Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 428-433
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doi:10.3906/vet-1504-67

Molecular characterization of the bovine noroviruses from diarrheic calves in Turkey

İrem GÜLAÇTI[1], İbrahim SÖZDUTMAZ[2,*], Hakan IŞIDAN[3]

[1]Pendik Veterinary Control and Research Institute, İstanbul, Turkey

[2]Department of Virology, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey

[3]Department of Virology, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, Turkey

Received: 20.04.2015 • **Accepted/Published Online:** 27.10.2015 • **Final Version:** 27.06.2016

Abstract: In this study, we performed a survey to detect bovine noroviruses (BNoVs) in Turkey between 2009 and 2011 using 235 fecal samples from neonatal calves with diarrhea that were analyzed by nested reverse-transcription RT-PCR, using primers located in the consensus sequences of the BNoV RNA-dependent RNA polymerase (*RdRp*) gene. The BNoV *RdRp* gene was detected in 1.7% (4/235) of samples by nested RT-PCR. The nucleotide sequences of partial *RdRp* fragments from the BNoV isolates including the newly identified Turkish isolates showed more than 86.3% nt and 96.8% aa identity with each other. Interestingly, in a comparison of the nt and aa sequences, one of the Turkish BNoV strains was found to be 100% aa identical with some Italian and Tunisian strains. In conclusion, BNoV is one of the pathogens that contributes to neonatal calf diarrhea cases in Turkey.

Key words: Bovine norovirus, molecular epidemiology, Turkey

1. Introduction

The bovine norovirus (BNoV) belongs to the genus *Norovirus* of family *Caliciviridae*. Caliciviruses are small, nonenveloped viruses of approximately 27–35 nm in diameter, with a positive-sense, single-stranded RNA genome (1–3). In gnotobiotic calves, BNoV induces nonhemorrhagic enteritis, mild diarrhea, transient anorexia, and malabsorption (4,5).

The first bovine enteric noroviruses were described in 1978 in Great Britain and are known as Newbury Agent 2 (6). Several studies indicated that RNA-dependent RNA polymerase (*RdRp*) and capsid genes can be used in the detection and classification of noroviruses; regarding this, *RdRp* regions are taken into consideration more often for the same purposes with their highly conserved utilities. Complete sequencing of the RNA-dependent *RdRp* and capsid genes has allowed the classification of noroviruses into five genogroups (G), and BNoVs fall into GIII (1,4,7,8). The first detection of BNoV in Turkey was reported in 2011 (9).

It is a field of interest to study human noroviruses; although there is a widespread occurrence of divergent human norovirus strains, there are only a few reports of the prevalence and genetic diversity of BNoVs (10,11). The current study aimed to examine the prevalence and genetic diversity of BNoVs in diarrheic calves in Turkey.

2. Materials and methods

2.1. Specimen collection

A total of 235 diarrheic fecal specimens were collected from calf herds with diarrhea from 28 provinces of Turkey between 2009 and 2011. Figure 1 indicates the location of samples.

2.2. RNA extraction

The fecal samples were diluted in 10× volumes of phosphate-buffered saline (pH 7.5) and centrifuged at 1000 × *g* for 1 min at room temperature. The supernatant was transferred to a new tube and a second centrifugation was conducted at 8000 × *g* for 5 min at room temperature. RNA was extracted from the supernatant using an RNeasy Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. For each extraction period, ddH_2O was used as a negative control.

2.3. RT-PCR

A previously described nested RT-PCR for BNoV detection was used with the primers (Table 1) targeting the BNoV *RdRp* gene. A One-Step RT-PCR Kit (QIAGEN) was used according to the manufacturer's instructions for the first step. The mixture for RT-PCR was incubated at 42 °C for 60 min; preheated at 94 °C for 5 min; subjected to 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 2 min at 72 °C; and finally incubated for 7 min at 72 °C. A Taq PCR Master Mix Kit (QIAGEN) was used according to the manufacturer's

* Correspondence: ibrahimsozdutmaz@hotmail.com

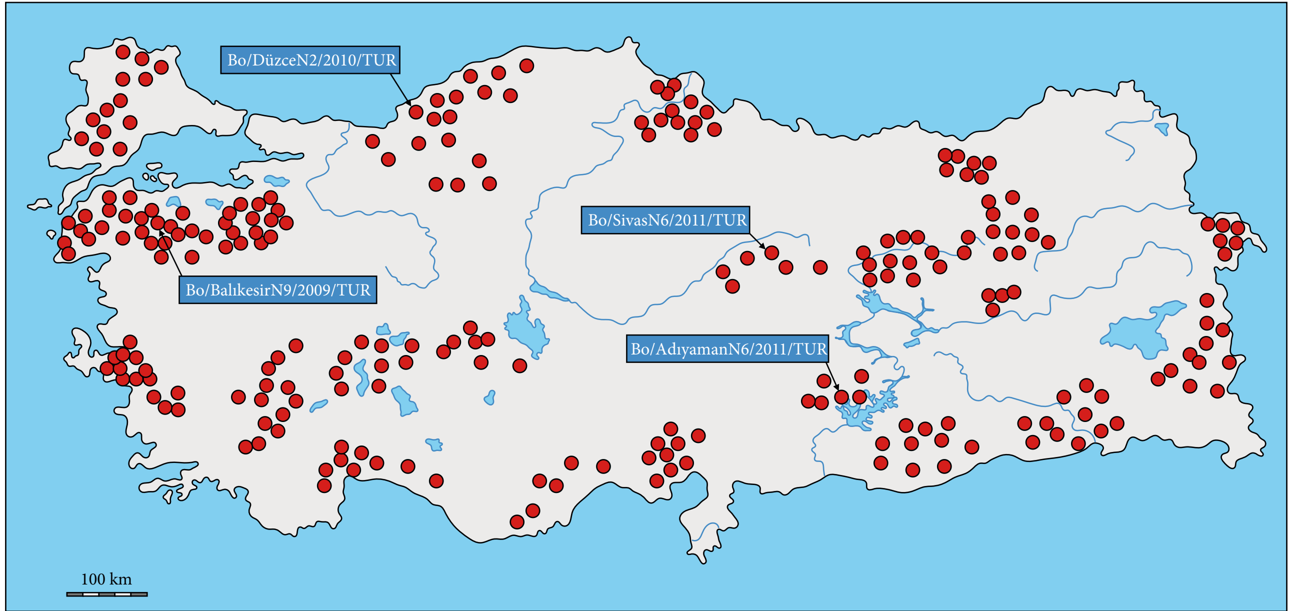

Figure 1. Distribution of BNoV-positive samples in Turkey. Black dots indicate individual samples and positive samples are indicated with labels.

instructions for the nested PCR, whose mixture was preheated at 94 °C for 5 min and then subjected to 30 cycles of, 1 min at 50 °C and 1 min at 72 °C, with a final 7 min of incubation at 72 °C. For each reaction, ddH_2O was used as a negative control. PCR products were detected by electrophoresis on 1.5% agarose gel and visualized by irradiating the ethidium bromide-stained samples with UV light (11,12).

2.4. DNA sequencing

The nested RT-PCR products corresponding to the BNoV *RdRp* open reading frames were directly sequenced by the Pendik Veterinary Control and Research Institute, İstanbul, with the ABI Prism BigDye Terminator version 3.1 cycle sequencing kit and an Applied Biosystems 310 DNA analyzer (Applied Biosystems Inc., Foster City, CA, USA).

2.5. Molecular analysis

Phylogenetic and bootstrap (1000 replicates) analyses based on the nt and aa alignments were constructed using the neighbor-joining method and the unweighted-pair group method with the average linkages of Molecular Evolutionary Genetics Analysis (MEGA, Version 5.2) with pairwise distance (13,14). A sequence similarity search was carried out for BNoV and partial *RdRp* gene sequences using the LALIGN query program of the GENESTREAM network server at the Institut de Génétique Humaine, Montpellier, France (http://xylian.igh.cnrs.fr/bin/lalign-guess.cgi), and using the BLOSUM50 parameters.

3. Results

We conducted a survey of diarrheic calves in Turkey and collected 235 diarrheic calf stool samples between 2009 and 2011. Four (1.7%) out of 235 fecal samples tested positive with nested RT-PCR, targeting a 326-bp fragment of the *RdRp* region of BNoV (Figure 1). The nested RT-PCR products were directly sequenced in both directions. The genetic diversity of the BNoVs was investigated by sequencing 326 bp of the *RdRp* region from the four

Table 1. Oligonucleotide primer pairs used for RT-PCR.

Primer direction	Sequence (5'-3')	Region	Product size (bp)	Reference
Forward	AGTTAYTTTTCCTTYTAYGGBGA	4543–5074	532	(12)
Reverse	AGTGTCTCTGTCAGTCATCTTCAT			
nForward	GTCGACGGYCTKGTSTTCCT	4690–5015	326	(11)
nReverse	CACAGCGACAAATCATGAAA			

BNoVs detected from the diarrheic fecal samples. The molecular analyses were conducted together with some of the sequences provided from GenBank, which belonged to GIII.1 and GIII.2 (Table 2). Additionally we conducted a sequence similarity search with the use of BLOSUM50 parameters for the 285-bp nt sequence and for 95 aa sequences (Table 3).

4. Discussion

Noroviruses are major etiological agents of gastroenteritis in humans (2,15,17). Studies also indicated that the contribution of BNoVs to gastroenteritis in cattle is frequent (5,11,18). The sequences of noroviruses show that they are highly divergent because of their high number of recombinations (19). This makes it difficult to assess the genetic analyses of noroviruses. To avoid confusion and to provide clear criteria for classification of BNoVs, we analyzed 95 deduced aa sequences of the BNoV *RdRp* gene and we also used only strains that belong to GIII.1 and GIII.2, provided by GenBank, for phylogeny due to the proper substitution of strains on clades. All the Turkish strains were clustered in genotype GIII.2 (Figure 2). This is similar to a previously reported study (9). However, we could not use those strains for the phylogenetic analysis because no data had been entered in GenBank. Furthermore, the previous study indicated that 6/70 (8.6%) diarrheic calves obtained from the

Table 2. GenBank accession numbers of the reference strains of BNoVs used in nucleotide and amino acid sequence comparisons, and phylogenetic analysis.

Designation	Country	Year	Accession number	Reference
BEC195/IT	Italy	2004	HM745906	
Bo/MonastirB46/2006/TUN	Tunisia	2006	JN418484	(5)
Bo/MonastirB76/2007/TUN	Tunisia	2007	JN418486	(5)
BEC537/IT	Italy	2004	HM745907	(4)
Bo/Aberystwyth24/00/UK	United Kingdom	2000	AY126475	
Bo/Newbury2/1976/UK	United Kingdom	1976	AF097917	(8)
Bo/NoV/JN-MA156/04/Korea	South Korea	2004	DQ912792	(11)
bovine/DijonA311-1/08/FR	France	2008	GU259574	(15)
Bo/Dumfries/94/UK	United Kingdom	1994	AY126474	(4)
Bovine/BV24/2007/BEL	Belgium	2007	EU877969	(10)
Bovine/BV52/2007/BEL	Belgium	2007	EU877970	(10)
Bovine/GIII.2/300_0250/2006/NOR	Norway	2006	FM242187	(3)
Bovine/GIII.2/471_0790/2005/NOR	Norway	2005	FM242193	(3)
Bo/Nov-6/USA/2010	United States	2010	JN585033	
Bo/Nov-46/USA/2010	United States	2010	JN585061	
Isolate_Jena	Germany	1984	AJ011099	(16)
CV500-OH	United States		AY151258	(12)
Bo/MonastirB169/2010/TUN	Tunisia	2010	JN418492	(5)
Bovine/Wasme/B199/2003/Be	Belgium	2003	AY686493	
BEC483/IT	Italy	2004	HM745909	
Bo/Penrith55/00/UK	United Kingdom	2000	AY126476	(4)
Bo/Nov-4/USA/2010	United States	2010	JN585031	
Bo/Nov-50/USA/2010	United States	2010	JN585063	
Bovine/DijonA358/07/FR	France	2007	GU259578	(15)
Bo/BalikesirN9/2009/TUR	Turkey	2009	KF218822	This study
Bo/AdiyamanN6/2011/TUR	Turkey	2011	KF218825	This study
Bo/SivasN6/2011/TUR	Turkey	2011	KF218824	This study
Bo/DuzceN2/2010/TUR	Turkey	2010	KF218823	This study

Table 3. Nucleotide (below the diagonal) and amino acid (above the diagonal) similarities of the partial (326-bp) *RdRp* gene sequences.

	Isolate Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	Bo/BalikesirN9/2009/TUR		98.9	96.8	96.8	100	100	96.8	100	100	96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3	88.4
2	Bo/DuzceN2/2010/TUR	88.4		97.9	96.8	98.9	98.9	97.9	98.9	98.9	96.8	98.9	98.9	98.9	97.9	97.9	98.9	97.9	98.9	98.9	96.8	96.8	97.9	97.9	97.9	96.8	97.9	97.9	86.3	86.3	85.3	88.4
3	Bo/SivasN6/2011/TUR	90.1	88.4		98.9	96.8	96.8	100	96.8	96.8	96.8	96.8	96.8	96.8	97.9	95.8	96.8	97.9	96.8	96.8	96.8	96.8	97.9	97.9	97.9	98.9	97.9	97.9	86.3	86.3	85.3	88.4
4	Bo/AdiyamanN6/2011/TUR	86.3	88.1	87.0		96.8	96.8	98.9	96.8	96.8	97.9	96.8	97.9	96.8	98.9	95.8	97.9	96.8	97.9	97.9	97.9	97.9	98.9	98.9	98.9	100	98.9	98.9	87.4	87.4	86.3	89.5
5	BEC195/IT	97.5	89.1	89.1	86.3		100	96.8	100	100	96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3	88.4
6	BEC537/IT	97.5	88.8	89.1	85.6	97.2		96.8	100	100	96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3	88.4
7	BEC483/IT	88.0	86.0	88.4	87.0	87.4	87.0		96.8	96.8	96.8	96.8	96.8	96.8	97.9	95.8	96.8	97.9	96.8	96.8	96.8	96.8	97.9	97.9	97.9	98.9	97.9	97.9	86.3	86.3	85.3	88.4
8	Bo/MonastirB46/2006/TUN	97.2	88.4	90.2	86.0	96.8	97.5	87.4		100	96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3	88.4
9	Bo/MonastirB76/2007/TUN	96.1	88.1	89.1	86.0	95.8	95.8	87.0	97.5		96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3	88.4
10	Bo/MonastirB169/2010/TUN	90.1	85.6	86.3	88.1	89.5	89.1	91.2	89.5	88.4		96.8	100	98.9	100	97.9	98.9	98.9	96.8	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	97.9	97.9	86.3	86.3	85.3
11	Bo/Aberystwyth24/00/UK	96.1	88.4	88.1	86.0	95.4	96.8	87.0	95.1	94.7	88.1		96.8	97.9	96.8	96.8	95.6	97.9	96.8	97.9	97.9	98.9	97.9	97.9	98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
12	Bo/Newbury2/1976/UK	91.2	86.0	87.0	86.7	90.5	90.5	87.4	90.9	89.8	87.0	88.1		98.9	100	97.9	98.9	96.8	98.9	98.9	98.9	97.9	96.8	96.8	97.9	97.9	97.9	96.8	97.9	86.3	86.3	85.3
13	Bo/Dumfries/94/UK	89.8	86.3	86.7	86.0	89.8	90.9	86.7	89.8	89.5	86.7	87.0	90.5		98.9	98.9	97.9	100	96.9	100	100	98.9	97.9	97.9	98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
14	Bo/Penrith55/00/UK	91.2	87.0	89.5	88.4	90.2	90.2	95.4	90.5	89.5	93.7	86.7	90.9	88.4		97.8	98.9	98.9	96.8	98.9	97.9	96.8	96.8	96.8	97.9	97.9	97.9	96.8	97.9	86.3	86.3	85.3
15	Bo/NoV/JN-MA156/04/Korea	89.4	84.9	86.0	86.7	89.5	90.5	86.3	89.5	88.8	87.0	93.7	89.5	89.8	87.7		96.8	98.9	97.9	98.9	98.9	100	98.9	98.9	100	100	100	98.9	100	88.4	88.4	87.4
16	Bovine/DijonA311-1/08/FR	91.5	88.1	86.3	87.4	91.2	91.2	88.1	90.2	90.2	88.4	87.0	91.2	88.4	94.0	87.4		97.9	95.8	97.9	96.8	95.8	95.8	95.8	96.8	96.8	96.8	95.8	96.8	85.3	85.3	84.2
17	Bovine/DijonA358/07/FR	87.4	88.7	90.8	86.3	87.0	87.7	85.9	88.0	85.6	86.3	88.4	91.2	91.6	88.4	91.2	89.8		96.8	100	100	98.9	97.9	97.9	98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
18	Bovine/BV24/2007/BEL	90.5	85.6	87.4	86.7	89.1	88.8	86.3	88.4	87.4	86.7	86.3	87.3	83.8	87.7	87.0	86.3	86.6		96.8	96.8	97.9	96.8	96.8	97.9	97.9	97.9	96.8	97.9	86.3	86.3	85.3
19	Bovine/BV52/2007/BEL	90.5	87.4	88.4	87.0	89.5	90.2	86.7	89.8	88.8	88.4	86.7	89.8	88.8	92.6	86.7	90.9	87.4	87.3		98.9	97.9	97.9	97.9	98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
20	Bovine/Wasme/B199/2003/Be	90.5	86.7	87.7	89.5	90.5	90.2	93.3	89.1	88.1	93.7	88.4	90.5	89.8	94.0	89.1	92.3	88.8	88.4	95.8		98.9	97.9	97.9	98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
21	Bovine/GIII.2/300_0250/2006/NOR	90.9	86.3	88.4	85.6	89.4	89.1	89.1	88.7	87.7	89.4	93.7	89.5	89.1	87.4	97.2	87.7	91.9	85.9	86.7	88.1		98.9	98.9	100	100	100	98.9	100	88.4	88.4	87.4
22	Bovine/GIII.2/471_0790/2005/NOR	91.6	85.6	88.4	87.0	91.2	90.1	87.5	89.1	89.4	88.7	89.4	88.0	86.6	86.3	90.8	85.2	87.7	87.4	87.7	87.7	90.1		97.9	98.9	98.9	98.9	97.9	98.9	89.5	89.5	88.4
23	Bo/Nov-6/USA/2010	91.9	86.7	87.7	87.7	90.2	89.5	90.5	89.1	88.4	92.6	88.7	89.1	88.4	88.4	90.1	86.6	87.7	86.0	89.1	87.7	89.8	94.4		98.9	98.9	98.9	97.9	98.9	87.4	87.4	86.3
24	Bo/Nov-46/USA/2010	90.5	85.3	88.4	88.1	88.8	88.8	89.1	89.1	88.1	90.5	92.6	88.4	88.8	88.1	93.7	88.1	89.5	86.3	88.1	90.2	92.3	91.2	90.5		100	100	98.9	100	88.4	88.4	87.4
25	Bo/Nov-4/USA/2010	86.3	86.0	87.4	95.1	85.6	86.3	86.3	86.7	86.3	87.0	90.5	88.1	85.3	87.7	93.0	88.1	87.7	85.9	87.0	89.1	91.6	89.8	90.5	95.8		100	98.9	100	88.4	88.4	87.4
26	Bo/Nov-50/USA/2010	87.0	87.7	88.8	97.2	87.0	87.0	87.7	87.4	87.7	87.7	87.0	86.7	88.1	86.0	88.4	86.7	87.7	88.4	86.0	87.0	88.8	86.3	86.3	87.4	86.7		98.9	100	88.4	88.4	87.4
27	CV500-OH	90.1	86.7	89.1	89.5	88.8	88.4	92.3	88.8	88.4	91.2	87.7	87.4	87.4	87.4	89.1	88.8	88.8	88.0	86.7	87.7	89.5	86.3	87.7	88.8	88.8	97.2		98.9	87.4	87.4	86.3
28	Isolate_Jena	75.7	76.1	77.8	76.4	76.8	76.4	73.9	76.1	76.1	72.5	91.2	89.1	88.4	86.7	96.1	87.7	89.5	85.9	87.4	88.1	94.0	90.5	90.5	94.0	93.3	88.8	90.2		88.4	88.4	87.4
29	Bo/DijonA273/2007/FR	75.4	76.8	77.5	76.8	75.4	75.4	75.4	74.3	75.7	74.6	72.5	78.2	75.4	76.1	74.6	77.1	76.4	76.8	75.7	75.0	74.6	75.4	76.8	73.2	74.6	76.1	77.8	75.4		100	96.8
30	Bovine/DijonA036/06/FR	76.4	73.9	75.4	75.4	76.1	75.0	73.6	75.4	76.1	73.2	74.6	75.0	73.9	75.0	77.5	75.2	75.4	75.7	74.6	75.7	77.1	77.5	77.5	77.1	77.8	76.4	78.2	77.5	89.8		96.8
31	Cattle/BV416/2008/BEL	77.9	76.4	75.7	75.0	76.8	77.8	76.1	76.8	77.5	76.1	73.2	75.7	75.7	75.0	75.4	74.3	74.3	72.5	74.6	74.3	75.4	75.7	77.1	75.7	77.1	73.6	75.4	75.4	88.4	89.1	

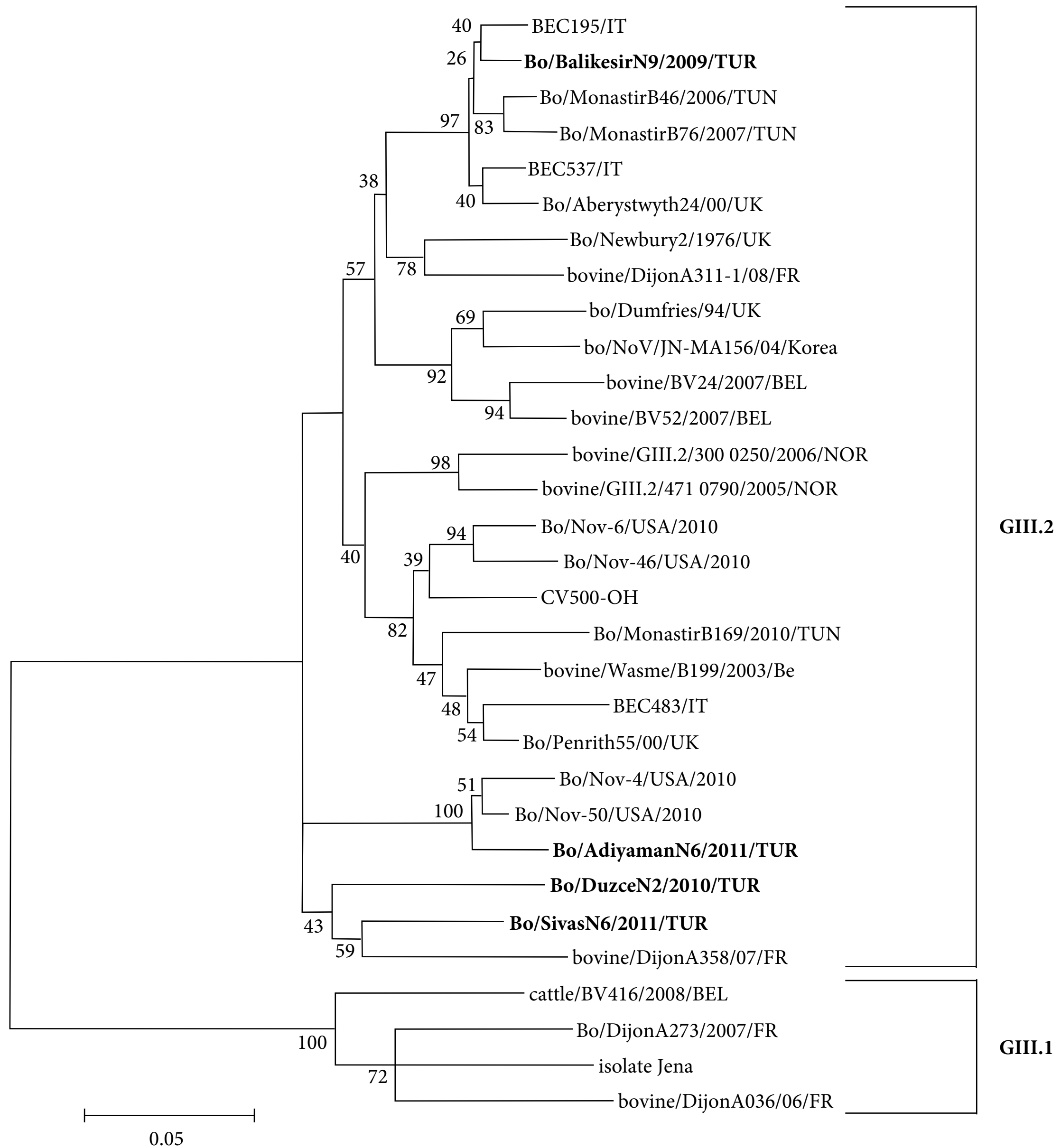

Figure 2. A phylogenetic tree of the partial 285-bp nucleotide sequence of the *RdRp* gene of BNoV strains was made using the neighbor-joining method of MEGA (13). The names of the viruses used are listed in Table 2.

Marmara region were detected as positive by SYBR Green-based real-time PCR assay, while our study encompassed the whole of Turkey as shown in Figure 1. In spite of adequate sampling and being conducted in the same period of time, the positive results of our survey were five times lower than those of the previous report. There are several explanations for the inconsistency between these studies. First, the rare and nonuniform distribution of BNoVs among cattle herds may decrease the ratio. Second, the different RT-PCR procedures followed in these two studies may cause variation. The sensitivities of different methods must be compared in order to obtain further reliable data.

The alignment indicated that the Turkish Bo/BalikesirN9/2009/TUR strain clustered slightly distantly from the other three Turkish strains (Bo/SivasN6/2011/TUR, Bo/DuzceN2/2010/TUR, and Bo/AdiyamanN6/2011/TUR) within GIII.2. These results indicate that there are different strains circulating in Turkey. Furthermore, only the detection of genotype GIII.2 strains confirms genotype GIII-2 as a main genotype in BNoV (4,12,20).

The similarity analysis (Table 3) indicated that Turkish BNoVs vary from 86.3% to 90.1% nt and 96.8% to 98.9% aa similarities among each other. Interestingly, a comparison

of the nt and aa sequences among the BNoVs revealed that all the analyzed GIII.2 BNoVs shared 85.2% to 96.8% nt and 96.8% to 100% aa. GIII.1 strains shared 86.3% to 89.8% nt and 87.4% to 100% aa with each other. GIII.1 and GIII.2 strains vary between 72.5% to 78.6% nt and 84.2% to 100% aa. On the other hand, while the similarity of the Turkish Bo/BalikesirN9/2009/TUR strain was found as 96.1% to 97.5% nt and 100% aa identical with some of the Italian and Tunisian strains, there was no relation between the other three Turkish strains and any of the remaining strains.

In conclusion, based on the partial sequence of the BNoV *RdRp* gene, one of the Turkish BNoV strains (Bo/BalikesirN9/2009/TUR) was more closely related to the Italian and Tunisian BNoVs than to the other BNoVs. All reported strains of Turkish BNoV belonged to genotype GIII.2. This study shows that BNoV infections are rarely circulated between diarrheic calves in Turkey.

Acknowledgment

We thank Dr Eray Atıl from the Pendik Veterinary Control and Research Institute (İstanbul, Turkey) for sequencing the PCR products.

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