Investigation of Rotavirus with Various Methods in Children with Acute Gastroenteritis and Determination of Its Molecular Epidemiology in Kayseri Province, Turkey

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Background: In this study, the fresh stool samples from 254 children under 5 years of age with acute gastroenteritis which were delivered between October 2012 and December 2013 were collected. **Methods:** In the stool samples, rotavirus antigens were investigated using two different immunochromatographic methods which are routinely used at different times, namely the RIDA[®]QUICK Rotavirus/Adenovirus Combi Test (R-Biopharm AG, Germany) and the Genx[®] Rotavirus Test (Diamed-Lab, Turkey), in addition to the Rotavirus Ag (Stool) ELISA (DRG, Germany) kit. The results were compared with reverse transcriptase PCR (RT-PCR). **Results:** When the Genx[®] Rotavirus Test and RIDA[®]QUICK Rotavirus/Adenovirus Combi Test immunochromatographic methods were compared with RT-PCR, their sensitivity and specificity were found

as 97.1%, 100%, and 80.4%, 72%, respectively. As to the Rotavirus Ag (Stool) ELISA method, on the other hand, its sensitivity was found to be 95.1% and its specificity was 86.5%. The most common genotype was G9P[8] (40%), which was followed by the G1P[8] (18.7%) and G3P[8] (9.6%) genotypes. **Conclusion:** Consequently, it was revealed that the sensitivity of ELISA and immunochromatographic methods, which provide results in a short time and are used in the investigation of rotavirus antigen, was high and their specificity was low; further studies to determine the distribution of G and P genotypes will contribute to establishing strategies for vaccine development for rotavirus in the world. J. Clin. Lab. Anal. 31:e22030, 2017. © 2016 Wiley Periodicals, Inc.

Key words: ELISA; immunochromatographic methods; molecular epidemiology; rotavirus

INTRODUCTION

Rotavirus, the most important member of the Reoviridae family, is the major cause of serious gastroenteritis in children under 5 years of age, both in developed and developing countries (1, 2). Rotaviruses are nonenveloped, double-stranded RNA viruses with 11 segments. Viral RNA is surrounded by a three-layer capsid, including core capsid, inner capsid, and outer capsid. Based on the antigenic VP6 protein, rotaviruses have been subdivided into eight groups (A–H) by the International Committee on Taxonomy of Viruses (ICTV) (3). The vast majority of human rotaviruses are included in group A. VP7 and VP4 (proteinase-sensitive protein), the two structural proteins of the

outer capsid, determine the G and P genotypes of a virus, respectively (4, 5). Due to the segmented structure of viral RNA, genomic segment changes may occur between strains, and as a consequence, many G and P genotypes may occur (6, 7). In epidemiological studies, it was reported that the most common genotypes causing disease in children over the last 20 years have been G1P[8], G2P[4], G3P[8], and G9P[8] (2, 8).

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G12 is reported to be a recently emerged genotype in the world (2, 4).

While electron microscopy, cell culture, and nucleic acid amplification tests are also used in the laboratory diagnosis of rotavirus infections, ELISA, latex agglutination, and immunochromatographic methods are the most commonly used methods, as they are fast, they do not require special technical expertise and tools, their specificity and sensitivity are high, and they are easy to use (9).

In this study, our aim was to compare immunochromatographic and ELISA methods, the most commonly used methods in diagnosis, with the reverse transcriptase PCR (RT-PCR) method, and to contribute to vaccine and surveillance studies to be conducted in the future by determining common rotavirus genotypes in the Kayseri region.

MATERIALS AND METHODS

Sample Selection

As the aim of this study was to compare the methods used in diagnosis and of investigating rotavirus genotype distribution, fresh stool samples from 254 children under 5 years of age with acute gastroenteritis, which were delivered to the Erciyes University Hospital – Central Laboratory Serology Unit between of October 2012 and December 2013, were collected. The stool samples that were infested with parasites and bloody were excluded from the study. The stool samples collected from children with acute gastroenteritis were stored at -80°C until testing.

Rotavirus Antigen Detection

Rotavirus antigen was investigated in the stool samples with two different immunochromatographic methods, which are routinely used at different times, namely the RIDA[®]QUICK Rotavirus/Adenovirus Combi Test (R-Biopharm AG, Darmstadt, Germany; a total of 101 samples) and the Genx[®] Rotavirus Test (Diamed-Lab, Istanbul, Turkey; a total of 153 samples). In addition, Rotavirus antigen was also investigated by the ELISA (DRG, Marburg, Germany) in 254 stool samples.

Rotavirus PCR and Genotyping

All the antigen-positive and -negative samples (a total of 254) were transferred to the Public Health Agency of Turkey's, Molecular Microbiology Research Laboratory, and were tested for the determination of genotypes G and P, using the seminested multiplex

polymerase chain reaction (PCR) method, which was performed with consensus and genotype specific primers. Viral RNA was extracted using the EZ1 virus MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions (10). RT-PCR was carried out using the Superscript OneStep RT-PCR Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA,) using consensus primers VP7-forward/VP7-reverse (5'-ATGTATGGTATTGAATATACCAC-3'/5'-AACTTGCCACATTTTTC-3') and VP4-forward/VP4-reverse (5'-TGGCTTCGCCAT TTTATAGACA-3'/5'-ATTTCGGACCATTATAACC-3') to amplify the VP7 and VP4 genes, respectively. For amplification of the VP7 gene, the amplification conditions were 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. For amplification of the VP4 gene, the thermal cycling conditions were carried out as follows: denaturation at 95°C for 3 min, 35 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min, and final extension at 72°C for 10 min (10–12).

Seminested type-specific multiplex PCR was carried out for the determination of the genotypes P and G. For G genotyping, using a mix of specific primers (G1–4, G8–10), VP7-consensus primer and first-round PCR product were used. For P genotyping, specific primers VP4-F, P(4), P(6), and P(8–11) were used. Amplification was performed as 35 cycles at 95°C for 45 s, 45°C for 45 s, and 72°C for 1 min after denaturation. The amplification product was submitted to electrophoresis through a 2% agarose gel, and genotypes were determined by the sizes of the amplicons (10, 13). The PCR amplicons were purified with the Agencourt AMPure kit (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA) and the sequencing was performed using consensus primers for the VP7 and VP4 genes. The sequencing reaction was performed as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 96°C for 20 s, annealing at 55°C for 20 s, and elongation at 60°C for 4 min. The sequence data were provided from an automated analysis and sequencing system (Beckman Coulter CEQ 8000). The obtained sequences were genotyped using the National Center for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD) Basic Local Alignment Search Tool (BLAST) server on GenBank database.

Statistical Analyses

The data obtained in this study were statistically analyzed using SPSS 15 (Statistical Package for Social

Sciences, Chicago, IL). The agreement of the RT-PCR method with RIDA[®]QUICK Rotavirus/Adenovirus Combi and Genx[®] Rotavirus immunochromatographic methods, and ELISA were measured using the Cramer's Phi coefficient of association. Additionally, the sensitivity and specificity values were calculated for all methods.

Ethical Approval

The study was approved by Erciyes University Research Ethics Committee (Resolution number: TYL-2015-5610), Kayseri, Turkey.

RESULTS

One hundred and thirteen (73.9%) of 153 stool samples using the Genx[®] Rotavirus Test, 70 (69.3%) of 101 samples using the RIDA[®]QUICK Rotavirus/Adenovirus Combi Test immunochromatographic methods, 169 (66.5%) of 254 samples using the Rotavirus Ag (Stool) ELISA method, and 165 (65%) of 254 samples were detected as positive (Table 1). When the Genx[®] Rotavirus Test and RIDA[®]QUICK Rotavirus/Adenovirus Combi Test immunochromatographic methods were compared with RT-PCR, their sensitivity and specificity were found as 97.1%, 100% and 80.4%, 72%, respectively. However, the sensitivity and specificity of Rotavirus Ag (Stool) ELISA method was found to be 95.1% and 86.5%, respectively. The compatibility was found to be significant in all the three methods. The RIDA[®]QUICK Rotavirus/Adenovirus Combi Test method was found to be moderately compatible (Cramer's $V = .773$, $\Phi = 0.773$), whereas the other methods, namely the Genx[®] Rotavirus Test

(Cramer's $V = 0.810$, $\Phi = 0.810$) and Rotavirus Ag (Stool) ELISA (Cramer's $V = 0.826$, $\Phi = 0.826$), were found to be highly compatible.

The most common rotavirus genotype G9P [8] ($n = 66$) had a frequency rate of 40% and it was followed by G1P [8] ($n = 31$) at 18.7%, G3P [8] ($n = 16$) at 9.6%, G2P [4] ($n = 11$) at 6.7%, G9P [4] ($n = 9$) at 5.4%, G2P [8] ($n = 8$) at 4.9%, G4P [8] ($n = 5$) at 3%, and others (G3P[4], G9P[4]P[8], G2P[4][8], G3P[4][8], G3G2P[4], G1P[4], G8P[4], G4P[4]P[8], G1P[4][8], G2G9P[8], G10G4P[8], G9P[10], and G9G1P [8]) ($n = 19$) at 11.5%. The G9 genotype (48.5%) was the most common G genotype, followed by G1 (20%), G2 and G3 (13.3%), G4 (3.7%), and G8 and G10 (0.6%). The most common P genotype was P[8] with a frequency rate of 77.5%, followed by P[4] at 21.8% and P[10] at 0.6%.

DISCUSSION

Rotavirus is one of the most important viral etiologic agents of acute gastroenteritis in children under 5 years old. It is estimated that annually 453,000 children die from gastroenteritis associated with rotaviruses and most of the deaths occur in developing countries (14).

ELISA and latex agglutination tests are widely used in the detection of rotavirus antigen in stool samples (15). These tests are not very expensive, they allow quick diagnosis, and their sensitivity and specificity are high (16). In a study in which 86 stool samples were tested with three different immunochromatographic methods and compared with RT-PCR, it was reported that the sensitivity rates of the IP-Rota V, Dip Stick Eiken ROTA, and ROTA-ADENO test kits were 97.2%, 95.8%, and 88.7%; their specificity rates were 100%, 93.3%, and 100%, respectively (16). In a study in which four immunochromatographic methods and three ELISA methods were compared with in-house PCR methods, it was reported that the sensitivity rates of the test kits were between 80% and 100%, and their specificity ranged between 99.4% and 100%, except for an immunochromatographic method its specificity was found to be 54.3% (17). In another study in which an immunochromatographic method (RIDAQuick Rotavirus kits, Darmstadt, Germany) and ELISA method (RIDASCREEN[®] kit, Darmstadt, Germany) were compared with RT-PCR, their sensitivity and specificity were found to be 90%, 88% and 100%, 100%, respectively (18). However, in our study, the sensitivity and specificity of the Genx[®] Rotavirus Test and RIDA[®]QUICK Rotavirus/Adenovirus Combi Test immunochromatographic methods were found to be 97.1%, 100% and 80.4%, 72%, respectively. The

TABLE 1. Comparison of Various Methods for the Detection of Rotavirus

	Immunochromatographic methods	Rotavirus Ag (Stool) ELISA Test (%)	RT-PCR (%)
	Genx [®] Rotavirus Test (%)		
Positive	113 (73.9)	109 (42.9)	107 (42.1)
Negative	40 (26.1)	44 (17.3)	46 (18.1)
Total	153 (100)		
	RIDA [®] QUICK Rotavirus/Adenovirus Combi Test	Rotavirus Ag (Stool) ELISA Test (%)	RT-PCR (%)
Positive	70 (69.3)	60 (23.7)	58 (22.9)
Negative	31 (30.7)	41 (16.1)	43 (16.9)
Total	101 (100)	254 (100)	254 (100)

Data are presented as n (%). RT-PCR, Reverse transcriptase PCR.

sensitivity of the Rotavirus Ag (Stool) ELISA method was 95.1% and its specificity was 86.5%. Frühwirth et al. (19) reported that the ELISA was false positive for 12% of the samples compared with the results of RT-PCR. In our study, when compared with the results of RT-PCR, the false positivity of ELISA was found as 2.4%.

The prevalence of rotavirus genotypes varies according to years and regions (20). Epidemiological studies revealed that the G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] genotypes were the most common cause of the disease in children over the past 20 years (8, 21). In the United States, it was reported that the most common genotypes are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G9P[6] (22). In a study in which 16 laboratories from 15 European countries participated, similar to the study in the United States, the most common genotype in Europe was reported to be G1P[8] and it was followed by the G2P[4], G3P[8], G4P[8], and G9P[8] genotypes (23). Antunes et al. (24) investigated rotavirus genotypes in an unvaccinated population in northern Portugal in 2007, and they reported the most common genotype was G2P[4] with a rate of 68.6% and it was followed by G9P[8] with a rate of 14%. In South Korea, India, Japan, and Indonesia, the most common genotype was G1P[8]; it was followed by the G4P[6] genotype in South Korea, by G9P[8], G2P[4], and G9P[4], respectively, in India, by G3P[8] and G9P[8] in Japan, and by G1P[6] and G2P[4] genotypes in Indonesia (25–28). The most common genotype in Brazil was reported to be G2P[4], and it was followed by the G1P[8] genotype (28). It was reported that the most common genotype in Nigeria was G4P[8], and although it varies according to years, the most common genotypes in Cameroon were G9P[8], G1P[8], and G3P[6] (30, 31).

In studies from Turkey, Durmaz et al. (12) reported that the most common genotype was G9P[8] (40.5%) and it was followed by the G1P[8] (21.6%), G2P[8] (9.3%), and G2P[4] (6.5%) genotypes. In our study, the most common genotype was similarly found to be G9P[8] (40%) and it was followed by the G1P[8] (18.7%), G3P[8] (9.6%), and G2P[4] (6.7%) genotypes. In a study conducted in a province in the northeast of Turkey, the most common genotype was found to be G9P[8] and the second was G2P[4] genotype (10). However, in a study covering nine provinces in the west and southeast of Turkey, the most common genotype was found to be G4P[8] (32).

There are two oral attenuated rotavirus vaccines, developed with the purpose of preventing of rotavirus gastroenteritis, and these have been licensed and used in many countries since 2006; they are Rotarix™ (GlaxoSmithKline, Belgium) and Rotateq™ (Merck &

Co., NJ). While the Rotarix™ vaccine provides a high level of protection against rotavirus gastroenteritis caused by G1, G3, G4, and G9 types, its protection is low for the G2 type (33). It was reported that the Rotateq™ vaccine, which is applied in three doses, provides a high level of protection against the G1–G4 and G9 types (34). Rotavirus vaccines are available in our country; however, they have not been included in the national vaccination program. It can be said that the coverage ratio of current rotavirus vaccines for the genotypes detected in our country is quite high.

As a result, we conclude that the sensitivities of ELISA and immunochromatographic methods, which provide fast results and are used to investigate rotavirus antigen, is high; their specificity is lower; the most common rotavirus antigen is G9P[8] and determining the distribution of G and P genotypes will contribute to establishing strategies for vaccine development for rotavirus in our country and in the world.

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