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Emergence of rotavirus G9 in 2012, as the dominant genotype in Turkish children with diarrhea, in a university hospital in Ankara

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Abstract

***Introduction**: Rotavirus infection is a major cause of morbidity and mortality in infants and young children with diarrhea throughout the world. **Material and Methods**: In this study, we aimed to determine the detection rate of rotavirus infection in 181 children less than 5 years of age presenting with acute gastroenteritis and admitted to a tertiary care hospital in Ankara, Turkey, from April to November 2012. We documented the epidemiological data by elucidating the prevalent genotypes. Stool specimens were collected, and rotavirus antigen in the samples was detected using ELISA. G and P genotypes were determined by RT-PCR via type specific primers. The nucleotide sequence of the concerned genes was determined by Sanger sequencing and phylogenetic analysis was performed by neighbor-joining method. **Results**: Of the 181 samples, 28 (15.5%) were positive for the rotavirus antigen. Twenty-seven samples were positive for G genotypes and 21 were positive for P genotypes. Genotypes G1 (7.1%), G2 (7.1%), G3 (7.1%), G4 (3.6%), G9 (71.5%) and P4 (3.6%), P8 (71.4%) were identified. Genotype G9P[8] (50%) was predominant in the combination of G and P genotypes. Most of the G9 strains of this study formed an independent cluster in Lineage III, except two strains which clustered with an Ethiopian G9 strain of 2012. **Conclusions**: It seems that during 2012 season, genotype G9P[8] increased significantly in Ankara due to a new circulating strain of G9.*

***Keywords:** children, genotype, rotavirus infection, Turkey*

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Introduction

Globally, diarrhea remains the second most common cause of death among children under five years of age (1,2). In Turkey, every year, there are 1.25 million births and the annual number of diarrhea episodes in children under five is estimated to be 13,371,800 (3). Rotavirus (RV) is the most significant cause of severe gastroenteritis in children of the age group mentioned before. The latest WHO estimate of rotavirus deaths every year around the world declined from 453,000 in 2008 to 215,000 in 2013 (4,5).

RV is a non-enveloped, double-stranded (ds) RNA virus belonging to the *Reoviridae* family. The RV genome consists of 11 dsRNA segments and encodes six structural proteins (VP1–4, VP6, and VP7) and 6 non-structural proteins (NSP1–6) (6). According to the classification system based on the gene sequence of VP6, an inner capsid protein, RVs are currently categorized into ten groups (A, B, C, D, E, F, G, H, I and J). Most RV infections in humans are caused by the Group A RV (6,7). Outer capsid proteins, VP7 (glycoprotein) and VP4 (protease-sensitive protein) respectively determine the G and P genotypes. To date, at least 36 G-genotypes and 51 P-genotypes have been identified in humans and other animals according to the last release of the Rotavirus Classification Working Group (8,9).

RV genotypes that cause the majority of infections worldwide are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (10). First discovered in 1995, the G9 genotype occured all over the world and became the fifth most commonly detected human RV (10). Furthermore, G9 frequency has increased of late, and it is the predominant genotype found in recent studies conducted in Turkey (11,12).

In Turkey, RV vaccination is currently not included in the national immunization program. Surveys on the prevalence of RV in Turkey show that the responsible genotypes vary considerably according to the year and the studied groups (13-15). Therefore, it is necessary to identify the RV genotypes circulating in Turkey before including them in the national vaccination program. Moreover, the growing divergence of RVs and the emergence of G9 strains emphasize the need for continued RV surveillance in Turkey. In this study, we attempted to determine the detection rate of G9 genotype in Turkish children with acute gastroenteritis admitted to a tertiary care hospital in Ankara.

Material and Methods

Collection of Stool Samples

Stool samples were collected prospectively from 181 children (younger than 5 years of age) with acute gastroenteritis admitted to the Gazi University Hospital, Ankara, Turkey, from April to November 2012. The samples were sent to microbiology laboratories from various pediatric clinics. The samples were first examined routinely using the native-lugol method for screening of leucocytes, erythrocytes, and parasites. After the samples with leukocytes, erythrocytes, and parasites were excluded, the stool samples were stored in different aliquotes at −80 °C for RV detection.

Ethical Approvement

This study was approved by the Ethics Committee of the Gazi University Ethics Committee (2010/01-152). Informed consent was obtained from the child's guardian prior to sample collection.

Detection of RV Antigen (Ag) in Stool Samples

The samples were diluted to 10% in phosphate-buffered saline, and RV group A antigen was identified using a commercially available ELISA kit (Rotaclone, Meridian Diagnostics Inc., Cincinnati, Ohio, USA) according to the manufacturer's instructions. Spectrophotometry was used to measure the optical density (OD)

of the ELISA microplate at 450 nm. The cut-off value was set at 0.150, and if the OD of the sample was equal to or higher than the cutoff value, then the sample was designated as positive, and negative if the OD was lower.

Extraction of dsRNA

RV genomic dsRNA was extracted from the ELISA-positive samples by using a commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Germany) using the manufacturer's instructions.

VP7 and VP4 Amplification via RT-PCR

For VP7 and VP4 gene amplification, extracted RNA was transcribed to cDNA using AccessQuick™ RT-PCR kit (Promega Corporation, Madison, WI, USA) and consensus primers Beg9 and End9 for VP7 gene, consensus primers con-2 and con-3 for VP4 gene (16,17). The primer sets used for PCRs are listed in Table 1. RT-PCR was performed in a 50 µl volume: 1X Master Mix, 1 µM concentrations of primers, 5 U reverse transcriptase and 2 pg of template RNA. RT-PCR for VP7 and VP4 amplification was carried out in a Thermal Cycler (Thermo-Hybaid PCR Px2, England) with the following conditions, respectively: reverse transcription of VP7 gene for 45 min at 45°C, 2 min at 95°C, 1 min at 50°C, 1.5 min at 72°C; 39 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; post-extension for 5 min at 72°C; reverse transcription of VP4 gene for 45 min at 45°C, 2 min at 95°C, 30 s at 50°C, 1 min at 72°C; 29 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min; post-extension for 5 min at 72°C.

G and P Genotyping

For G and P specific genotyping, PCR Master Mix (Promega Corporation, Madison, WI, USA) and primers for the most common genotypes were used. G genotyping was done by genotype specific primers for G1, G2, G3, G4, and G9. P

Table 1. G and P typing consensus and type-specific primers

Primers	Sequences (5'-3')	Location (nt)	Amplicon Sizes (bp)
G Typing			
1st round consensus primers			1062
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	
End9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	
2nd round			
VP7-R	AACTTGCCACCATTTTTTCC	914-932	-
G1	CAAGTACTCAAATCAATGATGG	314-335	618
G2	CAATGATATTAACACATTTTCTGTG	411-435	521
G3	ACGAACTCAACACGAGAGG	250-269	682
G4	CGTTTCTGGTGAGGAGTTG	480-498	452
G9	CTTGATGTGACTAYAAATAC	757-776	179
P Typing			
1st round consensus primers			887
Con2	ATTTCGGACCATTTATAACC	868-887	
Con3	TGGCTTCGCCATTTTATAGACA	11-32	
2nd round			
HumCom5	CTCTCGATGGTCCATATCAACC	200-221	-
P[4]	ATATATTGCCTATTTGTTTGAC	347-368	186
P[6]	GTATTACAGTTTCTACTTCAGA	592-613	381
P[8]	TGTACGTCTATTATAAAATTCATTT	456-480	280
P[9]	CGTCGCTCCTTGATACCAGT	533-552	350

typing was done by genotype specific primers for P[8], P[4], P[6], and P[9] (Table 1) (17-20). PCR was performed in a 50 µl volume: 1X Master Mix, 0.2 µM concentrations of primers and 1 µl of template cDNA. PCRs for G and P genotyping were carried out with the following conditions, respectively: 30 cycles of 1 min at 95°C, 2 min at 42°C, 1 min at 72°C, post-extension for 5 min at 72°C; and 30 cycles of 10 s at 94°C, 30 s at 42°C, 30 s at 72°C, post-extension for 5 min at 72°C. Amplification products were analysed according to amplicon size in 2% agarose gel electrophoresis.

Sequence Analysis for VP7 and VP4

The nucleotide sequences of VP7 and VP4 genes were determined using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and the products were analyzed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

Multiple sequence alignment was achieved using ClustalW algorithm belonging to BioEdit Sequence Alignment Editor version 7.1.3 as described by Hall TA (21), and phylogenetic analysis was performed using MEGA 6 software and a distance based neighbour-joining method and Kimura's 2-parameter model (22).

Results

RV antigen was detected in 15.5% (28/181) of the samples as per ELISA. The age of the children ranged from 1 to 60 months, and the majority of RV infections were detected in children aged between 13 and 24 months (28.6%), followed by 0–6 month (21.4%) old. The infection was lowest in children aged 37–48 months (3.6%) (Figure 1).

The boys:girls ratio of RV-positive samples was 3:4. Sixteen (57.1%) of the RV Ag-positive children were girls and 12 (42.9%) were boys. Although the duration of our study did not include the entire year, RV was found to be mainly prevalent in April (33.3%) and May (25.9%). Thirteen samples were collected in June and 35 in November; however, they did not show positive results (Figure 2).

As a result of VP7 and VP4 amplification of 28 RV Ag-positive samples, G and P genotypes were detected in 27 and 21 samples, respectively. G9 (71.5%; 20/28), G1 (7.1%; 2/28), G2 (7.1%; 2/28), G3 (7.1%; 2/28), and G4 (3.6%;

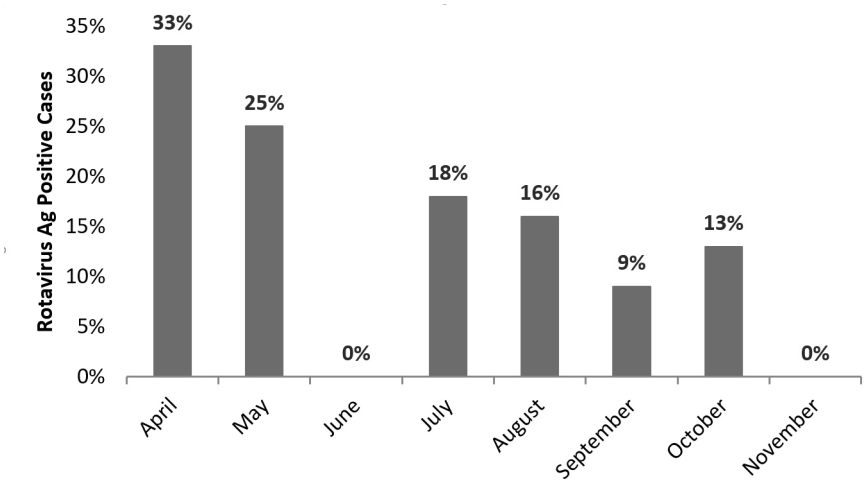

Fig. 1. The range of patients with rotavirus diarrhoea distributed according to age.

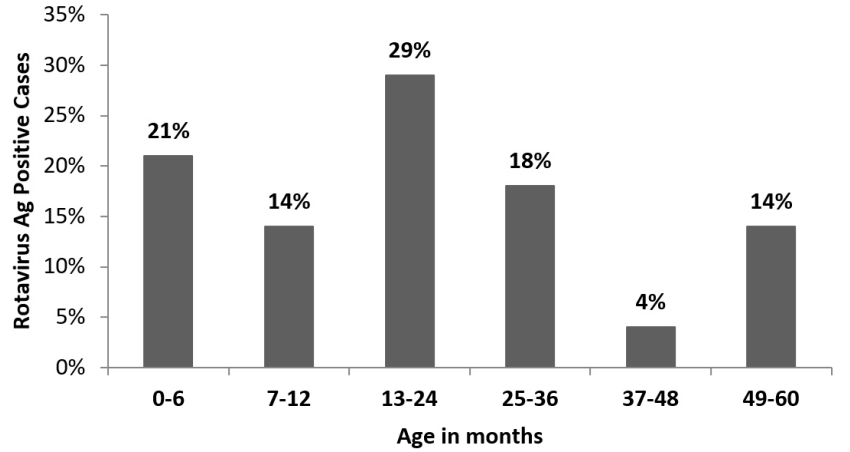

Fig. 2. The monthly occurrence of rotavirus diarrhoea among the children in Turkey. The monthly occurrence is represented by the percentage of rotavirus cases detected among the diarrheal cases of each month.

1/28) constituted the G genotypes; 3.6% (1/28) of the samples, however, were untypable. P[8] (71.4%; 20/28) and P[4] (3.6%; 1/28) comprised the P genotypes; 25% (7/28) of the samples were untypable. Among the samples typed successfully, genotype G9P[8] (50%) was predominant (Table 2).

G9-positive samples were then used for sequence analysis for phylogenetically analyzing the G9 strains. All the Turkish G9 strains belong to lineage III, and of the 13 G9 strains detected in this study, 11 formed an independent cluster. The other two strains formed a cluster with an Ethiopian G9 strain detected in 2012 (Figure 3).

Discussion

Our results showed that G9 and P[8] were the most common RV genotypes. Because the stool samples used in the present study belonged to 2012, an increase in G9 detection rate was expected. Following the implementation of RV vaccine worldwide, the prevalence of genotype G9 is increasing of late. Before 1990, G1P[8], G2P[4], G3P[8], and G4P[8] were the most common genotypes. Since then, however, G9P[8] has emerged as the fifth most common type around the world (23). This situation is similar to the genotype distribution in Turkey. G1P[8] tended to be the predominant genotype in earlier studies in Turkey (13,14,24). Genotype G9, which is not included in the current vaccination program, started being detectable at very low frequencies in the late 1990s. It remains unclear whether widespread implementation of RV vaccines causes the emergence of non-vaccine genotypes. Recent regional epidemiological studies have confirmed the high prevalence of the genotype G9,

Table 2. Numbers and Percentages of Rotaviruses with Different G and P Types Combinations

Genotypes	G1	G2	G3	G4	G9	Gnt	Total
P[4]	0	1 (3.6%)	0	0	0	1 (3.6%)	2 (7.1%)
P[8]	2 (7.1%)	1 (3.6%)	2 (7.1%)	1 (3.6%)	14 (50%)	0	20 (71.5%)
Pnt	0	0	0	0	6 (21.4%)	0	6 (21.4%)
Total	2 (7.1%)	2 (7.1%)	2 (7.1%)	1 (3.6%)	20 (71.5%)	1 (3.6%)	28 (100%)

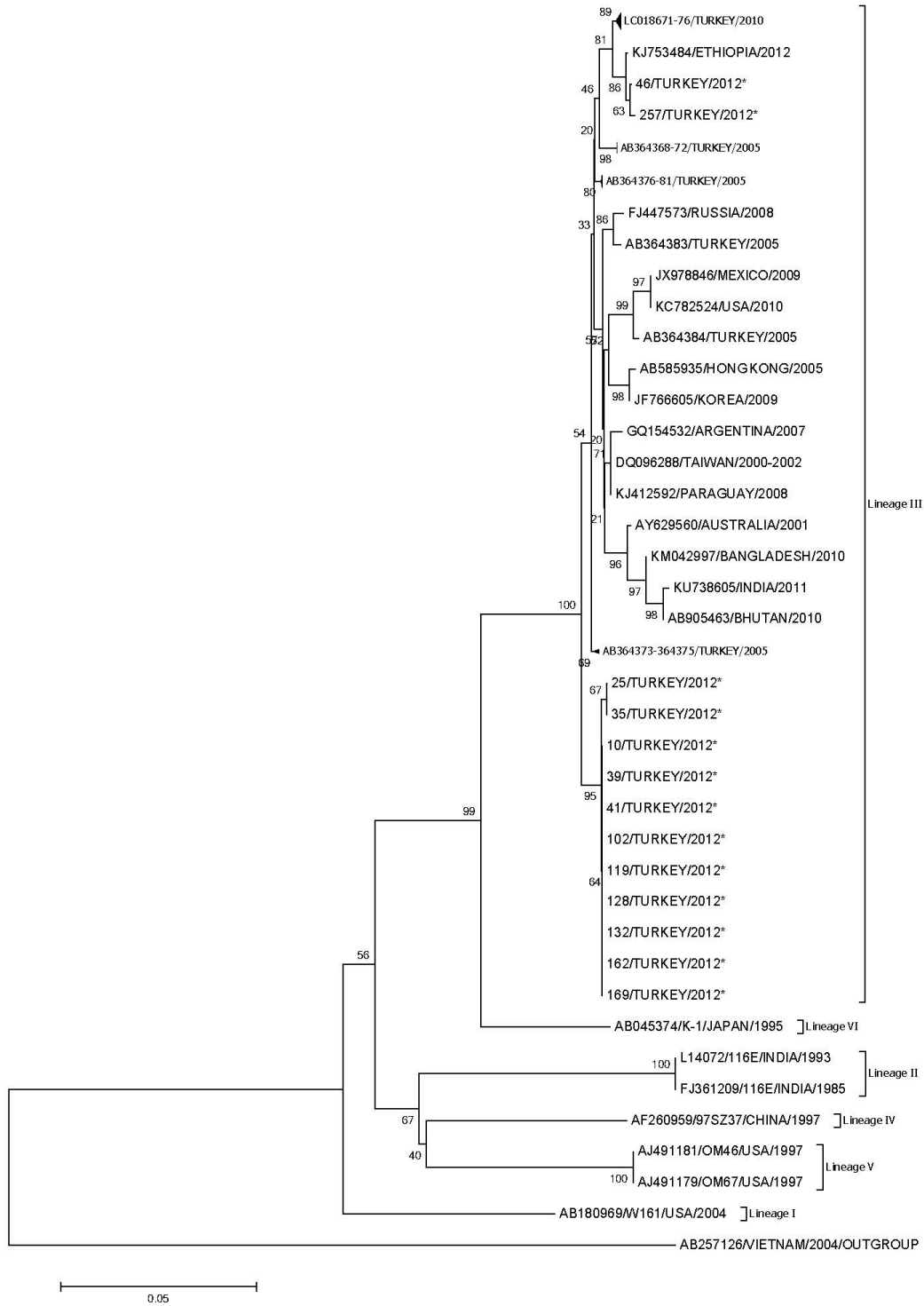

Fig. 3. The phylogenetic tree was constructed using the neighbour-joining method. Bootstrap analysis of 1,000 replicates was conducted to identify the significance of branching of the constructed tree. Bootstrap values of >70 are shown at branch nodes. Scale bar shows genetic distance expressed as nucleotide substitutions per site.

reaching 50%–90% in some circumstances (25). RV genotype diversity, especially that of G9 and G12, is a great challenge for current vaccination programs (11). Similar to our findings, low levels (19.7%) of RV infection with higher proportion of G9 were detected in Argentina in 2005 (58.0%) and 2006 (61.5%) (26). In Denmark, G9 increase has been documented during 2009–2013 (27). Moreover, the emergence of G9 and G12 in 2010 in Bhutanese children has also been reported (28).

A Brazilian study (29) investigating changes in the epidemiology of RV during 2011–2012 found that after the monovalent RV vaccine was included in the national immunization program, RV was detectable in 1.7% (6/348) of the cases. RV positivity rates decreased to 88% in 2011 and 78% in 2012 compared with those in 2005/2006 (29). A study in Scotland found that the changing of the molecular epidemiology of RV infection after introduction of monovalent RV vaccination in 2012–2015. A decrease was seen in the prevalence of G1P[8] strains (from 72.1% to 15%) after the introduction of the vaccine. Genotype G2P[4] was the predominant strain (21.9%) with increase in G9P[8] (12.9%) in 2013-2015 (30).

In 2004, G9 emerged in Turkey and increased substantially in 2005 to 17.2% of the samples. Its frequency continued to increase, and it has since been confirmed to be the predominant genotype by recent studies. G9 was absent in the samples acquired from 2006 and 2007, but it then re-emerged in 2008 and increased gradually (11,14). A pilot study showed that G9[P8] continued its dominance since 2008 as the primary genotype among children in Ankara. G9P[8] prevalence was 21.2% in 2008, increasing to 34.8% in 2009, 44.3% in 2010, 40% in 2011, and finally to over 70% of the strains including G9 (11). A previous study developed by "The Turkish rotavirus surveillance network" showed that during 2012–2014, genotype G9 was the most dominant genotype not only in Ankara but also all over Turkey, except the Eastern part (15). Another study in Central Anatolia (Afyon) revealed that G9P[8] (48.7%) was the most common genotype during 2012–2013, followed by G9P[4] (17.5%), similar to the findings of our study (31).

Currently, there are two oral, live attenuated RV vaccines: Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq (Merck & Co., Inc., Whitehouse Station, NJ, USA). The WHO has recently recommended the inclusion of rotavirus vaccination of infants in all national immunization programs (32). Although RV vaccines have not been introduced in the national vaccination programs in Turkey, both vaccines are commercially available (15). Following the implementation of the vaccination programs, some studies have reported an increase in genotype G9 prevalence in recent years. In 2009, Tapisiz et al. found in their study in Ankara that G9P[8] was the most frequently occurring genotype in 19 patients (19%), followed by G1P[8] and G4P[6], each in 7 (7%) patients (12).

In the present study, we found that the proportion of genotypes G1, G2, and G3 was 7.1% and that of G4 was 3.6%. The total number of RV-positive samples was not high, and most of the positive samples belonged to genotype G9 (71.5%). In the same geographic region, a similar study that included stool samples collected more recently (November 2016 and February 2018) from another tertiary care hospital which has lower income patient profile was conducted by Kahraman et al (33). Seventeen percent of 476 diarrheic stool samples were RV Ag positive. Genotype G1 (31%) was the most prevalent genotype followed by G12 (20%) and G9 was detected in 10%. Although these results do not reflect the society incidence, a decrease can be seen in G9 frequency (33). Also a rotavirus surveillance study from Turkey in 2014-2016 reported that the most prevalent genotype was G1P[8] (24.6%) followed by G3P[8] (19.6%) and G9P[8] (12.2%) (34). The samples of the

present study belong to year 2012, which is one of the limitations of our study, however it can be seen from the literature that genotype G9 was the predominantly circulating genotype around 2012. Currently, G9 frequency is lower, but still circulating. Therefore, our results are important in terms of showing the predominance of G9 in 2012. Also, the sample collection period (8 months) of our study is another limitation.

Conclusion

It is not clear why the dominance of G9 is persistent in the absence of widespread vaccine use in Turkey. Our phylogenetic analysis showed that G9 strains of two new lineages were circulating in Ankara. We assume that these strains of genotype G9 infected the immunologically naïve population and, as a result, spread rapidly and increased the proportion of G9 strains.
We found that RV G9P[8] strains increased considerably in Ankara in 2012. This increase might be due to introduction of a new dominant strain of G9 in the population. We believe this information is important for policy-makers before the implementation of a national RV immunization program in Turkey.

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Conflicts of interest

The authors declare that there is no conflict of interests.

Authors' contribution

AAK, investigation, writing original draft preparation, visualization; MA, investigation, writing original draft preparation; TM, investigation, data analysis; TY, investigation; BD, resources; GB and KA, methodology, writing-review and editing, supervision.

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