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A Comparison of Bovine Torovirus Strains Based on Partial Membrane Glycoprotein (M) Gene Sequences

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Abstract: Bovine torovirus (BoTV), which was recently been separated from bovine coronaviruses, is one of the important causative agents of diarrhea in newborn calves. Although the epidemiological data are limited throughout the world, it has been reported in some countries of the world since the early 80's. In this study, stool samples (n: 150) were taken from 0-30 days old diarrhoeic calves from Elazig, Sivas, and Malatya provinces and were screened by nested RT-PCR method. In 6% (9/150) of the studied samples, 409 bp partial membrane glycoprotein (M) gene was successfully amplified. Some of the positive samples were further sequenced and the 383 nt length data of nine samples were subjected to bioinformatic analysis. The multiple sequence comparison and phylogenetic analyses together revealed that these novel strains presented close identity to previously reported strains from Turkey. The nucleotide identity of the strains was found between 97.13% and 100%. Furthermore, valine-isoleucine substitution (V→I) at 114th position was detected in Turkish strains only (MN717266; MF687255-60), whereas the same substitution at the 144th position was shared between the Turkish (KF188708-11 and KF188714; MF687255-60; MG957145 and MN717266-67) and some of the Chinese-originated isolates. Moreover, four silent mutations were detected in the novel isolates subjected in this study. As a result, we demonstrated the contribution of BToV in the pathogenesis of calf diarrhea and presented new data concerning on the molecular characteristics and the epidemiology of the bovine toroviruses in Turkey.

Keywords: Bovine torovirus, Phylogenetic analysis, sequencing, Turkey.

Bovine Torovirus Suşlarının Kısmi Membran Glikoprotein (M) Gen Dizilerine Göre Karşılaştırılması

Öz: Bovine torovirus (BoTV), sığır koronavirüslerinden yakın bir zamanda ayrılmış olup yenidoğan buzağıların önemli viral ishal etkenlerinden birisidir. Epidemiyolojik veriler dünya çapında kısıtlı olmakla beraber, bu virüs diğer bazı ülkelerde 80'lerin başından itibaren bildirilmiştir. Bu çalışmada Elazığ, Sivas ve Malatya illerinden 0-30 günlük ishalli buzağılardan alınan dışkı örnekleri (n:150) nested RT-PCR metodu ile tarandı. Çalışılan örneklerin % 6'sında (9/150) 409 bç. kısmi M geni başarılı bir biçimde amplifiye edildi. Pozitif örneklerin bazıları seçilerek sekanslandı ve 9 adet örneğin 383 bazlık sekansı biyoinformatik analizde kullanıldı. Çalışmada GenBank'ta yer alan verilerle birlikte yapılan filogenetik analizler yeni bulunan izolatların da daha önce Türkiye'den bildirilenlerle benzer olduğunu ortaya koymuştur. Dizi analizi yaptırılan izolatların nükleotid benzerliği 97.13% ile 100% arasında bulunmuştur. Valin-izolöysin (V→I) mutasyonu 114. pozisyonda yalnızca Türk suşlarında mevcutken 144. pozisyonda bazı Türk ve Çin suşlarında gözlemlenmiştir. Öte yandan sadece bu çalışmada ortaya çıkarılan yeni Türk izolatlarında 4 adet sessiz mutasyon olduğu ortaya konuldu. Sonuç olarak, ortaya konulan bu bilimsel veriler ile sığır toroviruslarının moleküler karakteristiği ve epidemiyolojisine katkı sağlandı.

Anahtar Kelimeler: Bovine torovirus, DNA dizi analizi, Filogenetik analiz, Türkiye.

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INTRODUCTION

Bovine torovirus (BoTV) is one of the 3 species (cattle, horse and pig) in the genus torovirus, in family *Tobaniviridae*, order *Nidovirales*, according to the current version published by the International Committee of Virus Taxonomy (ICTV) (https://talk.ictvonline.org). The viruses belonging to the genus Toroviruses are enveloped and positive single-stranded RNA viruses (1-5). The BoTV genome has a size of about 28 kilobases (kb). The genome of BoTV, together with large ORF (ORF1a and ORF1b) encoding 2 nonstructural proteins; it consists of 4 structural genes that encode spike glycoprotein (S) gene, membrane glycoprotein (M) gene, hemagglutinin esterase (HE) gene, and nucleocapsid phosphoprotein (N) gene (6).

Toroviruses are the cause of gastroenteritis in mammals and have been detected in cattle, horses, pigs and humans. Horse and cattle toroviruses are serologically related to each other (7). Toroviruses were first isolated from a horse with diarrhea in Switzerland in 1972 (3). A few years after that, BoTV was firstly detected in a calf with diarrhea in the USA in 1979 (8). Since the first isolation of the BoTV in 1992, The virus has been reported all over the world, including Turkey (9-17). Recent epidemiological studies have evidenced that the BoTV is one of the viral agents that contribute to neonatal calf diarrhea (18, 19).

Bovine torovirus was firstly reported by Gulacti et al. in Turkey (17) and since then, BToVs has increased their importance throughout the world. Recently we conducted a full-length sequence analysis of the virus (Isidan et al., unpublished data). The purpose of this study was to expand the knowledge on the role of BoTV on diarrhea by comparing sequences based on M gene between the GenBank provided sequences and the circulating strains in the studied area.

MATERIALS and METHODS

Samples

A total of 150 stool specimens, which some of them were studied and published for bovine astrovirus (20), bovine hunnivirus, bovine aichivirus and bovine enterovirus (21) previously, were collected from diarrheic calves, between 1 to 30 days of age from the region covering Sivas, Malatya and Elazig provinces (Ethics Decision Number: 04-498). The collected samples were delivered to the laboratory as soon as possible and stored at -80 °C until RNA isolation. The distribution of the collected samples by province is shown in Figure 1.

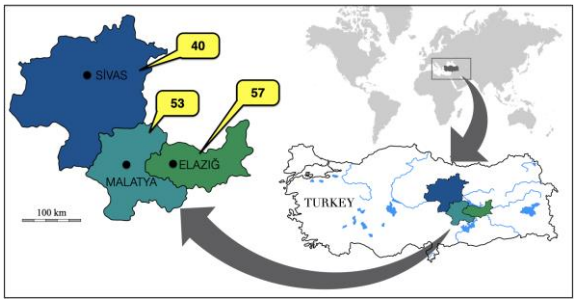

Figure 1. The distribution of the collected samples by province.

Şekil 1. Toplanan örneklerin illere göre dağılımı.

RNA Isolation

The fecal samples were diluted 1/10 in 1M PBS, centrifuged at 5000 rpm for 5 minutes to precipitate coarse particles and large cellular debris. After centrifugation, the supernatants were submitted to RNA extraction with the GF-1 Viral Nucleic Acid Extraction Kit (Vivantis, Selangor, Malaysia) in accordance with the manufacturer's directives. The obtained RNAs were stored in a -80 ºC freezer until used.

RT-PCR, Sequencing and the Phylogenetic Analysis

Due to the cDNA synthesis, a mixture was prepared with 4 µl RNA extract, 10 mM deoxynucleotide triphosphate (dNTP), 2.5 µl 10 x RT buffer (50 mM Tris-HC1 (pH 8.3 at 25 ° C), 75 mM KCl, 3 mM MgCl2 and 10 mM DTT), 50ng random hexamer, 40 U RNasin, 200 U M-MuLV Reverse-Transcriptase RNase H (Vivantis, Selangor, Malaysia) and completed with nuclease-free water to 25 µl final volume. Reverse transcription was applied for 1 hour at 37 °C.

Amplification of partial M gene was performed by nested primer set (see Table 1) as described by Park et al. (22) previously with using T100 Thermal Cycler (BioRad). The PCR was conducted in a 50 µl final volume using 5 µl of the RT reaction mixture as a template, along with 5 µl 10 × PCR buffer, 10 mM dNTP, 10 pmol/µl of each step's sense/antisense primers of the nested set (Table 1), and 5 U of Taq DNA Polymerase (Vivantis, Germany). Amplification steps (first step and the nested) followed by 95°C/2 minutes for predenaturation step, 40 cycles 94°C /45 sec, 50°C /45 sec, 72°C /60 sec and a final extension step 72°C /10 minutes were used in both first and second step of the amplification. 409 bp nested RT-PCR products were visualized in 1.5% agarose under UV illumination by using LB-16 Ultrabright Led transilluminator (Maestrogen, Taiwan).

Table 1. Primer set used in the detection and sequencing.
Tablo 1. Tespit ve dizilemede kullanılan primer seti.

Target Gene	Name	Sequence (5'-3')	Position*	Product
BoTV M Gene	F	TTCTTACTACACTTTTTGGA	25855-26457	603 bp
	R	ACTCAAACTTAACACTAGAC		
	nF	TATGTACTATGTTTCCAGCT	25909-26317	409 bp
	nR	CCAACACAAATCCGCAACGC		

* Positions were calculated according to the strain Breda 1 (GenBank Accession Number: NC_007447.1).

The amplicons obtained by nested RT-PCR are sequenced (ABI 3100) using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) after cleaning with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) kit. All sequencing products were used for phylogenetic data. Partial sequencing data of 409 bp length of the BoTV M gene was compared to all available BoTV partial sequencing data provided by the National Center for Biotechnology Information (NCBI) (Table 2). Sequence alignment and phylogenetic analysis based on partial nucleotide sequences of length were created using Geneious Prime Version 2020.2.2 software (23). Sequencing data were deposited in GenBank under the accession numbers MG957145, MG957146; MN717264 – MN717270.

Table 2. The list of sequences used in the study.
Tablo 2. Çalışmada kullanılan dizilerin listesi.

	GenBank Accession Code	Isolate Name	Year	Host	Country	References		GenBank Accession Code	Isolate Name	Year	Host	Country	References
1	NC_007447	Breda 1 (RefSeq)	1982	Cattle	USA	Draker et al., 2006	48	MH697550	CHN/HN-1	2018	Cattle	China	Unpublished
2	AJ575374.1	B6	1990	Cattle	Italy	Smits et al., 2003	49	MH697551	CHN/HN-2	2018	Cattle	China	Unpublished
3	AJ575375.1	B145	1998	Cattle	Netherlands	Smits et al., 2003	50	MH697552	CHN/HN-3	2018	Cattle	China	Unpublished
4	AJ575376.1	B150	1998	Cattle	Netherlands	Smits et al., 2003	51	MH697553	CHN/HN-4	2018	Cattle	China	Unpublished
5	AJ575377.1	B155	1998	Cattle	Netherlands	Smits et al., 2003	52	MN073058	SC-1 Sichuan/2018	2018	Cattle	China	Unpublished
6	AB270905.1	K-567	2004	Cattle	Japan	Ito et al., 2007	53	MN073059	SC-2 Sichuan/2018	2018	Cattle	China	Unpublished
7	AB270908.1	K-637	2004	Cattle	Japan	Ito et al., 2007	54	MN073060	SC-1	2018	Cattle	China	Unpublished
8	AB285126.1	Aichi/2004	2004	Cattle	Japan	Kuwabara et al., 2007	55	MN073061	SC-2	2018	Cattle	China	Unpublished
9	DQ778041.1	K34	2004	Cattle	South Korea	Park et al., 2008	56	MN073062	SC-3	2018	Cattle	China	Unpublished
10	DQ778042.1	K49	2004	Cattle	South Korea	Park et al., 2008	57	MN073063.1	SC-4	2018	Cattle	China	Unpublished
11	DQ778043.1	K96	2004	Cattle	South Korea	Park et al., 2008	58	MN073064.1	SC-5	2018	Cattle	China	Unpublished
12	DQ778044.1	K110	2004	Cattle	South Korea	Park et al., 2008	59	MN073065.1	SC-6	2018	Cattle	China	Unpublished
13	DQ778045.1	K119	2004	Cattle	South Korea	Park et al., 2008	60	MN073066.1	SC-7	2018	Cattle	China	Unpublished

Table 2. The list of sequences used in the study (Continue).
Tablo 2. Çalışmada kullanılan dizilerin listesi (Devamı).

	GenBank Accession Code	Isolate Name	Year	Host	Country	References		GenBank Accession Code	Isolate Name	Year	Host	Country	References
14	AB270911.1	K-645	2005	Cattle	Japan	Ito et al., 2007	61	MN073067.1	SC-8	2018	Cattle	China	Unpublished
15	AB270913.1	K-674	2005	Cattle	Japan	Ito et al., 2007	62	MN073068.1	SC-9	2018	Cattle	China	Unpublished
16	AB270915.1	K-676	2005	Cattle	Japan	Ito et al., 2007	63	MN073069.1	SC-10	2018	Cattle	China	Unpublished
17	AB270917.1	K-683	2005	Cattle	Japan	Ito et al., 2007	64	MN073070.1	SC-11	2018	Cattle	China	Unpublished
18	DQ778046.1	K374	2005	Cattle	South Korea	Park et al., 2008	65	MN073071.1	SC-12	2018	Cattle	China	Unpublished
19	DQ778047.1	K490	2005	Cattle	South Korea	Park et al., 2008	66	MN073072.1	SC-13	2018	Cattle	China	Unpublished
20	DQ778048.1	K501	2005	Cattle	South Korea	Park et al., 2008	67	MN073073.1	SC-14	2018	Cattle	China	Unpublished
21	DQ778049.1	K536	2005	Cattle	South Korea	Park et al., 2008	68	MN073074.1	SC-15	2018	Cattle	China	Unpublished
22	DQ778050.1	K537	2005	Cattle	South Korea	Park et al., 2008	69	MN073075.1	SC-16	2018	Cattle	China	Unpublished
23	DQ778051.1	K540	2005	Cattle	South Korea	Park et al., 2008	70	MN073076.1	SX-1	2018	Cattle	China	Unpublished
24	DQ778052.1	K546	2005	Cattle	South Korea	Park et al., 2008	71	MN073077.1	SX-2	2018	Cattle	China	Unpublished
25	DQ778053.1	K577	2005	Cattle	South Korea	Park et al., 2008	72	MN073078.1	LN-1	2018	Cattle	China	Unpublished
26	KF188708.1	T1	2009	Cattle	Turkey	Gülaçtı et al., 2014	73	MN073079.1	LN-2	2018	Cattle	China	Unpublished
27	KF188709.1	T2	2009	Cattle	Turkey	Gülaçtı et al., 2014	74	MN882587.1	BToVyak-XZ01/2019	2018	Yak	China	Unpublished
28	LC088094.1	BToVIshikawa/2010	2010	Cattle	Japan	Ito et al., 2016	75	MN882588.1	BToVyak-QH05/2019	2019	Yak	China	Unpublished
29	KF188710.1	T3	2010	Cattle	Turkey	Gülaçtı et al., 2014	76	MN882589.1	BToVyak-QH06/2019	2019	Yak	China	Unpublished
30	KF188711.1	T4	2010	Cattle	Turkey	Gülaçtı et al., 2014	77	MN882590.1	BToVyak-QH07/2019	2019	Yak	China	Unpublished
31	KF188712.1	T5	2010	Cattle	Turkey	Gülaçtı et al., 2014	78	MN882591.1	BToVyak-QH08/2019	2019	Yak	China	Unpublished
32	KF188713.1	T6	2010	Cattle	Turkey	Gülaçtı et al., 2014	79	MN882592.1	BToVyak-SC09/2019	2019	Yak	China	Unpublished
33	KF188714.1	T7	2011	Cattle	Turkey	Gülaçtı et al., 2014	80	MN882593.1	BToVyak-XZ04/2019	2019	Yak	China	Unpublished
34	KF188715.1	T8	2011	Cattle	Turkey	Gülaçtı et al., 2014	81	MN882594.1	BToVyak-XZ03/2019	2019	Yak	China	Unpublished
35	KF188716.1	T9	2011	Cattle	Turkey	Gülaçtı et al., 2014	82	MN882595.1	BToVyak-XZ02/2019	2019	Yak	China	Unpublished
36	KF188717.1	T10	2011	Cattle	Turkey	Gülaçtı et al., 2014	83	MN882596.1	BToVyak-SC10/2019	2019	Yak	China	Unpublished
37	KF188718.1	T11	2011	Cattle	Turkey	Gülaçtı et al., 2014	84	MN882597.1	BToVyak-XZ01/2019	2019	Yak	China	Unpublished
38	LC088095.1	BToVKagoshima/2014	2014	Cattle	Japan	Ito et al., 2016	85	MG957145.1	BToV-HT1-TUR	2016	Cattle	Turkey	This Study
39	MF687252.1	TR-Erz-Ask-8	2017	Cattle	Turkey	Aydın et al., 2019	86	MG957146.1	BToV-HT2-TUR	2016	Cattle	Turkey	This Study
40	MF687253.1	TR-Erz-Ask-13	2017	Cattle	Turkey	Aydın et al., 2019	87	MN717264.1	BoTV/TUR/1	2016	Cattle	Turkey	This Study
41	MF687254.1	TR-Erz-Azi-27	2017	Cattle	Turkey	Aydın et al., 2019	88	MN717265.1	BoTV/TUR/6	2016	Cattle	Turkey	This Study
42	MF687255.1	TR-Erz-Pal-30	2017	Cattle	Turkey	Aydın et al., 2019	89	MN717266.1	BoTV/TUR/7	2016	Cattle	Turkey	This Study
43	MF687256.1	TR-Erz-Yak-46	2017	Cattle	Turkey	Aydın et al., 2019	90	MN717267.1	BoTV/TUR/16	2016	Cattle	Turkey	This Study
44	MF687257.1	TR-Erz-Tor-48	2017	Cattle	Turkey	Aydın et al., 2019	91	MN717268.1	BoTV/TUR/20	2016	Cattle	Turkey	This Study
45	MF687258.1	TR-Erz-Tor-50	2017	Cattle	Turkey	Aydın et al., 2019	92	MN717269.1	BoTV/TUR/93	2016	Cattle	Turkey	This Study
46	MF687259.1	TR-Erz-Pas-52	2017	Cattle	Turkey	Aydın et al., 2019	93	MN717270.1	BoTV/TUR/96	2016	Cattle	Turkey	This Study
47	MF687260.1	TR-Erz-Pas-54	2017	Cattle	Turkey	Aydın et al., 2019							

RESULTS and DISCUSSION

Bovine torovirus-associated diarrhea in young calves has been reported from various countries. The presence of bovine torovirus in calves and adult cattle varies according to the disease table and the countries studied. For instance, 6.25% in Brazil (24), 5.2% in Austria (16), 5% in Germany (25), 3.6% in Hungary (11), 2.9% in diarrheal calves in Korea (15), 1.74% in cattle and calves in China (26), 2.25% in calves with respiratory symptoms in Japan (27), 8.4% in cattle with diarrhea (14) and 7% in their control samples, while BoTV was detected in 15.2% (28) in another study conducted in Japan. On the other hand, the 36.4 and the 43.2 per cents of BToV positivity from the clinically infected cattle were two examples of high-level of prevalence reported from Canada and Croatia, respectively; however, the 11.6% positivity was detected from asymptomatic calves in the same report from Canada (29,30).

Neonatal calf diarrhea is a major problem causing significant economic losses accounting for its increased mortality in Turkey. Previous studies have focused on the common viral agents of the gastro-intestinal system, such as rotaviruses and coronaviruses (31,32,33). Bovine toroviruses have already been reported from Turkey. Gulacti et al. (17) were reported BoTV 4.6% (11/238) in diarrheic calves all over Turkish land, and Aydin et al. (19) reported 16.7% (12/72) in Erzurum, which is located in eastern Turkey. In this study, we are reporting 6% (9/150) of the diarrheic neonatal calves' fecal samples as positive for the partial M gene amplicons. According to previous results, BoTVs were detected from 4.6 to 16.7 percent of the diarrheic calves in Turkey in three different studies (17,19).

In this study, when the partial M gene sequences of 9 newly identified BoTV strains and other 84 strains available in Genbank were compared, the nt similarity level was found between 95.56% and 99.48%, while the similarity between these novel strains was between 97.13% to 100%. When all sequence data were evaluated, it was determined that the similarity of strains varied between 93.99% and 100%.

The multiple sequence alignment was implemented to the partial M gene sequencing data of the strains retrieved from three independent studies conducted in Turkey. Our findings demonstrated twelve amino acid variations in total; however, the novel strains obtained in this study presented only two out of these eleven substitutions. On the other hand, a total of 18 nucleotide mutations, 2 of which cause amino acid changes, were detected. The first of the mutations that cause amino acid change, according to the reference sequence, Breda 1 strain (NC_007447.1), caused the GTT→ATT change in the first nucleotide of the codon encoding the 114th amino acid to Valine to Isoleucine (V→I). This mutation was found only in BoTV/TUR/7 (MN717266) strains from new isolates and Tr-Erz-30, 46, 48, 50, 52 and 54 (MF687255-60) strains from previous Turkish isolates. The other mutation caused the GTT→ATT change in the first nucleotide of the codon encoding the 144th amino acid to the Valine to Isoleucine (V→I) change. This mutation was found in 29 of 93 isolates (31.2%) whose M gene information was obtained in the GenBank database, and from Turkish isolates T1, 2, 3, 4 and 7 (KF188708-11 and KF188714), Tr-Erz-30, 46, 48, 50, 52 and 54 (MF687255-60), from new isolates BToV-HT1-TUR (MG957145), BoTV/TUR/7 (MN717266) and BoTV/TUR/16 (MN717267), and Chinese CHN/HN-2 and CHN/HN-3 (MH697551-52), LN-2 (MN073079) and all isolates isolated from yak in China (MN882587-97). On the other hand, it was seen that 4 of 16 silent mutations of the new isolates were found only in new isolates. These mutations are the T→C change at nucleotide 258 (BToV-HT1-TUR - MG957145), the C→A change at nucleotide 279 (BToV-21-TUR - MG957145), the T→C change seen at nucleotide 354 (BToV- HT1-TUR - MG957145 and BoTV/TUR/16 - MN717267), and the G→A change seen at nucleotide 441 (BoTV/TUR/20 - MN717268).

In the phylogenetic analysis of a total of 93 BoTV strains with 29 Turkish isolates at the partial M gene

level, it is seen that all Japanese and Korean strains are located on a different branch than other strains (Figure 2). Our results indicated the significant level of variations in Turkish strains at the nucleotide and amino acid levels. Considering the variations observed on the partial M protein residues, further study is required to identify the potential influence of these mutations on the pathogenicity of the BToVs.

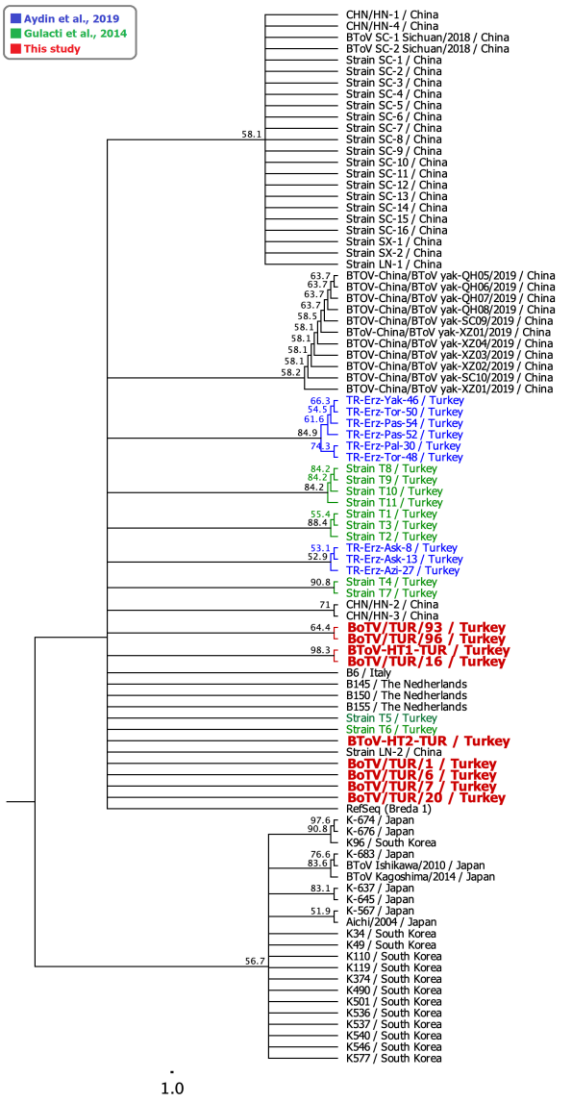

Figure 2. A cladogram representing the consensus (1 000 replicates) neighbour joining phylogenetic tree of the Bovine torovirus strains based on the 409 bp partial *M* gene.

Şekil 2. Bovine torovirüs suşlarının 409 bp kısmi M gen bölgesi temelinde consensus (1000 tekrarlı) neighbor-joining filogenik ağacını gösteren kladogram.

In conclusion, in this study, we sought to determine the frequency of the bovine toroviruses in samples obtained from diarrhoeic calves. We processed the partial genomic data of the M gene to compare with publicly available data and, therefore, revealed the genomic constellation of novel toroviruses in Turkey. We conjectured that the epidemiological and molecular aspects of this study might expand our knowledge on bovine toroviruses in Turkey.

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Conflict of interest

The authors declare that they have no conflict of interest.

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