



# Prevalence and Genetic Diversity of Norovirus in Acute Gastroenteritis Cases in Aydın, Turkey

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**Aims:** Noroviruses may cause both epidemic and sporadic acute gastroenteritis (AGE) globally. Thus, this study evaluated the prevalence of norovirus in stool samples of hospitalized patients with AGE in Aydın, Turkey using enzyme-linked immunoassay (ELISA) and real-time reverse transcription-polymerase chain reaction (rRT-PCR) and genotyped positive samples to detect which genotypes have currently circulated.

**Methods:** This retrospective descriptive study collected 92 stool samples from patients with AGE symptoms from Aydın Adnan Menderes University Hospital from September 2017 to May 2019. The samples were tested using the commercial Third Generation Ridascreen norovirus ELISA and rRT-PCR. Positive samples were

genotyped by sequencing of conventional positive RT-PCR products followed by phylogenetic analysis.

**Results:** Of the 92 samples, 5 (5.4%) using ELISA and 12 (13%) using rRT-PCR tested positive for norovirus. All positive samples were genogroup II (GII). Two norovirus positive samples were genotyped successfully using DNA sequencing of the nested conventional PCR products. One sample (GII/Hu/TR/2019/Aydın25) could be categorized as GII.3 and the other (GII/Hu/TR/2019/Aydın20) as GII.13.

**Conclusion:** rRT-PCR testing of stool samples is significantly more sensitive than Ridascreen ELISA. Two norovirus positive samples were successfully genotyped. Data from our study provide protocols on how to study the epidemiology of norovirus.

## INTRODUCTION

Enteric viruses, such as enteric adenovirus, rotavirus, norovirus, astrovirus, and sapovirus are the most common etiologic agents of childhood viral gastroenteritis.<sup>1</sup> Norovirus is also one of the most frequent causes of acute gastroenteritis (AGE) outbreaks in semi-closed settings, such as schools, hospitals, nursing homes, worship places, cruise ships, and military facilities.

Moreover, norovirus has emerged as the most frequent cause of AGE in children in countries with public vaccination programs including rotavirus vaccine.<sup>2</sup> Norovirus may lead to severe AGE in developing countries, with an estimated 70,000–210,000 deaths in children annually.<sup>3</sup>

Noroviruses belong to the family *Caliciviridae*. Norovirus generally show high genetic variability and are classified into 10 genogroups (GI to GX) based on the capsid (VP1) protein. They can be further divided into 48 capsid genotypes based on VP1 and 60 P-types.<sup>4</sup>

Robust cell culture or small animal model is unavailable; however, successful replication of human norovirus in human intestinal enteroids has been reported.<sup>5</sup> Norovirus can be detected by laboratory assays, such as enzyme-linked immunoassay (ELISA) and immunochromatographic assays, that detect the viral antigen, and by molecular tests, such as real-time reverse transcription-polymerase chain reaction (rRT-PCR), which is accepted as the gold standard for norovirus diagnosis.<sup>3</sup>

This study aimed to determine the presence of norovirus by ELISA and rRT-PCR methods in AGE cases in Aydın, Turkey. Additionally, positive samples were genotyped to understand which genotypes have currently circulated in our hospital.

## MATERIAL AND METHODS

This retrospective descriptive study was conducted between September 2017 and May 2019. Stool samples were collected from patients (0–83 years old) who are admitted with AGE symptoms

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to the Aydin Adnan Menderes University, School of Medicine Hospital. This study included 92 fecal samples, which are stored at  $-80^{\circ}\text{C}$  until laboratory analysis. This study was approved by the Clinical Research Ethical Review Committee of the Aydin Adnan Menderes University (No:2017/1229) and supported by Aydin Adnan Menderes University Research Fund (Project number: 17058).

Fecal samples of patients with AGE were tested for norovirus by Ridascreen norovirus Antigen ELISA kit (R-Biopharm, Darmstadt, Germany), which can qualitatively determine norovirus genogroup I and II viruses. Tests were performed following the manufacturer's instructions. The cut-off value was determined by adding 0.150 above the measured optical density (OD) value of the negative control. Samples with OD above the cut-off value were accepted as positive for the norovirus antigen. Co-infections with adenovirus and rotavirus were detected by an immunochromatographic test (Rotavirus and Adenovirus Combo Rapid Test, Hangzhou, China).

RNA extraction was done by Bosphore Viral RNA Extraction Spin Kit (Anatolia Geneworks, Istanbul, Turkey) using the Magnesia<sup>®</sup> instrument (Anatolia Geneworks, Istanbul, Turkey). Internal controls were included in each sample during the extraction step. Norovirus RNA was then amplified by the Bosphore<sup>®</sup> norovirus Detection Kit v1 in Montania 4896 instrument (Anatolia Geneworks). PCR amplification was performed following the manufacturer's instructions.

Conventional RT-PCR assays were performed to amplify a partial region of the VP1 gene using primer pairs NV-GIIF1 (5' GGG AGG GCG ATC GCA ATC T 3') and NV-GIIR1 (5' CCR CCI GCA TRICCR TTR TAC AT 3'), which amplify a 5'-region of VP1.<sup>6</sup> Additionally, JV12 (5' ATA CCA CTATGA TGC AGA TTA 3') and JV13 (5' TCA TCATCA CCA TAG AAA GAG 3') primers were used to amplify a partial region of the RdRp gene. Extracted RNA was first converted into complement DNA (cDNA) using the Thermo First Strand cDNA kit. The PCR conditions for partial VP1 gene amplification included initial denaturation of the nucleic acid for 5 min at  $95^{\circ}\text{C}$ , followed by 35 cycles at  $95^{\circ}\text{C}$  for 30 sec,  $49^{\circ}\text{C}$  for 30 sec,  $72^{\circ}\text{C}$  for 1 min, and a final extension of 7 min at  $72^{\circ}\text{C}$ . Nucleic acid was first denatured for 5 min at  $95^{\circ}\text{C}$  to amplify the partial gene, followed by 35 cycles of PCR at  $95^{\circ}\text{C}$  for 30 sec,  $44^{\circ}\text{C}$  for 30 sec,  $72^{\circ}\text{C}$  for 1 min, and a final extension of 7 min at  $72^{\circ}\text{C}$ . PCR products were separated and visualized on a 1% agarose gel and then purified using a commercial kit (Invitrogen Combo Purification kit). Purified products were processed to Sanger sequencing with ABI 3100 Genetic Analyzer (Applied Biosystems, USA)

Obtained sequences were checked in BLAST implemented in the GenBank database. The phylogenetic tree was constructed with norovirus reference strains using a computer-based tool (MEGA v10.0) to understand the genotypes of our sequences.

Descriptive statistics were shown as frequency, percentage, median (25<sup>th</sup>–75<sup>th</sup> percentile), and minimum and maximum values. The chi-square or Fisher exact test was used to compare the categorical variables. A  $p$ -value of  $<0.05$  was considered significant.

## RESULTS

During the study period, fecal specimens from 92 patients with AGE were collected, of which 55 (59.8%) were males and 37 (40.2%) were females. The age of patients ranged from 0 to 83 years with a median age of 2 years.

Of the 92 samples, 5 (5.4%) using ELISA and 12 (13%) using rRT-PCR tested positive for norovirus. Five patients with an ELISA positive result were all males, whereas 10 were males and 2 females in the 12 patients with positive real-time RT-PCR. A statistically significant difference was found between norovirus positivity by ELISA and age groups ( $p = 0.002$ ); however, positivity by rRT-PCR between age groups was not statistically significant ( $p = 0.182$ ) (Table 1). Samples from the 0–5 years age group most frequently tested positive for norovirus; however, no significant difference was found in the positivity rate between the 0–2 years and 3–5 years age group for either the samples tested by ELISA or by rRT-PCR ( $p = 1.000$  for both tests, data not shown). Overall, 12 samples tested positive for GII, of which two (GII/Hu/TR/2019/Aydin20 and GII/Hu/TR/2019/Aydin25) samples were successfully sequenced. Using BLAST, 1 sequence (GII/Hu/TR/2019/Aydin25) had the highest sequence identity with GII.3 viruses, whereas 20 was identical to GII.13 in sequence GII/Hu/TR/2019/Aydin (Figure 1). The following accession numbers were assigned by GenBank (GII/Hu/TR/2019/Aydin20) MT815529 (VP1), MW392526 (RdRp); GII/Hu/TR/2019/Aydin25 MT815530 (VP1), MW392525 (RdRp).

Norovirus was detected throughout the year, but its prevalence was highest in the winter and spring (Table 2). No statistically significant difference was found between norovirus positivity and seasons ( $p = 0.192$ ). Co-infections with rotavirus or adenovirus were detected in 9 cases.

Table 1. Detection of Norovirus by ELISA and Real-time RT-PCR According to Age Groups

Age groups	ELISA results (n,%)*		PCR results (n,%)**	
	Positive	Negative	Positive	Negative
0–5	3 (3.3)	68 (73.9)	8 (8.7)	63 (68.5)
6–17	-	16 (17.4)	2 (2.2)	14 (15.2)
≥18	2 (2.2)	3 (3.3)	2 (2.2)	3 (3.3)
Total	5 (5.4)	87 (94.6)	12 (13)	80 (87)

\* $p = 0.002$ ; \*\* $p = 0.182$

Table 2. Seasonal Distribution of Real-time RT-PCR Results for Norovirus

Season	PCR results (n,%)		
	Negative	Positive	Total
Winter	22 (75.9)	7 (24.1%)	29 (100%)
Spring	46 (92%)	4 (13.7%)	50 (100%)
Summer	5 (100%)	0	5 (100%)
Autumn	7 (87.5%)	1 (3.4%)	8 (100%)
Total	80 (87%)	12 (13%)	92 (100%)

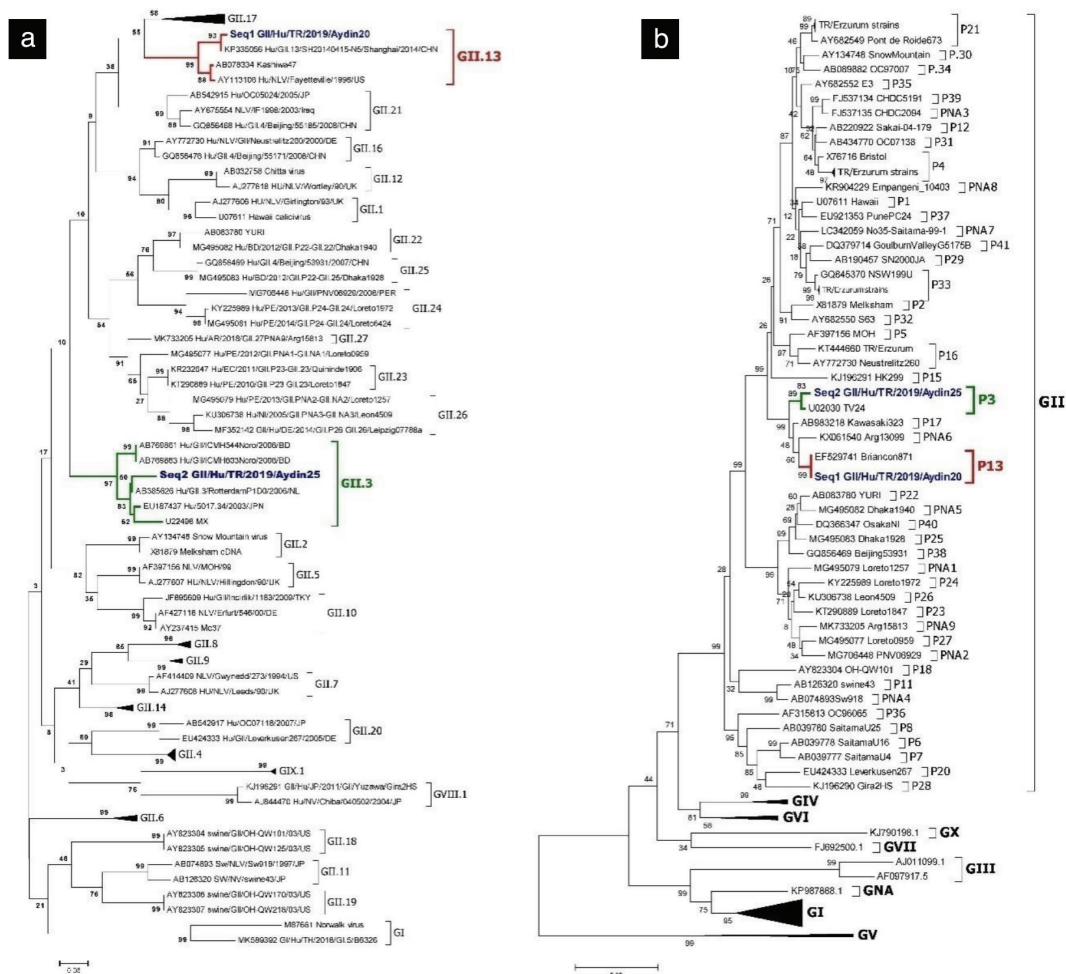


FIG. 1. (a) Molecular phylogenetic analysis based on VP1 by Maximum Likelihood method. (b) Molecular phylogenetic analysis based on RdRp by Maximum Likelihood method. Turkish new sequences, GII/Hu/TR/2019/Aydin20 and GII/Hu/TR/2019/Aydin25, were categorized as GII.13 and GII.3, respectively.

### DISCUSSION

The present study determined the prevalence and genetic diversity of norovirus that is detected in hospitalized patients with AGE in Aydin, Turkey. The Ridascreen ELISA had a sensitivity of 42% and a specificity of 100% compared to the rRT-PCR. All rRT-PCR positive samples (100%) were positive for GII. The two samples that were successfully amplified by conventional RT-PCR could be genotyped as GII.3 and GII.13.

Based on a systematic review, norovirus causes AGE in approximately 20%–24% of patients in community or outpatient clinics and 17% of hospitalized patients worldwide.<sup>3</sup> The first norovirus outbreak was reported from central Anatolia in 2008 in Turkey.<sup>7</sup> Several water and foodborne norovirus outbreaks have been reported since then.<sup>8-10</sup> Altindis et al.<sup>11</sup> reported norovirus in 17% of sporadic AGE in Turkey.

Previous studies used ELISA and revealed the rate of norovirus ranged from 9.7% to 26%.<sup>7,12-14</sup> Our study revealed 5 (5.4%)

stool samples that were positive for norovirus using ELISA, which was similar to a recent report from Canakkale.<sup>12</sup> Previous norovirus prevalence determined by rRT-PCR ranged from 15.1% to 33.4%.<sup>7,11-13,15,16</sup> Using rRT-PCR, a similar positivity rate was found in our study.<sup>11-13</sup> The sensitivity rate of the ELISA test was similar to other studies, which reported the sensitivity rates for Rida screen norovirus kit that range from 31.6% to 65.3%, respectively.<sup>17</sup> The ELISA kit showed a lower sensitivity than reported by Dimitriadis et al.<sup>18</sup> in Australia (71%), Aksuet al.<sup>12</sup> in Turkey (60%), and Zhang et al.<sup>19</sup> in China (96%), which was similar to a study conducted by Rovida et al.<sup>20</sup> Most studies, including ours, a >90% ELISA specificity.<sup>7,12,16</sup> Due to its high sensitivity and specificity, rRT-PCR is considered the gold standard for norovirus detection.

Globally, GII noroviruses are associated with 90%–100% of all infections.<sup>12,16,21</sup> The limited studies from Turkey that performed genotyping primarily detected GII.4, GII.6, GII.16, and GII.21 viruses.<sup>12,15-16</sup> To the best of our knowledge, this is the first report

on detected GII.3 and GII.13 genotypes of norovirus infections in Turkey.

Co-infections with two or more enteric pathogens are common, and the increasing use of molecular techniques has revealed even higher rates.<sup>22</sup> Our study detected co-infections in 9 (9.8%) norovirus rRT-PCR-positive samples. The most common co-infections were with rotavirus (n = 8) and adenovirus (n = 1).

Our study has several limitations. The number of participants with AGE was small and we could only successfully sequence 2 samples, which indicates that the used primers were suboptimal for robust genotyping.

In summary, our study detected and genotyped noroviruses in Aydın, Turkey with tourism and increased social interactions between the Aegean part of Turkey and Europe, thereby increasing the risk of norovirus transmission. Our pilot data will increase the knowledge of the importance of norovirus infections in Turkey.

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