ROTAVIRUS AND ADENOVIRUS IN CHILDREN WITH ACUTE GASTROENTERITIS AND THE MOLECULAR EPIDEMIOLOGY OF ROTAVIRUS

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ABSTRACT

Objective: *The aim of this study was to investigate the prevalence of the rotavirus and adenovirus related to the viral etiology of gastroenteritis as well as to determine the frequency of common rotavirus genotypes.*

Material and Method: *Fecal samples were obtained from 492 children under 6 years of age who had not been vaccinated with the rotavirus vaccine and who applied to the pediatric outpatient clinic of Afyon Kocatepe University Hospital with the complaint of diarrhea. All of the fecal samples were evaluated in order to determine the presence of bacterial and parasitic agents using conventional identification methods, all results were negative. Rotavirus and adenovirus antigens were examined in stool samples using the immunochromatographic method. Rotavirus G and P genotypes were determined by reverse transcription PCR using consensus primers detecting the VP7 and VP4 genes, followed by semi-nested type-specific multiplex PCR.*

Results: *It was found that 3.3%, and 20.3% of 492 children with acute gastroenteritis were positive for adenovirus and rotavirus, respectively. A total of six different combinations of G and P types were found, including combinations of the G1, G2, G4, G9, P[4], and P[8] genotypes. The most common rotavirus genotypes were G9P[8] (48.7%), followed by G9P[4] (17.5%). Other strains were G1P[8] (16.2%), G2P[8] (11.2%), G1P[4] (3.7%), and G4P[8] (2.5%). Sixty-six percent of the regional rotavirus genotypes were G9P[8] and G9P[4].*

Conclusion: *Regarding the high frequency of rotavirus infection, continuous monitoring is needed in gastroenteritis prevention programs for treatment and to provide information about the occurrence of new rotavirus strains.*

Keywords: *Rotavirus, adenovirus, rotavirus genotyping, vaccine.* ***Nobel Med 2016; 12(1): 87-93***

AKUT GASTROENTERİTLİ ÇOCUKLARDA ROTAVİRÜS, ADENOVİRÜSÜN SIKLIĞI VE ROTAVİRÜSÜN MOLEKÜLER EPİDEMİYOLOJİSİ

ÖZET

Amaç: Bu çalışmada, gastroenterit nedeni olan viral etkenlerden rotavirüs ve adenovirüs sıklığının belirlenmesinin yanısıra rotavirüsün yaygın genotiplerinin araştırılması amaçlanmıştır.

Materyal ve Metot: Çalışmaya, Afyon Kocatepe Üniversitesi Hastanesi Çocuk Hastalıkları Polikliniği'ne ishal yakınması ile başvuran, 6 yaşından küçük ve rotavirüs aşısı ile aşılanmamış çocuklardan toplanan, konvansiyonel yöntemler ile yaygın bakteriyel gastroenterit etkenleri ve parazit araştırılmış, her iki etken açısından negatif bulunan 492 dışkı örneği dahil edilmiştir. Ardından gaita örneklerinde immunokromatografik yöntem (ICT) ile rotavirüs ve adenovirüs antijenleri araştırılmış, rotavirüs pozitif örneklerde VP7 ve VP4 genlerini saptayan konsensus primerlerinin kullanıldığı reverse transkriptaz PCR sonrasında semi-nested type-specific multiplex PCR ile rotavirüsü G ve P genotipleri belirlenmiştir.

Bulgular: Akut gastroenteritli 492 çocukta adenovirüs ve rotavirüs pozitifliği sırasıyla %3.3 ve %20,3 olarak bulunmuş, G ve P genotiplerinin G1, G2, G4, G9 ve P[4], P[8] varyasyonlarını içeren toplamda 6 farklı kombinasyonu belirlenmiştir. En yaygın rotavirüs genotipleri G9P[8] (% 48,7) ve G9P[4] (% 17,5) olup bunları sırasıyla G1P [8] (% 16,2), G2P [8] (% 11,2), G1P [4] (% 3,7), G4P [8] (% 2,5) oranları ile takip etmişlerdir. Sonuçta, bölgemizdeki rotavirüs genotiplerinin %66'sının G9P[8] ve G9P[4] olduğu gösterilmiştir.

Sonuç: Rotavirüs enfeksiyonunun yüksek oranlarıyla ile ilgili olarak, gastroenterit önleme programları hakkında bilgilendirmenin yanı sıra yeni rotavirüs suşların ortaya çıkması hakkında bilgi sağlamak amacıyla sürekli izlem gereklidir.

Anahtar kelimeler: Rotavirüs, adenovirüs, rotavirüs genotiplendirme, aşı. **Nobel Med 2016; 12(1): 87-93**

INTRODUCTION

Acute gastroenteritis is the second most common cause of morbidity and mortality in children after lower respiratory tract infections. In terms of causative agents, viral enteropathogens are known to hold the first place, with a share of 30-70% in infectious diarrhea. Previous research has established that the rotavirus and enteric adenovirus serotypes 40-41 are the most common reason for gastroenteritis in children, especially in the age group of 0-5 years.[1] Almost half of all childhood diarrheas are caused by rotavirus.[2] Around the world, approximately 2 million children die from gastroenteritis each year, and rotavirus is the causative agent in 600,000 of these deaths.[3] The incidences of rotavirus infection are similar in developed and developing countries, although they differ in terms of outcomes. In developed countries, the number of deaths due to rotavirus infections are very small, whereas the hospitalization rate is quite high. In developing countries, on the other hand, mortality rates are also high, and the major factor involved in this situation is malnutrition. The similar incidences of rotavirus infection in developed and developing countries suggest that improvement in personal and communal hygiene or the development of sanitation applications are not very effective in the prevention of rotavirus infection; this highlights the importance of immunization.[4] In countries with moderate climatic conditions, like Turkey, rotavirus-induced diarrhea usually emerges in the winter and in infants aged 6-24 months.[2] Infections in humans may be caused by group A, B, and C rotaviruses; among these, group A is the most common infection, leading to 21-65% of cases of severe infantile gastroenteritis.[5]

The G and P genotyping of group A rotavirus strains is crucial in the detection of prevalent strains prior to rotavirus vaccine production, as well as during active monitoring studies after vaccination schedules and when genotypic changes are investigated. Moreover, before rotavirus vaccine routine use, rotavirus morbidity and mortality rates, the prevalence of rotavirus gastroenteritis, age-specific incidence, and hospitalization ratios must be analyzed for each individual country.[4,6] When it comes to adenoviruses, they are responsible for 5-15% of gastroenteritis cases in newborns and preschoolers, regardless of the season. Adenovirus types 40-41 may cause gastroenteritis, as well as types 2 and 31, although to a lesser extent.[7]

Our aim in this study is to determine the frequencies of rotavirus and adenovirus infections in children with acute gastroenteritis in our region, as well as the molecular epidemiology of rotaviruses. The results will contribute to future immunization and surveillance studies.

MATERIAL AND METHOD

Collection of Samples

Stool specimens obtained from children who aged 0–6 years and who were suspected to have viral

gastroenteritis in pediatric outpatient clinic of Afyon Kocatepe University between September 2012 and November 2013. Ethical permission was taken from Afyon Kocatepe University ethic committee (date: 06.12.2012 and no: B.30.2.AKÜ.0.20.05.04/64). The samples were sent to the microbiology laboratory and the relevant clinical and demographic data were collected through a questionnaire form.

Evaluation of Samples

Once the stool samples had been processed in the laboratory and upon macroscopic examination, softened or watery samples or samples which contained blood and mucous were included in the study. Direct microscopic examination was applied to search for the existence of erythrocytes, leukocytes, and intense yeast cells and/or pseudo-hyphae formation. Fresh slides or slides stained with Lugol's solution were checked for the presence of parasites. All samples were plated onto eosin methylene blue (EMB) agar and Salmonella shigella (SS) agar and inoculated into selenite F medium, and after incubation at 37°C for 24-48 h in the aerobic environment, they were evaluated for common enteropathogenic bacterial agents.

Immunochromatographic method (VIKIA Rota-Adeno Cassette Test, BioMérieux, France) was applied in accordance with the manufacturer's instructions to investigate whether rotavirus and enteric adenovirus antigens exist in fresh stool samples.

For stool samples with a positive result for the rotavirus antigen test, viral RNA extraction was performed using chloroform. Rotavirus G and P phenotypes were determined through reverse transcription polymerase chain reaction (RT-PCR) deploying consensus primers to amplify the VP7 and VP4 genes, followed by semi-nested specific multiplex PCR.

Genotyping

All antigen-positive samples were subjected to RT-PCR with consensus primers VP7-forward/VP7-reverse and VP4-forward/VP4-reverse to amplify the VP7 and VP4 genes, respectively.[8,9] For amplification of the VP7 gene, 5 ml of extracted RNA was reverse-transcribed and amplified using the Superscript one-step RT-PCR kit (Invitrogen) in the presence of 20 pmol of each primer, in particular, the VP7-forward and VP7-reverse primers described by Iturriza-Gómara et al.[8] Thermal-cycling was performed as follows: denaturation of dsRNA at 95°C for 5 min, reverse transcription at 45°C for 45 min, and then amplification of cDNA following the cycling parameters described by Iturriza-Gómara et al.[8] For amplification of the VP4 gene, cDNA was first synthesized with a random-hexamer primer using the first-strand cDNA synthesis kit (ThermoScientific, CA, USA). Then, the cDNA was amplified using 20 pmol of the VP4-forward/VP4-reverse primers described by Simmonds et al. in the PCR master mix (ThermoScientific).[9] The amplification conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Semi-nested type-specific multiplex PCR was used to identify P and G genotypes with the primers listed in Table 1. G typing was performed using 2 ml of the first-round PCR product, 20 pmol of each of specific primers targeted to G1, G2, G3, G4, G8, G9, and G10, and a VP7-R consensus primer in PCR master mix (ThermoScientific) following the cycling conditions described by Iturriza-Gómara et al.[8] P typing was performed using 2 ml of the first-round PCR product along with specific P[4] (10pmol), P[6] (5pmol), P[8] (15pmol), P[9] (5pmol), P[10] (5pmol), and P[11] (5pmol) primers with a VP4-F consensus primer (10pmol). Thermal-cycling was performed, including an initial denaturation at 95°C for 3 min followed by 35 cycles at 95°C for 45 s, 45°C for 45 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplification product was electrophoresed through a 2% agarose gel, and genotypes were determined by the sizes of the amplicons. Sequences of the primers used and the amplicon sizes of each genotype are shown in Table 1.

RESULTS

A total of 492 children younger than 6 years of age was admitted and pre-diagnosed with gastroenteritis. In this pediatric population, 20.3% were positive for rotavirus, 3.3% were positive for adenovirus, and 0.6% exhibited co-existing rotavirus and adenovirus infections, as determined via the immunochromatographic cassette testing method. Out of 100 rotavirus-positive cases, 61% were males and 31% were hospitalized, while 47% had applied to the emergency unit and 22% had visited the outpatient departments. The number of rotavirus infections began to rise in November and peaked in March (20%). Outside of the winter season, there was also a slight increase in April and June (8% and 9%, respectively). Adenovirus infections did not display a remarkable seasonality, but increases were observed in January and March (5% and 3%, respectively; Figure 3). In terms of the ages of patients, 78.8% were 0-24 months, 15% were 25-36 months, and 11% were 49-60 months; none had been previously immunized with rotavirus vaccine. Four different G genotypes of the rotavirus VP7 gene were determined; the most common G genotype was G9 (n=53), followed by G1

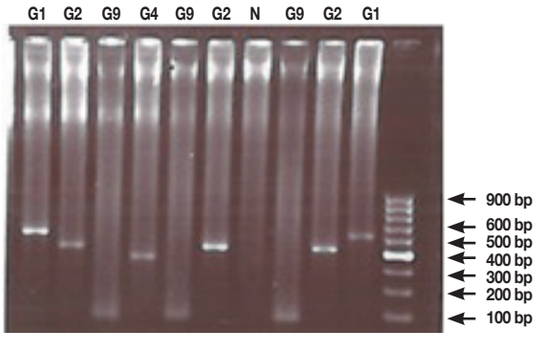

Figure 1: Gel electrophoresis of rotavirus G types

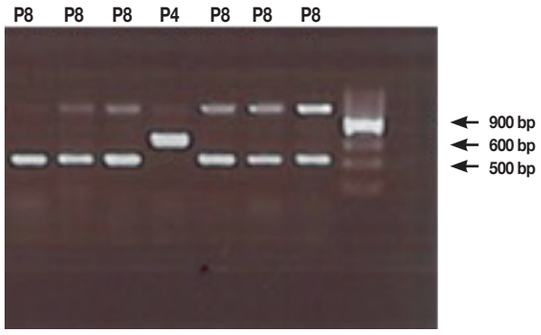

Figure 2: Gel electrophoresis of rotavirus P types

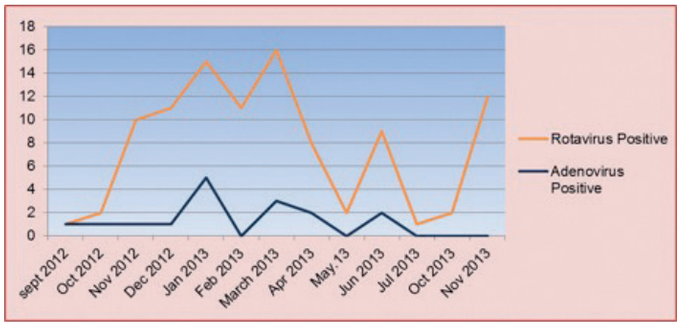

Figure 3: Seasonal distribution of the rotavirus and adenovirus(n)

(n=16), G2 (n=9), and G4 (n=2). Meanwhile, only 2 different P genotypes of VP4 gene were detected, namely P[8] (n=63) and P[4] (n=17; Figures 1 and 2).

When all rotavirus positive samples were checked for their VP7 (G genotype) and VP4 (P genotype) genotypes together, 6 various G/P combinations were encountered. In our study set, G9P[8] was the most predominant rotavirus combination detected in 39 cases corresponding to a ratio of 48.8% followed with the ratios of 17.5% (n=14), 16.3% (n=13), 11.3% (n=9), 3.8% (n=3), and 2.5% (n=2) by G9P[4], G1P[8], G2P[8], G1P[4], G4P[8], respectively (Table 2).

DISCUSSION

Acute gastroenteritis is a leading cause of morbidity and mortality in children. Each year, diarrhea emerges in 700 million of children under the age of 5, and diarrhea-related childhood deaths estimated to be 2,100,000.[10,11] Rotavirus infections globally cause 111 million diarrhea attacks and 25 million applications to the outpatient clinics consequently leading to hospitalization of 2 million children. Annually, more than 500 thousand child deaths are recorded due to severe rotavirus diarrheas, 85% taking place in developing countries.[12]

A review of different regional studies focusing on the rotavirus prevalence in 0-5 year-old children figures out a ratio of 44.8% in Bahrain, 10.7% in Greece, 34.3% in China for year 2013, and more recently 32.7% in Vietnam, 11.8% in Hong Kong and 36% in Sudan in year 2014.[13-18] Studies on rotavirus prevalence in Turkey revealed a positive result ratio of 18.1% in Izmir, in 2012; 28.3% in Ankara; 20.6% in Kayseri, and 20.3% in Mardin, in 2013.[19-22] Altindis et al. have conducted studies on rotavirus infections in our region in different years concluding ratios of 13.5%, 19.4% and 23.3% in years 2008, 2010 and 2011, respectively.[2,23,24] In our study the same ratio, rotavirus infections, was found to be 20.3%; a ratio similar to the results of studies across the world and in our country. The comparison of former and newer studies throughout the world points out a decline in the frequencies of rotavirus infections in certain developed countries, vaccination applications being responsible for this decline.[25] In our study, people with rotavirus infection were found out to be non-immunized individuals and rise has been noted in the regional infection rate over time. The adenoviral infection ratio in this study was calculated as 3.3% and was as well in consistency with the data available in the literature and in our region regarding the adenovirus prevalence.

Seasonal distribution of rotavirus infections is well-known. Various studies carried out in the world and in Turkey report a typical onset during early autumn which continues until the beginning of spring and continuation during the winter time is documented in Europe.[26] The infection has a seasonal distribution in countries with moderate climatic conditions, as in Turkey, and in general contracted most frequently during the winter and spring. Countrywide studies show rotavirus infections are most common in March (14.8%-20.1%) and then in January (14.4%-20.6%).[11,27] In parallel to the domestic data, in our study, rotavirus gastroenteritis cases were found to increase starting from November, reaching to peak values in January and March with decline commencing as of May. On contrary, our study involves a re-increase in rotavirus infections at the month of June (9%) which is attributable to high precipitation at this period in our region.

Studies across the globe and in Turkey have determined that rotavirus infections mostly occur during the first two years of life. In general, rotavirus infections emerging during the 0-24 months of age stand for the 71% of entire rotavirus infections. In a similar manner to the studies reflecting the whole country, regional studies in Turkey also report rotavirus gastroenteritis most predominantly during the age period of 12-24 months.[28] Our study, likewise, concluded that 78.8% of the cases with rotavirus infections were infants under two years old.

The information on the distribution of rotavirus serotypes by countries and regions plays an essential role in immunization strategies which are crucial in reducing the morbidity and mortality associated to rotavirus.[28,29] As vaccine use in involved in the agenda of diseases prevention, genotyping of the agents gains importance.[25] Taking into account that all cases in this study were non-immunized, the need to determine the predominant genotypes in our region appears clearly.

It is established that predominant strains in a given country may vary year after year.[30] A number of epidemiological studies have indicated that G1-G4 strains had a globally higher prevalence during early 1990s. Later, however, G9P[8] or G9P[6] strain had a higher appearance across the world. Increasingly determined rates of G8P[6] in Africa, G5P[8] in Brazil, and G10P[11] and G12P[6] in India refer to the newly emerging rotavirus genotypes. The great reassortment is evident by this trend and creates a concern as it poses a difficulty before the current and future studies related to rotavirus vaccine.[31] Than and Kim conducted a large-scale research on rotavirus strain in South Korea and identified the most predominant genotypes from 1989 to 2009. According to that study, most predominant genotypes found in 1989- 2007 and their corresponding ratios were as following: G1, G2, G3, G4, and G9 in 31%, 19%, 23%, 21%, and 5% along with P4, P6, and P8 in 22%, 24%, and 53%, respectively. Moreover, most predominant genotypes found in 2007-2009 and their corresponding ratios were as following: G1, G2, G3, G4, and G9 in 41%, 11%, 17%, 18%, and 12% along with P4, P6, and P8 in 10%, 32%, and 57%, respectively. The authors have highlighted the importance of further studies to figure out the long-term effectiveness of vaccines.[32]

The worldwide increase in the G9P[8] combination is as well concluded by this study. The ratio of G9P[8] in our region, according to the results of the study by Altindis et al., used to be 7.6% in 2009 which, in our study, was calculated as 48.8%. This nicely exemplifies how predominant strains may vary from year to year within a region and puts emphasize on the need of epidemiological studies in terms of vaccine content.

Table 1: Consensus and type specific primers for genotyping.

Primer	Sequence (5'-3')	Amplicon size(bp)
1. step (Consensus)		
VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	881
VP7-R	AAC TTG CCA CCA TTT TTT CC	
2. step (G-typing)		
G1	CAA GTA CTC AAA TCA ATG ATG G	618
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	521
G3	ACG AAC TCA ACA CGA GAG G	682
G4	CGT TTC TGG TGA GGA GTT G	452
G8	GTC ACA CCA TTT GTA AAT TCG	754
G9	CTT GAT GTG ACT AY[a]A AAT AC	179
G10	ATG TCA GAC TAC AR[b]A TAC TGG	266
VP7-R	AAC TTG CCA CCA TTT TTT CC	876
1. step		
VP4F	TGG CTT CGC CAT TTT ATA GAC A	876
VP4R	ATT TCG GAC CAT TTA TAA CC	
2. step (P-typing)		
P[4]	CTA TTG TTA GAG GTT AGA GTC	483
P[6]	TGT TGA TTA GTT GGA TTC AA	267
P[8]	TCT ACT GGR[b] TTR[b] ACN[c] TGC	345
P[9]	TGA GAC ATG CAA TTG GAC	391
P[10]	ATC ATA GTT AGT AGT CGG	583
P[11]	GTA AAC ATC CAG AAT GTG	312
VP4-F	TAT GCT CCA GTN AAT TGG	

a: Y = C or T, **b:** R = A or G, **c:** N= A, G, C or T.

Table 2: Genotype distribution of the rotavirus detected from Middle Anatolia.

G- Genotype (n)	P- Genotype (n)		TOTAL
	P4	P8	
G1	3	13	16
G2	-	9	9
G4	-	2	2
G9	14	39	53
TOTAL	17	63	80

Lately, studies across the world report an increase in all rotavirus G9 isolates.[33] The gradually increasing global rotavirus strain, G9, is similarly detected at a ratio of 66.3% in this study. G1, G3, G4, and G9 serotypes of rotavirus strain were associated to P[8] in almost all countries in the world whereas strains of G2 serotype are mostly stated to be associated with the P[4]

genotype.[32] Nevertheless, results in this study have associated all G2 strains with P8.

Recently, besides rotavirus G9 genotype, other uncommon novel rotavirus combinations such as G1P[4], G2P[8], and G6P[9] started to be detected and reported in quite high ratios in many countries. The ratio of such uncommon rotavirus types was found to range between 0-11.3% in European countries.3[3] In terms of lately reported novel strain combinations, this study detected G1P[4] and G2P[8] at a ratio of 3.8% and 11.3%, respectively.

According to the result of a multi-site study by Altindis et al. conducted between November 2006/ June 2007, taking only Central Anatolia region into account, out of 20 positive samples, 10 were G2P[4], 5 were G9P[8], and 1 sample was G9P[4] while 2 samples could not be typed and the remaining 2 were partially-typed.[23] Another study from 2009 in our region has evaluated 92 children under the age of 6 for viral agents, rotavirus being the most common viral agent with a ratio of 23.3%. The study also reported the genotyping results: G2P[4] was the most widespread rotavirus strain detected in 16 cases which corresponds to a ratio of 17.39% followed by 7 cases of G9P[8], 3 cases of G1P[8], 3 cases of G2P[8], 2 cases of G1+2P[8], 1 case of G9P[4], 1 case of G2+9P[8],1 case of G4+9P[6], and 1 case of G2P[4+8].[24] Another study, also conducted in the province of Afyon, in 2010, found G2P[4] (n=17) as the most common rotavirus sub-strain and in decreasing order the other genotypes were G9P[8] (n=9); G1P[8] (n=2), G2P[8] (n=2), G1+2P[8] (n=2), G9P[4] (n=1), G2+9P[8] (n=1), G4+9P[6] (n=1), and G2P[4+8] (n=1).[34]

In a similar manner to the studies performed in our region, studies carried out at the same site during different time periods may end up with diverging results. Results from our study indicates an increased rate of G9P[8] rotavirus strain in our region. The combinations of G9P[4], G1P[8] and G2P[8] have increased rates, as well. The G2P[4] strain, which used to be widely encountered in previous years holding the first place in terms of epidemiological significance was not detected at all in our study. Uncommon novel genotypes, namely, G2P[8] and G1P[4] as well as G2P[4] which had never been identified in former two studies in our region were isolated in this study.

CONCLUSION

As a result, a significant change was not concluded in our region regarding the rotavirus or adenovirus prevalence when compared to past studies while a remarkable change in terms of genotypes was detected. As it is proven that rotavirus genotypes may vary over time, continuity in this kind of studies is important to decide their conformability with the vaccine content and to provide contribution to national immunization policy.

* The authors declare that there are no conflicts of interest.

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