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Sequence analysis of Turkish field strains of bovine torovirus shows unique amino acid changes in the partial M gene

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ABSTRACT

Objective: To investigate the presence, prevalence and phylogenetic classification of bovine torovirus (BToV). **Methods:** Stool samples from 72 calves, which were negative for primary gastroenteritis agents, were examined with the nested PCR method by using BToV M gene-specific primers. **Results:** BToV was detected in 12 (16.7%) out of 72 samples. Phylogenetic analysis was performed using nucleotide and amino acid sequences. In the phylogenetic tree, European, American, Far East and Turkish strains were found to be divided into different branches. Interestingly, it was observed that Turkish strains were divided into two subgroups. Considering the amino acid sequences of these strains having differences at nucleotide level, the change at the 3rd amino acid of the partial M gene in Turkish strains has made Turkish strains different from all other strains. Similarly, the differences were observed in the 18th, 20th, 63rd and 93rd amino acids of the partial M gene only in Turkish field strains. **Conclusions:** This study revealed that Turkish strains of BToV constitute a separate phylogenetic group and can be divided into two subgroups. In addition, BToV was found to be a common pathogen causing diarrhea in calves in Turkey, and it is a necessity to consider BToV in cases of diarrhea with unknown cause.

1. Introduction

Bovine torovirus (BToV) is an enteropathogen that causes mild to moderate diarrhea, affecting cattle, horses, swines and humans. Toroviruses, a member of the *Coronaviridae* family in the Nidovirales order, have positive-sense, enveloped, and single-stranded RNA genome[1–3]. Genome of the torovirus is 25–30 kb long, which has a pleomorphic appearance and fringe-like peplomers on its surface, and encodes five different proteins consisting of RNA polymerase, spike (S), membrane (M), hemagglutinin-esterase (HE) and nucleocapsid (N) proteins. Although the torovirus is very similar to the coronavirus in electron microscope visually, there is no antigenic relationship between them[4,5].

Toroviruses have first been diagnosed in a foal with diarrhea in

Bern, Switzerland in 1972, and named as Berne virus. Seven years later, in the city of Breda which is in the state of Iowa in the United States, particles similar to Berne virus were detected in the electron microscope in the samples from calves with severe diarrhea, and named as Bredavirus. These viruses, which are prototypes of toroviruses, are now called as toroviruses and are expressed with the species name of the infected animal in taxonomy[6].

Toroviruses cause severe diarrhea in horses and cattle, whereas they have an asymptomatic characteristic in swines. In humans, it has been reported to cause nosocomial viral gastroenteritis,

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especially in the pediatric age group[7].

Among the causes of viral diarrhea, rotavirus and coronavirus are the first agents that come to mind. However, advances in technology and the development of various diagnostic methods have led to the identification of adenovirus, enterovirus, norovirus, and astrovirus as other diarrhea agents[8–11]. Although BToV has been detected many years ago for the first time, its prevalence and biology in the world have not been fully elucidated yet. Presence of torovirus in calves with diarrhea has been reported in various countries, such as USA[12,13], Austria[5], Brazil[14], Germany[15,16], Hungary[17], Japan[18,19] and Korea[20]. However, there is only one study that investigates torovirus in calves in Turkey[10]. Horse breeding is widespread in eastern region of Turkey, due to javelin sport in particular, and horses and cattle share the same barn or environment in this region. For this reason, it is necessary to identify the species of BToV, circulating in Turkey, and reveal these toroviruses with different genetic characteristics.

This pilot study aims to investigate the presence of BToV in eastern Turkey and to reveal its genetic diversity.

2. Materials and methods

2.1. Sampling

Stool samples were obtained between 2016 and 2017 from small family farms in eight different centers, namely Erzurum center and Yakutiye, Palandöken, Aziziye, Aşkale, Tortum, Pasinler, and Hasankale (Figure 1). According to the rapid test kit results, stool samples that have none of the common enteric pathogens (rotavirus, coronavirus, *Escherichia coli*, *Clostridium* and *Cryptosporidium*) were included in the study. Stool samples from a total of 72 calves with diarrhea were used in the study.



Figure 1. Locations of samples collected from the Province of Erzurum, and surroundings in Turkey.

White points represent the centers where the samples are collected.

2.2. RNA extraction

Stool specimens that were negative for the initial test (rapid test) were diluted 1/10 and vortexed. Then, they were centrifuged at 3 000 *g* for 20 min. After centrifugation, 200 μ L supernatant was transferred to a new tube and stored at -80 $^{\circ}$ C until the day of study.

The GF-1 Viral Nucleic Acid Extraction Kit (Vivantis, Malaysia) was used for the viral nucleic acid isolation in the stool samples according to the procedure recommended by the manufacturer.

2.3. cDNA synthesis and RT-PCR

Extracted viral nucleic acids were first converted into cDNA. For this purpose, RevertAidTM First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) was used. The procedure provided by the manufacturer company was used. The cDNA of the samples was used for detection of BToV by PCR. For the detection of toroviruses, nested PCR was performed using membrane (M) gene-specific primers as defined by the Park *et al* (Table 1)[21]. After gel electrophoresis was performed, the PCR products of 409 bp size under UV light were considered as positive. Positive samples with strong gel image were subjected to sequence analysis using the ABI Prism BigDye Terminator version 3.1 sequencing kit and Applied Biosystems 310 DNA analyzer (Applied Biosystems Inc., CA, USA) bidirectionally for the BToV M gene using PCR primers.

Table 1

RT-PCR and nested PCR primers for detection of BtoV.

Sequence (5' - 3')	Region	Product
F: TTCTTACTACACTTTTGGGA	98-700	603
R: ACTCAAACCTTAACACTAGAC		
nF: TATGTACTATGTTTCCAGCT	152-560	409
nR: CCAACACAAATCCGCAACGC		

F: forward primer for first PCR; R: reverse primer for first PCR; nF: forward primer for nested-PCR; nR: reverse primer for nested-PCR.

2.4. Sequencing and phylogenetic analyses

The raw data obtained after the sequencing was confirmed by GenBank (NCBI National Center for Biotechnology Information/BLAST). Sequences of reference strains and studied samples were aligned with BioEdit (version 7.0.5) program[22]. Phylogenetic and bootstrap (1000 replicates) analysis, based on the M gene nucleotide and amino acid sequence, was performed using the neighbor-joining method under the Molecular Evolutionary Genetics Analysis (MEGA, Version 6.0) program[23]. Table 2 shows the accession numbers from GenBank for the partial M gene sequences obtained in this study.

Table 2

Accession numbers of Turkish BToV partial M gene sequences.

Isolate name	Year/Sampling	Origin	Accession No
TR-Erz-Ask-8	March 2016	Aşkale	MF687252
TR-Erz-Ask-13	March 2016	Aşkale	MF687253
TR-Erz-Azi-27	April 2016	Aziziye	MF687254
TR-Erz-Pal-30	November 2016	Palandöken	MF687255
TR-Erz-Yak-46	February 2017	Yakutiye	MF687256
TR-Erz-Tor-48	February 2017	Tortum	MF687257
TR-Erz-Tor-50	February 2017	Tortum	MF687258
TR-Erz-Pas-52	March 2017	Pasinler	MF687259
TR-Erz-Pas-54	March 2017	Pasinler	MF687260

3. Results

After nested-PCR performed by targeting the partial M gene sequence

of 409 bp size of BToV, 12 (16.7%) out of 72 stool samples were found to be positive. Of the 12 positive samples, 9 samples with strong gel images were subjected to sequence analysis and phylogenetic analysis in order to determine their genetic diversity and similarity to other genogroups. Four different lineages were formed after phylogenetic analysis of the positive samples with the reference sequences obtained from GenBank. While all the strains from Turkey formed a group, strains from Italy and the Netherlands formed the European strains group. Similarly, Japanese and Korean strains and American Breda strains were distinctly clustered under different groups. And, the BToV strains identified in Turkey were further divided into two different subgroups (Sub-group 1, Sub-group 2) (Figure 2). When we look at the two subgroups of Turkish strains, it is seen that the strains collected

from the eastern and western regions of Erzurum are distinct from each other, and formed these two subgroups.

When we look at Figure 3, in which the similarity percentages of nucleotide sequences of Turkish strains and the reference strains are evaluated, we see that our study strains are similar among themselves by 96.4-100.0%. BToV strains of Turkey, which is considered a bridge between Europe and Asia, were found to be similar to the European strains by 97.4-99.0%, as expected. Nucleotide similarity between the studied strains and the Far East (Japan & Korean) strains was 95.1-98.7%. And, the relationship between the strains obtained in our study and the American origin Breda virus (Accession number: Ay427798) was most distant by 94.3-96.1% (Figure 3).

Considering the amino acid sequences of the M genes of BToV, it

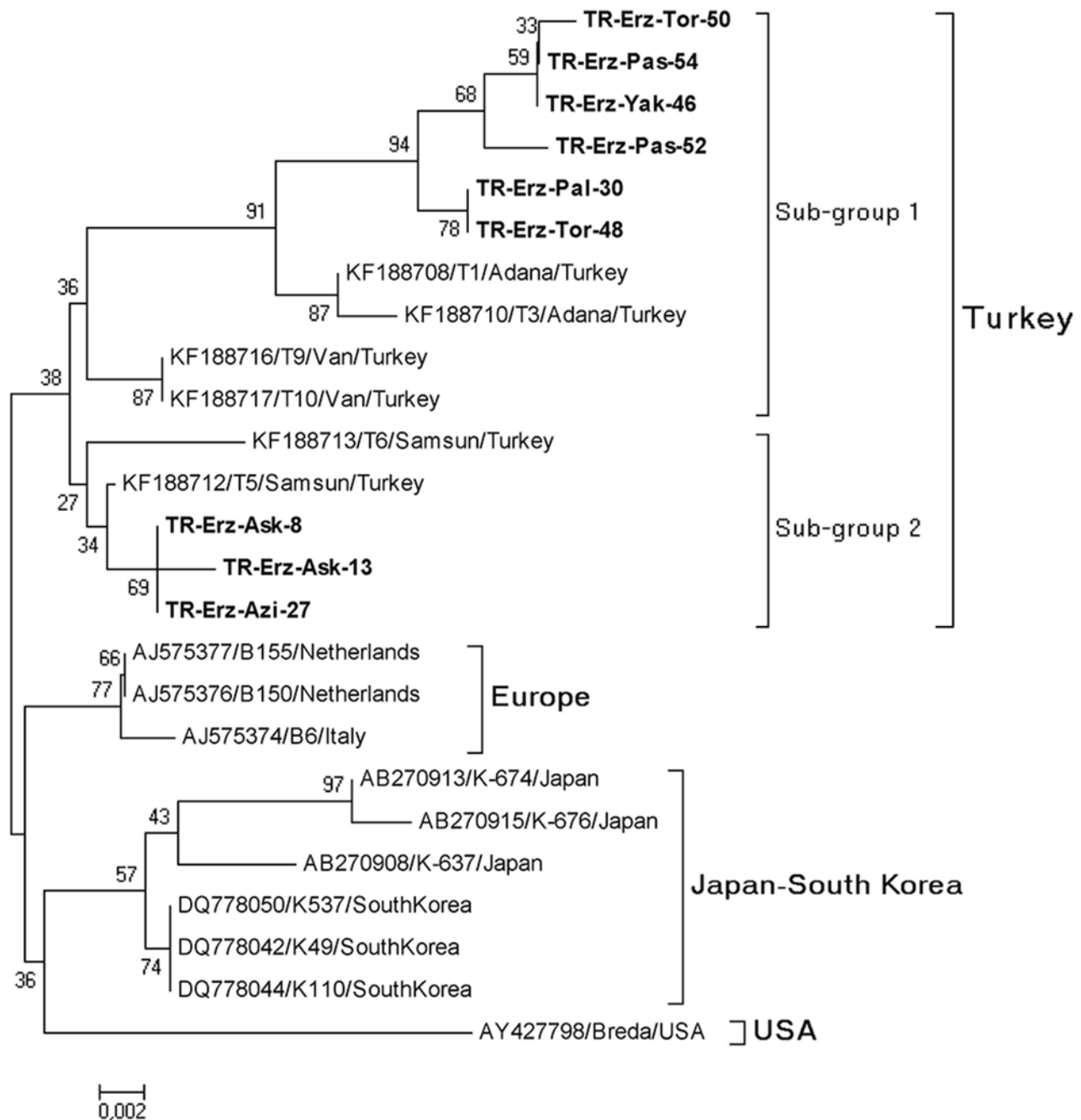


Figure 2. Neighbor-joining phylogenetic trees of 406-nt BToV membrane (M) gene sequences from 9 strains studied and 16 sequences form GenBank. Accession numbers and countries of the sequences obtained from GenBank are shown on the tree. BToV strains from this study are shown in bold. The bar graph shows the distance.

		Percent Identity																										
		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		
Divergence	1	■	96.4	96.4	96.4	95.6	96.1	96.4	96.4	96.6	96.6	96.1	96.4	96.4	96.4	95.6	95.3	96.1	95.8	96.1	95.1	95.1	95.1	94.3	95.1	95.1	1	USA
	2	3.7	■	100.0	100.0	99.2	98.7	99.0	98.4	98.7	98.7	98.7	99.0	98.4	98.4	97.7	97.4	98.7	98.4	98.7	97.1	96.6	97.1	95.8	96.6	96.6	2	SouthKorea
	3	3.7	0.0	■	100.0	99.2	98.7	99.0	98.4	98.7	98.7	98.7	99.0	98.4	98.4	97.7	97.4	98.7	98.4	98.7	97.1	96.6	97.1	95.8	96.6	96.6	3	SouthKorea
	4	3.7	0.0	0.0	■	99.2	98.7	99.0	98.4	98.7	98.7	98.7	99.0	98.4	98.4	97.7	97.4	98.7	98.4	98.7	97.1	96.6	97.1	95.8	96.6	96.6	4	SouthKorea
	5	4.6	0.8	0.8	0.8	■	98.4	98.7	98.2	98.4	98.4	97.9	98.2	97.7	97.7	96.9	96.6	98.4	98.2	98.4	96.4	95.8	96.4	95.1	95.8	95.8	5	Japan
	6	4.0	1.3	1.3	1.3	1.6	■	99.7	97.7	97.9	97.9	97.4	97.7	97.7	97.7	96.4	96.1	97.4	97.1	97.4	95.8	95.8	95.8	95.1	95.8	95.8	6	Japan
	7	3.7	1.0	1.0	1.0	1.3	0.3	■	97.9	98.2	98.2	97.7	97.9	97.9	97.9	96.6	96.4	97.7	97.4	97.7	96.1	96.1	96.1	95.3	96.1	96.1	7	Japan
	8	3.7	1.6	1.6	1.6	1.8	2.4	2.1	■	99.7	99.7	98.2	98.4	98.4	98.4	98.2	97.9	98.7	98.4	98.7	97.7	97.7	97.7	96.9	97.7	97.7	8	Italy
	9	3.5	1.3	1.3	1.3	1.6	2.1	1.8	0.3	■	100.0	98.4	98.7	98.7	98.7	97.9	97.7	99.0	98.7	99.0	97.4	97.4	97.4	96.6	97.4	97.4	9	Netherlands
	10	3.5	1.3	1.3	1.3	1.6	2.1	1.8	0.3	0.0	■	98.4	98.7	98.7	98.7	97.9	97.7	99.0	98.7	99.0	97.4	97.4	97.4	96.6	97.4	97.4	10	Netherlands
	11	4.0	1.3	1.3	1.3	2.1	2.6	2.4	1.8	1.6	1.6	■	99.2	98.7	98.7	97.9	97.7	99.0	98.7	99.0	97.4	96.9	97.4	96.1	96.9	96.9	11	Turkey
	12	3.7	1.0	1.0	1.0	1.8	2.4	2.1	1.6	1.3	1.3	0.8	■	99.5	99.5	98.7	98.4	99.7	99.5	99.7	98.2	97.7	98.2	96.9	97.7	97.7	12	Turkey
	13	3.7	1.6	1.6	1.6	2.4	2.4	2.1	1.6	1.3	1.3	1.3	0.5	■	100.0	98.7	98.4	99.2	99.0	99.2	97.7	97.7	97.7	96.9	97.7	97.7	13	Turkey
	14	3.7	1.6	1.6	1.6	2.4	2.4	2.1	1.6	1.3	1.3	1.3	0.5	0.0	■	98.7	98.4	99.2	99.0	99.2	97.7	97.7	97.7	96.9	97.7	97.7	14	Turkey
	15	4.6	2.4	2.4	2.4	3.2	3.7	3.5	1.8	2.1	2.1	2.1	1.3	1.3	1.3	■	99.7	98.4	98.2	98.4	99.0	98.4	99.0	97.7	98.4	98.4	15	Turkey
	16	4.8	2.7	2.7	2.7	3.5	4.0	3.7	2.1	2.4	2.4	2.4	1.6	1.6	1.6	0.3	■	98.2	97.9	98.2	98.7	98.2	98.2	98.7	98.2	98.2	16	Turkey
	17	4.0	1.3	1.3	1.3	1.6	2.6	2.4	1.3	1.0	1.0	1.0	0.3	0.8	0.8	1.6	1.8	■	99.7	100.0	97.9	97.4	97.9	96.6	97.4	97.4	17	TR-Erz-Ask-8
	18	4.3	1.6	1.6	1.6	1.8	2.9	2.6	1.6	1.3	1.3	1.3	0.5	1.0	1.0	1.8	2.1	0.3	■	99.7	97.7	97.1	97.7	96.4	97.1	97.1	18	TR-Erz-Ask-13
	19	4.0	1.3	1.3	1.3	1.6	2.6	2.4	1.3	1.0	1.0	1.0	0.3	0.8	0.8	1.6	1.8	0.0	0.3	■	97.9	97.4	97.9	96.6	97.4	97.4	19	TR-Erz-Azi-27
	20	5.1	2.9	2.9	2.9	3.7	4.3	4.0	2.4	2.6	2.6	2.6	1.8	2.4	2.4	1.0	1.3	2.1	2.4	2.1	■	99.5	100.0	98.7	99.0	99.5	20	TR-Erz-Pal-30
	21	5.1	3.5	3.5	3.5	4.3	4.3	4.0	2.4	2.6	2.6	3.2	2.4	2.4	2.4	1.6	1.8	2.7	2.9	2.7	0.5	■	99.5	99.2	99.5	100.0	21	TR-Erz-Yak-46
	22	5.1	2.9	2.9	2.9	3.7	4.3	4.0	2.4	2.6	2.6	2.6	1.8	2.4	2.4	1.0	1.3	2.1	2.4	2.1	0.0	0.5	■	98.7	99.0	99.5	22	TR-Erz-Tor-48
	23	6.0	4.3	4.3	4.3	5.1	5.1	4.8	3.2	3.5	3.5	4.0	3.2	3.2	3.2	2.4	2.6	3.5	3.7	3.5	1.3	0.8	1.3	■	98.7	99.2	23	TR-Erz-Tor-50
	24	5.1	3.5	3.5	3.5	4.3	4.3	4.0	2.4	2.6	2.6	3.2	2.4	2.4	2.4	1.6	1.8	2.7	2.9	2.7	1.0	0.5	1.0	1.3	■	99.5	24	TR-Erz-Pas-52
	25	5.1	3.5	3.5	3.5	4.3	4.3	4.0	2.4	2.6	2.6	3.2	2.4	2.4	2.4	1.6	1.8	2.7	2.9	2.7	0.5	0.0	0.5	0.8	0.5	■	25	TR-Erz-Pas-54

Figure 3. Percentage similarity and difference of nucleotides.

is noteworthy that Turkish strains clearly have different amino acid sequences compared to Europe, America and Far East strains (Figure 4). When the amino acid sequences obtained from the Turkish strains of BToV are examined, it is observed that methionine (M), the 3rd amino acid in partial M gene, is replaced by asparagine (N) amino acid. In addition, the amino acid sequences obtained in our study were found to differentiate from other Turkish strains through asparagine (N) to isoleucine (I) transformation in 18th amino acid, tryptophan (W) to glycine (G) in the 20th amino acid, and valine (V) to isoleucine (I) in 63rd and 93rd amino acids. When we look at the American strain “Breda”, we see that the serine amino acid (S) has been exchanged with arginine amino acid (R) change at the 70th amino acid of the partial M gene, constituting a unique sequence.

4. Discussion

There is no sufficient information about the prevalence, biology and genetics of BToV associated with the calf diarrhea. Although there are studies reporting that BToV is causing coronavirus-like winter dysentery in the bovine population, it has not yet been placed in the routine tests[4,12]. In our study, calf stool samples which were negative by routine diagnostic tests (cause not determined) were examined for BToV and their contribution in diarrhea etiology was evaluated for our region. To our knowledge, there is only one study on BToV in Turkey, and it has reported that BToV infection is sporadic with 4.7% prevalence in Turkey[10]. In our study which constitutes the second report from Turkey, the BToV prevalence was determined to be 16.7%. This rate is more than three times higher from the previous study, which suggests that the disease is widespread, rather than sporadic, in our regions of study. Furthermore, when we consider that the stool samples used in our study were negative for primary pathogens associated with diarrhea, the prevalence of BToV infection in diarrhea aetiology becomes more prominent. When we look at various studies on the prevalence of BToV in calf diarrhea in the world, we find prevalence rates of 36.4% in the USA[12], 5.2% in Austria[5], 43.2%

in Croatia[24], 6.5% in Japan[25], 6.4% in the Netherlands[26] and 2.9% in South Korea[20]. When compared with the findings from our study, we see that the prevalence of BToV is relatively higher than European countries and Far East (Japan & Korean).

Amino acid and nucleotide sequences of BToV positive amplicons were analysed between themselves and with previously reported sequences. Phylogenetic analysis of the M gene sequences of BToV field strains showed that America, Europe, Turkey and Far Eastern strains are grouped together, and divided into phylogenetic branches. However, it was observed that Turkish strains can be divided into two subgroups. This is thought to be a result of mutations frequently observed in RNA viruses. When we examined the nucleotide sequences of BToV M gene fragments obtained in our study, it was found that they are identical among themselves. Comparison of our study strains with BToV strains previously reported by Gulacti *et al.* in Turkey showed a sequence similarity rate of 96.1-99.7%[10]. Turkish strains, which form the most distant sequence relationship with American Breda strains by 94.3%, have closer nucleotide sequence similarities with European strains as expected. It is noteworthy that European strains have clustered together in the phylogenetic tree in various studies in Europe[5,24]. This shows that BToV strains circulated in Europe are in genetic stability, but there were differences in nucleotide levels observed in Turkish strains.

When we examine the amino acid sequence analysis of the reference strains and our study strains phylogenetically, it is observed that American Breda strain behaves as an out-group probably due to the arginine and serine amino acid exchange in the 70th amino acid. Supporting the hypothesis we have emphasized, Turkey strains are separated from the America, Europe and the Far East strains, especially due to the exchange of the methionine amino acid at the 3rd amino acid with the asparagine. In addition to creating a different cluster, Turkish strains that stand out in our study are divided into sub-groups within themselves. This is because, changes were found in 18th (N→I), 20th (W→G), 63rd (V→I), and 93rd (V→I) amino acids according to all other isolates. These differences identified at the M gene level of BToV cannot be neglected, and it is necessary to clarify this with multi-genomic studies that should be conducted in the future. This has the

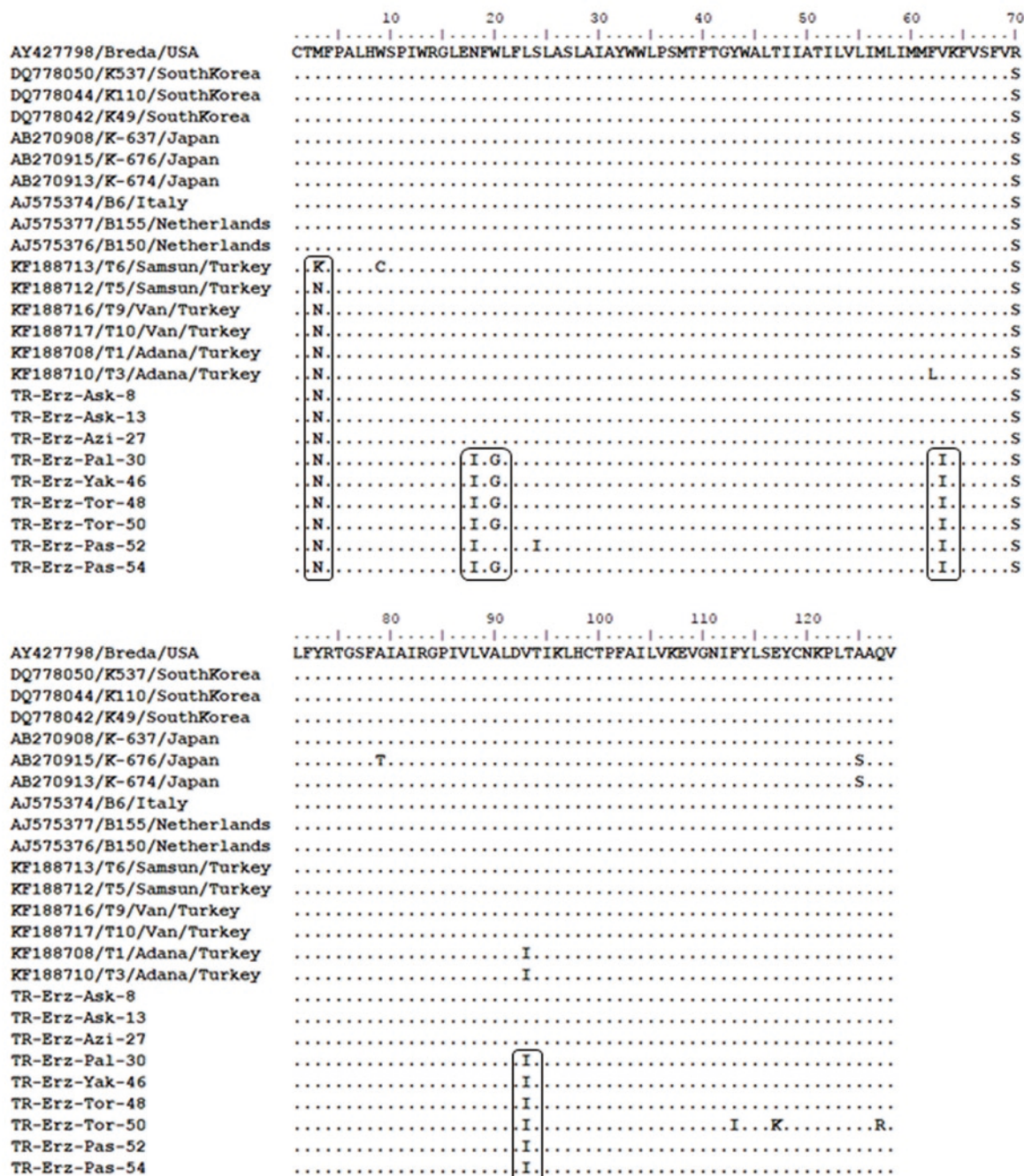


Figure 4. Amino acid sequences of the BToV M gene.

Mutation regions are shown in the box.

potential to affect the pathogenicity and virulence of the virus or the diagnosis of the disease.

Mutations are common in RNA viruses. These mutations, which are also common in coronaviruses, can eventually result in virulence-altered strains or different phylogenetic groups and even new species. The virus host spectrum can change and even gain zoonotic properties as a result of mutations[27,28]. As an example to mutations from the *Coronaviridae* family, the feline coronavirus type 2 virus was emerged as a result of recombination between feline coronavirus type 1 and canine coronavirus type 2[29]. The amino acid changes occurred in Turkish BToV field strains in our study are examples of small-scale

mutations.

Phylogenetic analysis, nucleotide homology and amino acid sequence analysis all support relationship among different strains. It is noteworthy that clusters are based on the continents or regions. Europe, Far East, Turkey and the America strains formed separate clusters. In addition, strains from western districts of Erzurum (Aziziye and Aşkale) and Samsun strains can be called as sub-group 2, and strains from other districts of Erzurum (Palandöken, Yakutiye, Tortum and Pasinler) and strains from provinces of Adana and Van can be named as sub-group 1. However, this investigation should not only be conducted on a single level of gene region, and further studies should be conducted to support

this finding.

As a result, the presence and prevalence of torovirus in north-eastern Anatolia region was investigated for the first time in the study, and positive strains were examined at amino acid and nucleotide level. The only study on BToV in Turkey emphasizes that BToV infection is a sporadic and rare infection in Turkey^[10], however, our rates show that it is far from being sporadic. Future studies should clarify the effect of BToV strains with different nucleotide/amino acid sequences on the clinical and pathological manifestation of the disease. Currently, there is no vaccine for protection against this infection. It was concluded that a vaccine to be developed at the M gene level in the future might be protective both in Europe and in Turkey. In addition, no diarrheal infections were found in horses during the study. Therefore, transition of toroviruses between cattle and horses in our region could not be clarified. It would be beneficial to clarify the cattle-horse and torovirus relationship in future studies.

Although BToV is known to cause an infection in the respiratory and gastrointestinal system in humans and animals, we do not yet have enough knowledge of the transition between species. Epidemiological and genetic studies are a necessity in order to establish a firm basis for the infections. The data obtained in this study provides not only information about the prevalence of BToV, but also important data in the genetic sense.

Conflict of interest statement

We declare no conflict of interest.

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