), but in South America, Asia and Africa, it is less common (20–30% of infections). In addition, G9 (with global rates ranging from 2% to 35%) has been emerging as an important strain in recent years and has been dominant in South America and Australia [26, 27]. Rotavirus vaccine is not included in the routine immunization schedule, and the vaccine cost is high in our country. Therefore, the vaccination rate is decreasing. In addition, our region being close neighbours with different countries such as Iran, Iraq, Armenia, Nakhichevan and Georgia increases the incidence of different rotavirus types. Interestingly, we determined that the high rate of the G12 genotype (15.6%) in our study had not been reported before in Turkey. In addition, the most frequently reported genotypes of G4, G2 and G3 in the world and in our country were identified at an unexpectedly low rate (1.8%) in our study [25, 27–30].

P[8] was determined to be the predominant P genotype in our region, followed by P[6] and P[4]. The P genotype diversity and rate were observed to be parallel between our country and the world [25, 29, 30]. Santos and Hoshino, in their study conducted in 52 countries in the continents of America, Asia, Africa, Europe and Australia/Oceania, reported the most dominant genotypes to be P[6], P[8] and P[4], with P[6] representing almost one-third of all identified types [22]. In a study containing a total of 100 states, the genotypes G1P[8], G2P[4], G3P[8], G9P[8], G4P[8] and G12P[8] were identified [28]. The G1P[8] and G9P[8] genotypes were observed to be the predominant combination of G and P genotypes in our study. These results are similar to the work done in our country and around the world [24, 25]. It is reported that more than 70% of rotavirus infections develop due to G1P[8] in North America, Europe and Australia, but this combination of infections is responsible for approximately 30% of cases in South America and Asia, and 23% in Africa [22]. In our study, the genotypes G12P[6] and G12P[8] were determined for the first time in Turkey. The G12 genotype in Europe was detected first in the UK in 2002, then in Belgium in 2003, and the incidence rates of this genotype are gradually increasing worldwide [31, 32]. The genotypes G9P[4] and G9P[6], which we determined in our study, have been observed rarely in our country.

For the Eastern Anatolia region where we conducted our research on the risk of infection with rotavirus, we took into account climatic factors such as the continental climate, the high altitude of over 1850 m, and the long and harsh winter, with an annual number of 50 days with snowfall and snow cover on the ground for 114 days. There is a relationship between these climatic conditions and the high rates of rotavirus (33.1%) identified in our study. Such environmental factors are also likely to create the appropriate conditions for survival and transmission of the virus.

This study determined six different G genotypes, three different P genotypes and 11 different G-P combinations in children aged 0–5 years with rotavirus gastroenteritis, with remarkable and valuable results. The dominant genotypes responsible for rotavirus gastroenteritis were G1P[8], G9P[8], G12P[6], G9P[4], G12P[8], G9P[6], G2P[4] and G4P[8]. Although there was a correlation between our data in Turkey and the world for the dominance of G1P[8] and G9P[8], the accordance for G2P[4], G3P[8] and G1P[4] was seen to a very limited extent. The most remarkable point in our study was determining the G12P[6] and G12P[8] genotypes, which were previously unreported in our country and are beginning to increase around the world. This situation suggests that a change in genotypes has begun in our country.

With this study, we determined the circulating genotypes of rotavirus, drew attention to new observed genotypes, emphasised that genotypes should be considered in vaccine studies, and contributed to the global rotavirus knowledge base. There is a need for further studies, including a full genome sequence, investigations into animal genetic diversity, and a determination of data on the possible emergence of human-animal reassortment strains, for a regional rotavirus genotype map.

Conclusions

The most remarkable point in our study was determining the G12P[6] and G12P[8] genotypes, which were previously unreported in our country and are beginning to increase around the world. This situation suggests that a change in genotypes has begun in our country.

Conflict of interest

The authors declare no conflict of interest.

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