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Frequency and molecular characterization of human norovirus in Erzurum, Turkey

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Background/aim: There are limited studies on genotyping and phylogenetic analysis of norovirus in Turkey, and this has not previously been studied in the Eastern Anatolia region. The aim of the present study was to determine the norovirus profile in this region with genotyping and phylogenetic analysis.

Materials and methods: Included in the study were stool samples obtained from 427 people from different age groups in Eastern Anatolia. The nucleic acid samples isolated by the automatic system and nucleic acid sequence reactions and phylogenetic analyses were performed on RNA samples.

Results: The presence of norovirus was detected in 86 (20.1%) of the 427 stool samples by RT-PCR analysis. Twenty-six samples selected randomly from norovirus–RNA positive samples were subjected to the sequence reaction. In 24 of the 26 samples, genogroup GII was determined, as well as one each from GI and GIV in sequence reactions. Four different genotypes were detected in genogroup GII, which were determined to be the dominant types. These were GII.1, GII.4, GII.16, and GII.21. The GI.6 and GIV.1 genotypes were determined in genogroups GI and GIV, respectively.

Conclusion: The high frequency and genetic diversity of these infections are risk factors for disease and so vaccine studies should be undertaken in consideration of this situation.

Key words: Genotype, norovirus, molecular epidemiology, viral gastroenteritis, Turkey

1. Introduction
Noroviruses have a privileged position compared to other viral gastroenteritis agents transmitted via the fecal–oral route since they infect people of all ages (1). Compared to other microbes causing diarrhea, noroviruses are the most common cause in both adults and children in areas where universal rotavirus vaccination has been introduced (2–6). The human reservoir for noroviruses is large, with multiple transmission routes such as person-to-person contact, waterborne, food-borne, and airborne droplets. Noroviruses are contagious at a relatively low 50% infective dose (7) and highly resistant to inactivation by freezing, heating, and detergent-based cleaning (8,9). Knowledge of the extent of norovirus infections as a cause of diarrhea is important for establishing prevention and treatment profiles.

The norovirus virion, which is a nonenveloped and single-chain RNA virus with positive polarity, is approximately 30 nm in diameter. Norovirus is one of the five genera of the family Caliciviridae (10). The other genera in this family are Lagovirus, Nebovirus, Sapovirus, and Vesivirus. Norovirus, which affects both human and nonhuman species, is divided into six different genogroups based on the determined sequences of their RNA-dependent RNA polymerase (RdRp) and/or major capsid protein (VP1) genes: I, II, III, IV, V, and VI (11). GI, GII, and GIV types in these genogroups have been identified in humans (12). The GI genogroup is detected in humans only; GII in humans and porcines; GIII in cattle; GIV in humans, cats, and dogs; GV in murines; and GVI in dogs (13). Transmission between different animal species was also reported but there are some studies referring to zoonotic transfer of GII.4-like norovirus strains to the human population from either a swine or bovine source (14) and GIV.2 strains from a dog source (15). At least 40 genotypes of human noroviruses have been identified (16), the majority of them in recent years (17). The most identified genogroup in the world is GII, specifically genotype GII.4 (18). GI is divided into nine genotypes, GII into 22, and GIV into two, based on complete capsid gene sequences (19). Sequencing methods provide the possibility of identifying new genotypes of...
pathogenic agents including viruses and sequencing of the RNA-dependent RNA polymerase gene region has good detection performance (16,17).

A recent publication described human norovirus replication in cell culture, although in a very crude and insensitive system. However, it is a start (20). Noroviruses were responsible for 42% of food-borne infections in the United States in 2009 and 2010 (21). There are limited numbers of studies on norovirus gastroenteritis conducted in Turkey (22–24).

In the present study, the regional profile of norovirus is intended to reveal the molecular characterization and phylogenetic analysis of norovirus in patients among various age groups admitted to hospital with complaints of gastroenteritis in Erzurum, Turkey.

2. Materials and methods

2.1. Study population and specimens

This molecular study was approved by the Ethics Committee of Atatürk University School of Medicine (date/protocol number: 12.11.2012/133) and supported by Atatürk University Scientific Research Projects Coordination (project number: 2012/361) in Erzurum, Turkey. Stool specimens from 427 patients, including 262 males and 165 females, were investigated. The specimens were obtained from patients admitted in 2012 and 2013 with complaints of gastroenteritis (including diarrhea, vomiting, nausea, and stomach upset) to the Faculty of Medicine Research Hospital and to Erzurum Regional Training and Research Hospital, representing the most patient potential in East Anatolia.

2.2. RNA extraction

Ribonucleic acid (RNA) isolation from stool specimens was performed using the QIAsymphony SP extraction device (Qiagen, Germany) and the QIAsymphony DSP Virus/Pathogen Midi Kit (Qiagen, Germany). The isolation process was performed according to the manufacturer's instructions, and 85 µL homogenate of nucleic acid was finally obtained.

2.3. Reverse transcription and PCR

Extracted RNA samples were converted into complementary DNA (cDNA) by reverse transcription (RT) before the PCR procedure. The RT process used a First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) and the reaction was carried out as stipulated by the manufacturer. For the PCR process, Taq DNA polymerase (Thermo Fisher Scientific, USA) and primer pairs JV12/ JV13, defined by Vinje and Koopmans (25), were used. The PCR reaction was also carried out according to method described by Vinje and Koopmans (25). Briefly, 5 µL of cDNA, 25 µL of 2X Thermo-Start PCR Master Mix, 1 µL of forward primer (10 pmol/µL), 1 µL of reverse primer (10 pmol/µL), and 18 µL of nuclease-free water were added to the test tube and the tube was placed into a thermal cycler for the PCR cycles. Samples were denatured for 3 min at 94 °C and subjected to 40 cycles at 94 °C for 1 min, 37 °C for 1 min 30 s, and 74 °C for 1 min. Agarose gel electrophoresis was performed and amplicons with a length of 327 bp were considered positive for norovirus.

2.4. PCR clean-up and sequence analysis

Clean-up of the amplicons obtained after PCR was performed with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's recommendations. We had sequence reactions of the purified PCR amplicons done by a commercial company (Pendik Veterinary Control Institute, İstanbul, Turkey).

PCR products were sequenced with Sanger sequencing methods, briefly the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) on ABI 3110xl DNA analyzer (Applied Biosystems, USA), and sequenced at least twice in both directions. Sequencing was done with both forward (JV12 (ATACCACTATGATGCAGATTA) and reverse (JV13-TCATCATCACCATAAGAGG) primers. Amplicon length was 4549–4875st nucleotide (Accession number: KF306212) by 327 base pairs. Raw data received in dendrogram-based form were reorganized and transferred to the MEGA 5.1 program for phylogenetic analysis (26). Phylogenetic analysis was performed according to the partial nucleotide sequence of the RNA-dependent RNA polymerase gene. The phylogenetic map was constructed by comparing the gene sequences of our strains with the gene sequences of strains previously entered into the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). In order to determine the alignment and degree of similarity among sequences at the nucleotide and amino acid levels in the strains, BioEdit version 7.2.5 software was used (27).

3. Results

The presence of norovirus was detected in 86 (20.1%) of the 427 stool samples included in the study by RT-PCR analysis. Twenty-six specimens, selected randomly from those exhibiting a good band in agarose gel electrophoresis of PCR products of 327 bp length, were subjected to sequencing. All norovirus genogroups infecting humans (GI, GII, and GIV) were found in the phylogenetic analysis of our region. In 24 of 26 samples, genogroup GII was identified, along with one each from GI and GIV. The gene sequences of our strains with the gene sequences of strains previously entered into the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). In order to determine the alignment and degree of similarity among sequences at the nucleotide and amino acid levels in the strains, BioEdit version 7.2.5 software was used (27).

Phylogenetic analysis was performed to evaluate the genetic relationships between norovirus-positive samples that had a 327 bp sequence and 33 published reference
strains (Figure). Since we had only one strain in the GI genogroup (GI/Hu/TR/2013/GI.6/Erzurum384), we compared this strain within the GI genogroups. In this comparison, the identities at the nucleotide level the farthest from the GI.7 genotypes (69.4%) and the closest to its own group of GI.6 genotypes (97.0%) were identified. The amino acid sequence similarities of this group were found to be 77.5% and 97.5% at the lowest and the highest values in the GI.7 and GI.6 groups, respectively. The GIV.1 genotype (GIV/Hu/TR/2013/GIV.1/Erzurum427), identified as only one strain in our study, was compared with the GIV.1 and GIV.2 strains, identified as limited to the GIV genogroup. This strain showed a similarity of 90.3%–68.6% with these strains for nucleotide sequences and 97.5%–67.5% for amino acid sequences. In our study, within the GII genogroup, identified as dominant in Turkey as well as the rest of the world, four different GII genotypes have been identified. Eight GII.1 strains from our study were determined to be 95.3%–100%. The similarities of these strains with the GenBank strains were found to be 90.0%–100% for amino acid sequences and 90.0%–100% among themselves.

The nucleotide similarity ratios of our GII.4 strains to the KF738705 GenBank strain were 94.1%–95.8% and 95.8%–100% among themselves. These ratios were 95.0%–96.2% and 98.7%–100% for amino acid sequence similarities, respectively. In addition, similarities for nucleotide sequences identified within the same genotype in a study previously conducted in Turkey by Altindis et al. (22) and our strains were 96.2%–97.9%, and 97.5%–98.7% for amino acid sequences, respectively. The GII.16 genotype, detected in our region as a single strain (GI/Hu/TR/2013/GII.16/Erzurum424), was similar to the GII.16/KJ145841 GenBank strain at 85.7% for nucleotide sequences and 90.0% for amino acid sequences. In addition, the GII.21 genotypes detected in the present study were similar to the JN899245 GenBank strain at 93.3%–94.5% for nucleotide sequences and 96.6%–100% among themselves. These ratios were 96.2%–98.7% and 96.2%–100% for amino acid sequence similarities, respectively. The similarity for nucleotide sequences identified within the same genotype in a study previously conducted in Turkey by Ozkul et al. (23) and our strains were 94.5%–95.8% for nucleotide sequences and 97.5%–100% for amino acid sequences. Sequences have been deposited in GenBank under accession numbers KT444636-KT444661.

4. Discussion
Noroviruses are important pathogens in humans, causing epidemics and sporadic gastroenteritis in both children and adults, and can also affect nonhuman species (13). It is reported that more than 260 million cases of norovirus occur every year (28). There are limited numbers of studies that report cases of norovirus in Turkey, and there is no study revealing the molecular diversity of noroviruses in the Eastern Anatolia region. The present study was intended to clarify both the presence/frequency and the genotype distribution of human noroviruses in this region.

Noroviruses are divided into six genogroups (GI–GVI) and human strains are grouped into GI, GII, and GIV (12,30). Noroviruses are indirectly transmitted to humans from contaminated food and water sources and can also be transmitted directly from person to person (31,32). GII noroviruses, especially the GII.4 strains, are the predominant cause of gastroenteritis, both in outbreaks and in sporadic cases worldwide (30). The prevalence and outbreak activity of the GI genogroup, which is the second most predominant genogroup, are limited (33), and reports of GIV around the world are also rare (34).

Noroviruses are a significant problem in both the American continent and Europe, and continue to be circulated despite all precautions. Between 2009 and 2013, reports of 3960 outbreaks caused by norovirus were entered into the Calicinet database for the American continent (35) and it was reported that the same agent caused two out of seven outbreaks during the same years in Argentina (36). Norovirus outbreaks in Europe have an important place epidemiologically. In the Netherlands (37)
Figure. Phylogenetic tree of norovirus strains from Erzurum. Phylogenetic tree analyses were inferred using MEGA version 5.1, distances were calculated by Kimura’s 2-parameter method and a phylogenetic tree was plotted by the neighbor-joining method based on a partial RNA-dependent RNA polymerase (RdRp) coding region (327 nt) of 26 novel norovirus strains detected during the study in Erzurum and from human reference strains in GenBank. Turkish GII strains are the round (●) shapes, Turkish GI strains are square (□) shapes and Turkish GIV strains are represented by diamond (♦) shapes. The scale bar represents the phylogenetic distance expressed as expected nucleotide substitutions per site. Bootstrap values (1000 replicates) are shown. Reference strains were selected from GenBank and the accession number and genotype are indicated. Accession numbers in GenBank: KF039736 (GI.1), EU085488 (GI.2), KC119510 (GI.3), KJ420357 (GI.4), JN241965 (GI.7), EU299761 (GI.8), KF586507 (GI.9), JN797508 (GI.10), KJ017778 (GI.12), U02030 (GI.13), KF738705 (GI.4), JX989075 (GI.6), JX48323 (GI.11), HQ664990 (GI.12), KJ196276 (GI.13), GI017903 (GI.14), KJ145841 (GI.16), KC597139 (GI.17), AY823304 (GI.18), AY823306 (GI.19), JN899245 (GI.21), AB083780 (GI.22), JN197579 (GI.1e), KJ145834 (GI.1g), EU794903 (GI.2), JX536251 (GIV.1), JF781268 (GIV.2), JN975491 (GV) and for the reference strains are available upon request. In this study, the strain’s name designation is our laboratory sample number.
between 1994 and 2005, norovirus was identified in 78.1% of stool specimens. A similar situation was demonstrated in studies conducted in Hungary (38), Spain (39), and Germany (40). In our study, this rate was 20.1%. Although noroviruses can cause gastroenteritis in all age groups, the course of this infection in childhood is more serious than in adults due to the insufficient development of the immune system in young children. In various studies on the relationship between age and genotype, evidence of such a relationship has not been detected. However, GII.4 genotype dominance worldwide in all age groups is noteworthy. In contrast to the predominance of genotype GII.4 norovirus all over the world, GII.21 (37.5%) and GII.1 (33.3%) were the dominant genotypes in our study. In addition, the G1.6 and GIV.1 genotypes are rarely reported in studies. This situation raises questions about transmission occurring due to human–animal contact. The determination in our study of genogroup GIV, which included dog–human transmission, suggests that the increased pet-breeding habits of humans could cause this condition. Furthermore, determined as single-strain, the G1.6 genotype is a group resulting from the human–pig relationship, and the absence of pig farming and pork consumption in Eastern Anatolia was evaluated a factor to be investigated for this region. This situation suggests that relations between wildlife and humans may cause such transmissions. Detecting the presence of a zoonotic infection requires taking a good anamnesis of the patient and questioning the history of children’s parents, as well as undertaking complete virus genome studies. Indeed, norovirus infections are not yet accepted as zoonotic (41) and it should be investigated in detailed future studies to determine whether they have such potential.

In Turkey, there are limited numbers of studies on the molecular epidemiology of noroviruses. A study conducted in 2008 and 2009 in Istanbul, Turkey’s most populous province, identified only the GII genogroup and its GII.4, GII.16, GII.b, and GII.e clusters. Özkul et al. reported that the dominant type was GII.4 and argued that systematic surveillance should be performed (23). In 2006 and 2007, Altındiş et al., in a province of Central Anatolia, identified GII.b as the predominant genotype, and also detected the genotypes GII.4 and GII.6 (22). A study conducted to determine the distribution and molecular characteristics of enteric viruses in children with diarrhea in Turkey and Bangladesh determined the G1 and GII.4 genotypes in Turkish children (24).

In the present study, the alignment and degree of similarity among sequences at the nucleotide and amino acid levels between our strains and the reference strains obtained from GenBank were demonstrated. It is possible to detect a proximity of 90% or higher in general in the PCR, sequencing reactions, and molecular characterization studies when targeting the RdRp gene region of the viruses. We evaluated the correlation between our findings on this analysis and other studies’ findings and found proportional similarities. The alignments of the G1.6 and GIV.1 genotypes were determined but we could not discuss the degree of similarity of the strains because of limited studies. The worldwide-dominant genogroup, GII, has also been reported previously in this country. These strains were not very similar, although they were close to each other in terms of nucleotide and amino acid sequences.

The present study is the first analysis of norovirus genotypes in the province of Erzurum and the Eastern Anatolia region in Turkey; norovirus GII has been identified as the most frequent genogroup responsible for gastroenteritis in this area. Contrary to expectations based on studies done previously in this country and around the world, genotypes GII.21 and GII.1 were identified as predominant. In addition, the identification in our study of the GI and GIV genogroups, which are rarely seen in humans, suggests the possibility of zoonotic transmission, a discussion that will continue around the world. Considering the unusual diversity of norovirus genotypes in this study, it is necessary that regional and territorial norovirus surveillance studies be performed periodically. In addition, considering that norovirus gastroenteritis was identified in second place after rotavirus diarrhea in a project involving this region (data not shown), there is a need for the selection of a reference test for noroviruses, as well as future vaccine studies.

The obtained data showed that norovirus infections, reported many times previously in Turkey, are endemic. This study is the first report on Eastern Anatolia about the diversity of norovirus genotypes, in addition to previous reports for the Central Anatolia, Aegean, and Marmara regions. This study has shown that the Eastern Anatolia region should be included in surveillance studies conducted in Turkey. In addition, it is thought that it will be beneficial to create a bank of virus strains obtained from further molecular epidemiological studies for national vaccine development, with this region’s strains compared to the strains found worldwide.
References


