Re-emergence of Bovine Ephemeral Fever in Turkey in 2020 after an 8-Year Absence: A Molecular Analysis Study

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ABSTRACT

Bovine ephemeral fever (BEF) is a vector-borne disease of ruminants in tropical and subtropical areas, leading to significant economic losses to the cattle and milk industry in many countries, including Turkey. Cattle showing high fever, stagnation, and recumbency have been extensively reported in Turkey's south and south eastern regions in 2020. Here, the genetic analysis and molecular epidemiology of the virus obtained from the last BEF outbreak were investigated. Of 32 symptomatic cattle selected from three provinces in Turkey, 23 were positive for bovine ephemeral fever virus (BEFV), and three entire G genes were identified by sequencing. The new BEFV isolates were genetically similar to some Turkish and Israeli isolates from 2008 and 2012 (>98 nucleotide (nt), 97% amino acid (aa)), and phylogenetic analysis based on the surface glycoprotein (G) gene revealed that BEFV isolates are of Middle Eastern origin. The G protein amino acid alignment showed that BEFV circulated in the same region with minor differences over the years. In this context, we recommend closely monitoring BEF outbreaks in neighbouring countries and prompt vaccination of susceptible cattle in areas at risk for BEFV in Turkey in the event of an outbreak.

Kew words: BEF; Genetic Characterization; Epidemic; Report; Turkey.

INTRODUCTION

Bovine ephemeral fever, also known as BEF, 3-day sickness, 3-day fever, bovine enzootic, bovine influenza, stiffseitke, or dragon boat) is an acute febrile illness of cattle, water buffaloes, and occasionally other ruminants, transmitted by arthropod vectors such as mosquitoes and biting midges (*Culicoides* spp.) (1). Bovine ephemeral fever, is widespread in tropical and subtropical areas of Africa, Asia, Australia, and the Middle East, causing an acute systemic inflammatory response characterized by biphasic fever, lymph node enlargement, salivation, nasal and ocular discharges, subcutaneous edema, tachycardia, respiratory distress, muscle stiffness and tremors, lameness and paralysis (2). In the epidemic season, infection and morbidity rates are typically high (nearly 100%). Nevertheless, mortality rates generally remain low (<2%), except for a few reports in Turkey and China, where they reached 10–20% (3, 4). The economic burden of BEF may be considerable and are due primarily to a sudden drop in milk production in dairy cattle, loss of condition and infertility in beef cattle and the immobilization of water buffalo used for draught power (5).

Bovine ephemeral fever virus (BEFV), the etiological agent of BEF, belongs to the family Rhabdoviridae, genus Ephemerovirus, and species Bovine ephemeral fever virus (5). It has a single-stranded, negative-sense RNA genome of 14,900 nucleotides (nt) in size that consists of 10 ORFs, which are 3'-N-P-M-G-[GNS-α1-α2-β-γ]-L-5' (6). The BEFV virion, which typically displays rhabdovirus

bullet-shaped morphology, contains five structural proteins: Nucleoprotein (52 kDa), tightly associated with the viral genome and most abundant in the virion; phosphoprotein (43 kDa), essential for transcription and replication. Matrix protein (29 kDa), which plays a critical role in virus maturation and budding; glycoprotein (81 kDa), which is a virion membrane surface protein and contains the major neutralizing antigen; and L protein (180 kDa), which is a multifunctional enzyme (1, 5). Apart from the structural proteins, the viral genome encodes five non-structural proteins, except for α1, which acts as a viroporin, and α3, which plays a role in apoptosis. The functions of other proteins (GNS, α2, and β) have not yet been elucidated (7, 8).

The BEFV's glycoprotein (G) gene has been sequenced most frequently. A phylogenetic analysis of this gene showed that the isolates fall into four groups based on where they were isolated: Africa, Asia, Australia, and the Middle East (9). BEF has been documented in Iran, Israel, Jordan, Syria, and Iraq, as well as Saudi Arabia and Turkey, and has been described in Egypt and Palestine (10).

The first reported BEF outbreak in Turkey was documented in 1985 in Anatolia's central, southern, and south-eastern regions, followed by outbreaks in 1999, 2003, 2008, and 2012 (4, 11). Although most outbreaks occurred in the southern part of Anatolia, the seroprevalence of BEF was reported as 2.5-15.3% and 13.5% in the western provinces of European Turkey and the central Black Sea region, respectively (12, 13). BEF outbreaks in Turkey occurred periodically every 4-5 years from 1999 to 2012, (4, 11) and each time they had the potential to spread rapidly in the south and southeast of Turkey, threatening cattle life and causing significant yield and economic losses. Therefore, BEF may be considered one of the important seasonal disease in the southern and south-eastern coastal regions of Turkey. While it seems more or less predictable when BEF will emerge, the situation in the last outbreak was unexpected. Eight years later, in 2020, the previous BEF outbreak in Turkey was markedly reported by veterinarians in Turkey's southern, south-eastern, and eastern provinces in late summer and early autumn, when temperatures exceeded 40°C and mosquito populations increased.

This study investigated the genetics and molecular epidemiology of the virus that was isolated in Turkey during the BEF epidemic of 2020.

MATERIALS AND METHODS

Sample collection and preparation, ethical statement

From the beginning of September to mid-December 2020, a total of 32 tubes of heparinized blood were collected from the jugular vein of symptomatic (high fever, sternal recumbency, stiffness, and oral and nasal discharge) cattle populations in Kahramanmaraş, Hatay, and Elazığ provinces located in the southern and eastern Anatolian region of Turkey. The blood samples were transferred in a constant cold chain to the Virology department of the Faculty of Veterinary Medicine of Firat University. Heparinized blood was centrifuged at 4500 rpm at 4°C for 15 minutes using a Hettich 32R centrifuge (Hettich, Tuttlingen, Germany) to separate the plasma. The obtained plasma was stored at -80°C until virus isolation and viral genome detection were carried out.

The Animal Experiments Ethics Committee of Firat University, gave permission No. 423048, for all procedures that were performed on the live animals.

Viral genome detection and virus isolation

Viral RNA isolation from plasma samples was performed with the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. One-step RT-PCR was applied to detect the positivity of clinical samples and performed as previously described with specific partial G gene primers (14). BEFV was separated from the processed blood cells in the same way as descibed by Abayli *et al.* (15). Briefly, African green monkey kidney epithelial cells (Vero E6, obtained from the American Type Culture Collection (ATCC, CRL 1586) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, MO, USA) supplemented with 15 mM/L HEPES (Thermo Fisher, MA, USA), 1.5 gr/L sodium bicarbonate (Merck, Darmstadt, DE) L-glutamine (Sigma-Aldrich, MO, USA), 100 U/mL penicillin (Sigma-Aldrich, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich, MO, USA), and 10% fetal bovine serum (Gibco, MA, USA) at 37°C in 5% CO2. After removal of the medium, 70% confluent Vero E6 cells were washed twice with PBS, inoculated with processed blood cells, and incubated for one hour at 37°C. At the end of the incubation, the inoculum was replaced with DMEM supplemented with 1% FBS (Gibco, MA, USA). Vero E6 cells were blind passaged six times and examined under the microscope daily for cytopathic effects.

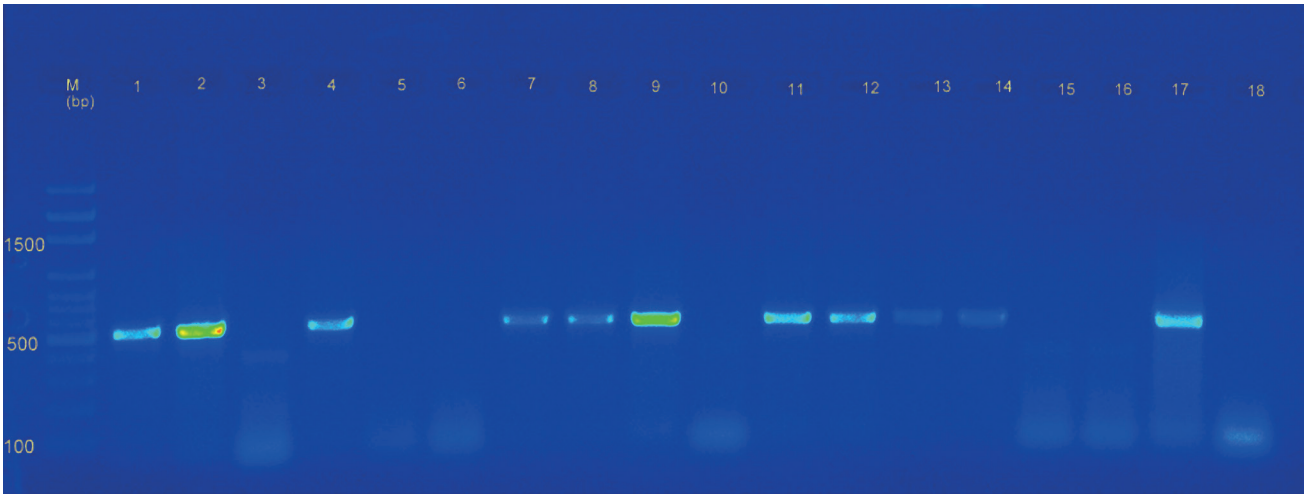

Figure 1. Agarose gel (1.5% w/v) electrophoresis after PCR screening from field samples. **M:** Solis Biodyne 100 bp DNA ladder.

RT-PCR for the full-length G gene, sequencing, and phylogeny

RNA from viruses were used to make a one-step RT-PCR kit that amplified the full-length G gene (Thermo Fisher, MA, USA). Briefly, the assay was carried out in a 50 μL reaction mixture containing five microliters of viral RNA (50 ηg/uL), three microliters of each primer (20 ρmol), ten microliters of the 5× buffer, two microliters of 10 mM dNTPs, two microliters of a mixture of SuperScript III reverse transcriptase and platinum Taq DNA polymerase and ultrapure water (all the reagents were provided in the kit) (15). After the reverse transcription step at 50°C for 30 min, amplifications were carried out at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min. The amplification products were analyzed in 1.5% agarose gel electrophoresis with ethidium bromide (Sigma-Aldrich, MO, USA) using a 100 bp DNA ladder (NEB, MA, USA). Electrophoresis was performed in an agarose gel electrophoresis system (Thermo Fisher, MA, USA) for 40 min at 120 volts, and the PCR products were visualized under UV light. The PCR fragments were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced in both orientations with the complete G gene primers. This stage was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems, CA, USA) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). The resulting bidirectional nt sequences were aligned, edited, verified with BLASTN, and submitted to the GenBank database (OQ134925-7).

Thereafter, nucleotide and amino acid sequences (aa) were aligned and compared with strains selected from GenBank using Clustal W software. Sequence data which had been submitted to the GenBank Nucleotide Sequence Database. Phylogenetic trees were generated using Molecular Evolutionary Genetics Analysis software version X (MEGA X), and the Maximum Likelihood method with 1000 bootstrap replicates (16).

RESULTS

Viral genome confirmation and virus isolation

After RT-PCR, of 32 heparinized blood samples, 23 (71.8%) had the predictable DNA fragment size (520 bp). After agarose gel electrophoresis, some of the positive samples are shown in Fig 1. Of 23 PCR-positive samples, 12 were confirmed by sequencing. Sequencing results were categorized into three. G genes randomized three samples from different categories were amplified by RT-PCR and deposited in GenBank by editing [TR-Hatay-2020-BEFV (OQ134925), TR-Maras-2020-1-BEFV (OQ134926), TR-Maras-2020-2-BEFV (OQ134927)].

No viral RNA and CPE could be detected at the end of the 6th blind passage in Vero E6 cells.

BEFV G gene sequencing

Based on G gene sequence analysis, three new BEFVs were identified (TR-Hatay-2020-BEFV, TR-Maras-2020-1-BEFV, and TR-Maras-2020-2-BEFV) (99.5-99.9% nt and 99.0-99.8% aa). The new BEFVs were genetically very similar to the Turkish isolate, the BEFV/Ad12/TUR (99.4-99.9%

Table 1: The nucleotide identity of existing new BEFV strains with some Israeli, Iranian, Egyptian, and Indian strains

	BEFV stains/isolates (Accession numbers)	TR-Hatay-2020-BEFV	TR-Maras-2020-1-BEFV	TR-Maras-2020-2-BEFV
Israeli	2008-Israel (JN646090)	99.0	98.8	98.4
	ISR00_2000 (JN833630)	97.0	96.9	96.5
	ISR01_2001 (JN833631)	97.1	97.0	96.7
	ISR04_2004 (JN833632)	97.0	96.9	96.6
	BEFV/Israel/2006 (MN078236)	97.3	97.2	96.9
	ISR10/1_2010 (JN833633.1)	96.5	96.4	96.0
	ISR10/2_2010 (JN833634)	96.4	96.2	95.9
Iranian	Khuzestan-2018 (MZ511169)	97.4	97.3	97.0
	2018-Ahvaz (MT274593)	96.2	96.2	96.2
Egyptian	EGY12 (KJ729108)	95.1	95.0	94.8
	Damietta2/Egy/2017 (MH939251); Damietta5/Egy/2017 (MH939254.1); Dakahlia3/Egy/2017 (MF968902); Dakahlia1/Egy/2017 (MF968900); Kafr_El-sheikh2/Egy/2017 (MF968904.1)	96.0	95.9	95.5
Indian	IND/JBL/BEV/2018 (MH933863.1); IND/BH/BEV/2018 (MH933862.1)	97.2	97.2	97.2
	IND/IDR/BEV/2018 (MH933864)	96.9	96.9	96.9
	IND/INDR/BEV/2019 (MN688612.1)	95.7	95.56	95.1

nt and 97.9-99.9% aa), followed by the other Turkish isolates (2008/TR/CP77, 2008/TR/CP62, and 2008/TR/CP60) with high identity ratio (98.7-99.2% nt and 97.5-99.2% aa). The new BEFVs had lower (96.9-98.9% nt and 96.8-98.8% aa) genetic relatedness to the two BEFVs reported by different investigators in the 2020 epidemic (TR/NO3/URF/2020 and TR/D2/URF/2020) (17).

The nucleotide identity of existing new BEFV strains, when compared with BEFVs from different geographic regions, shared a high identity with some Israeli, Iranian, Egyptian, and Indian strains (>95.0% nt; >97.0% aa) (Table 1). According to the same analysis results, this rate was 89.0-90.5% for the Australian reference strain named BB7721 (AF234533) and other Australian strains (MN026882-3; MN026898-9; MN026888); 85.3%-90.2% for Saudi Arabian strains (LC017738); 86.0-87.0% for South African strains (MN026880-1; MN026884; MN026887; MN026890-1; MN026896; MW463337; MW512963); 91.5-92.4% for Iraqi strains (MW600731-3).

Amino acid substitutions

The glycoprotein amino acid sequence of the new BEFV strains had substitutions at some positions compared to the Australian reference strain (BB7221): 16 (Leu-Phe), 18 (Lys-Glu), 72 (Ala-Asp), 83 (Arg-Lys), 198 (Glu-Lys), 200 (Ile-Val), 216 (Asn-Ser), 224 (Lys-Thr), (Lys-Glu), 223 (Asp-Glu), 237 (His-Arg), 249 (Lys-Arg), 250 (Asn-Ser), 305 (His-Pro), 311 (Thr-Pro), 333 (Arg-Ser) 360 (Arg-Lys), 366 (Asn-Ser), 399 (Val-Ile), 410 (Gln-Leu), 419 (Gly-Arg), 426 (Thr-Ser), 435 (Asn-Thr), 436 (Arg-Lys), 459 (Leu-Ile), 465 (Asp-Glu), 480 (Val-Ile), 486 (Arg-Lys), 499 (Asn-Ser), 503 (Lys-Thr), 567 (Ser-Asn), 570 (Arg-Ser), 580 (Thr-Ile), 581 (Thr-Ala), 583 (Glu-Gly) and 586 (Arg-Lys).

The amino acid sequence of new BEFVs was compared with those of Turkish isolates and determined substitutions. Lys18Glu was seen only in three new BEFVs and BEFV/Ad12/TUR. Some amino acid substitutions were unique for TR-Maras-2020-2 (Asp223Glu, Lys224Pro, Thr311Pro, Arg333Ser, and Arg360Lys) and for TR-Maras-2020-1 and TR-Maras-2020-2 (His305Pro). Some amino acid substitutions (Ile98Thr and Thr503Ala) in isolate BEFV/Ad12/TUR could not be detected in other Turkish strains/isolates. Ala429Thr was found in both TR/NO3/URF/2020 and TR/D2/URF/2020 isolates obtained in 2020, whereas substitution Ala427Glu was detected only in TR/D2/URF/2020. The G protein amino acid alignment with the

new Turkish BEFV strains and some others is shown in Fig 2.

Phylogenetic analysis

With the G gene nt data of 161 BEFV strains/isolates from the Middle East, East Asia, South Africa, and Australia/Philippines, a phylogenetic tree was prepared (Figs. 3 and 4). The four phylogroups/lineages were grouped as the Middle East, East Asia, South Africa, and Australia/Philippines. Turkish strains and isolates were found to be divided into two lineages (Middle East and East Asia). The Middle East lineage was found in all Turkish strains and isolates between 2008 and 2020. Some strains/isolates from 2012 were included in the East Asian lineage, exhibiting distant genetic characteristics from other Turkish BEFVs. According to the results, Turkish strains/isolates clustered in the East Asian lineage were 2012/TR/CU16, 2012/TR/CU15, 2012/TR/CP3, Mersin/Silifke6251-1/Turkey2012, TR/ADA-2/2012, TR-Etlik-2-BEF-2012, TR/ADA-1/2012, Adana5918-15/Turkey/2012, and Mersin/Silifke625-6/Turkey2012. BEFVs obtained in 2020 were in the same branch (Middle East lineage) but were in different sub-clusters. New Turkish BEFVs were sub-clustered along with BEFV/Ad12/TUR, 2012/TR/ADYMN, 2012/TR/Skr.1, 2008/TR/CP77, 2008/TRCP62, 2008/TR/CP60 2008-Israel, and IND/INDR/BEV/2019.

DISCUSSION

Bovine ephemeral fever occurs seasonally in a significant area of the world, including most of Africa, the Middle East, Asia, and Australia, posing a serious economic burden to the livestock industry in many countries, including Turkey (1, 4, 9, 11). The glycoprotein (G) of BEFV has four independent antigenic regions (G1, G2, G3, and G4) that cause the host to produce neutralizing antibodies (18-20). This has led to its extensive study in phylogenetic studies, making it a representative of the whole genome (1, 15, 21). In this study, the G gene of viruses from the last BEF epidemic in Turkey in 2020 was sequenced and analysed. When the G gene nucleotide and amino acid sequences were examined, the new BEFV strains showed high similarity to the strains of Egypt, Israel, Iran, and India (>95.00% nt, >97.00% aa). These results were also supported by the phylogenetic analysis, which revealed that BEFVs included four phylogroups/lineages (Middle East, East Asia, South Africa, and Australia/Philippines). As previously, most of the viruses from the countries above were included in the Middle Eastern lineage in the phylogenetic tree (4, 15). These results confirm a significant correlation between geographical distance and phylogenetic relationships (4, 22-24). The three new BEFVs shared a high genetic close relatedness (>96% nt) with the Israeli and Iranian strains. It is interesting that Indian strains also exhibited a high genetic relatedness to them, which is a significant finding in terms of the epidemiology of the virus. These results support the scenario of the inter-continental BEFV virus spread (22, 24). Wind transport of infected vectors over long distances is a scenario considered for past BEF epizootics (24-26). In the worst-case scenario, an outbreak in Africa or the Middle East could spread to Turkey via Egypt, Israel, and the Syrian corridor. Similarly, the virus could be transmitted to India and China via Iran and complete its cycle in the Africa-Middle East-East Asia triangle. In this context, vector control should be one of the measures taken to prevent the spread of BEFV. BEFV can be spread through uncontrolled animal movements or the animal trade (1, 24). In the 2012 epidemic, BEFV strains from two different origins circulated, in Turkey, suggesting the recent animal trade from China to the Middle East (1). Increasing virus controls with BEFV screening in the host and limiting free animal crossings between Turkey and border countries may limit the spread of BEFV.

Vaccination is recognized as the most effective measure to prevent BEF and is used with attenuated live vaccines for this purpose in Turkey (1, 27). It would be appropriate to follow up on BEF outbreaks in neighboring countries and to vaccinate susceptible cattle in regions that pose a risk for BEFV in Turkey at the time of the BEF outbreak. Similarly, it is essential to follow up on the antigenic characteristics of the viruses in the epidemic. Here, epitopic regions of BEFVs from the last BEF epidemics were also analyzed. G protein aa alignment of the three new BEFVs with 12 selected BEFVs showed that epitopes other than G3b were mainly conserved. Turkish BEFVs from the last epidemic had substitutions at positions 223 (Asp to Gly) and 224 (Lys to Thr; Lys to Pro) of the G protein corresponding to the G3b epitope. It has been demonstated that there is a single serotype of BEFV present worldwide (1, 2), however heterologous BEFV isolates obtained from different geographic areas or at other times exhibit different cross-neutralization (28, 29). Although BEFV is the only serotype, minor changes can be seen in epitopic regions (11, 30, 31). For instance, in Australia, four

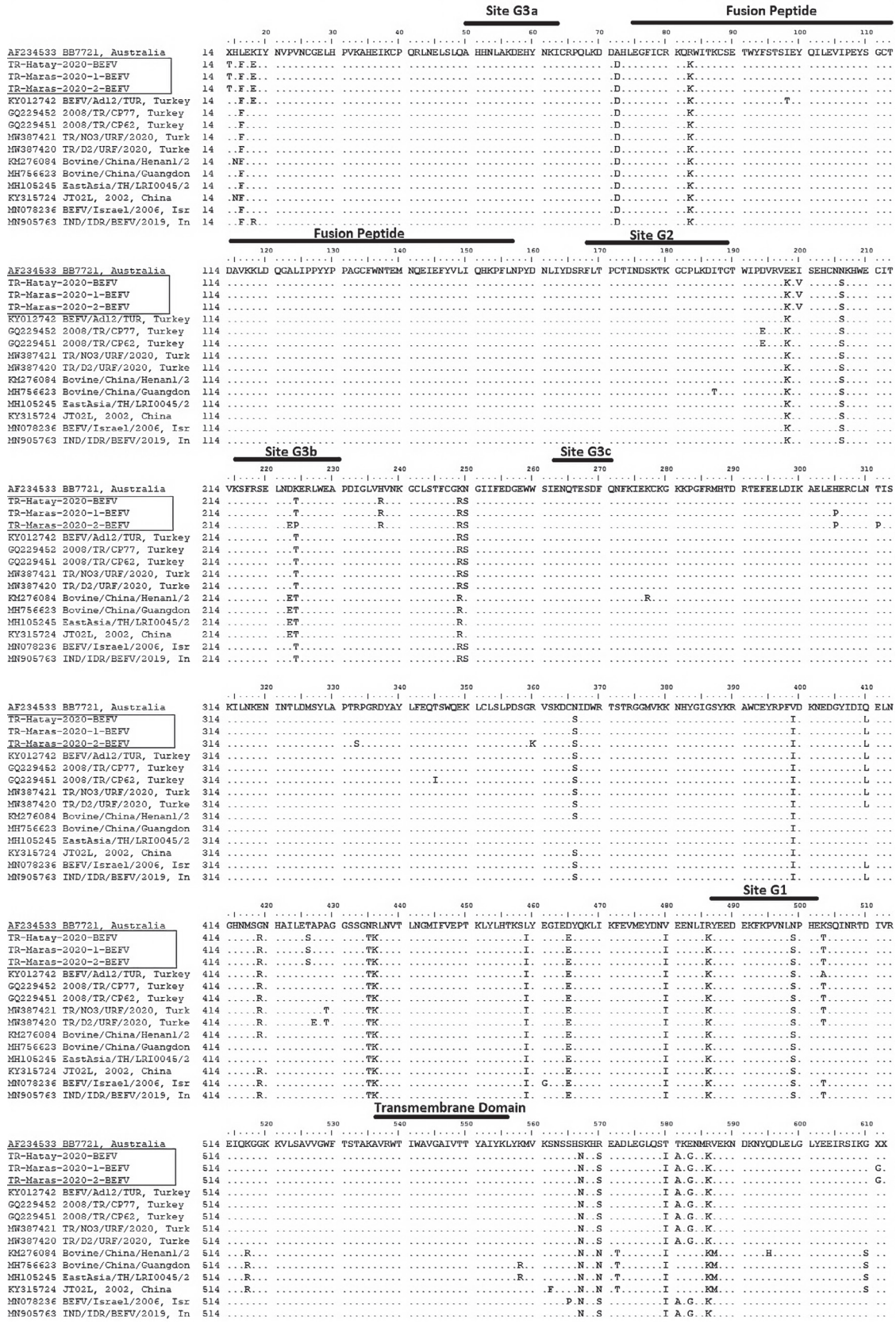

Figure 2. The G protein amino acid alignment with the new Turkish BEFV strains and some others

Figure 3. Phylogenetic tree constructed with G gene nucleotide data of 161 BEFV strains/isolates selected from Genbank.

Phylogenetic tree was created with Mega X software in 1000 replicates using Maximum Likelihood method and Tamura-Nei model. Filled circles indicate BEFV strains obtained from this study, and unfilled circles indicate other Turkish strains/isolates

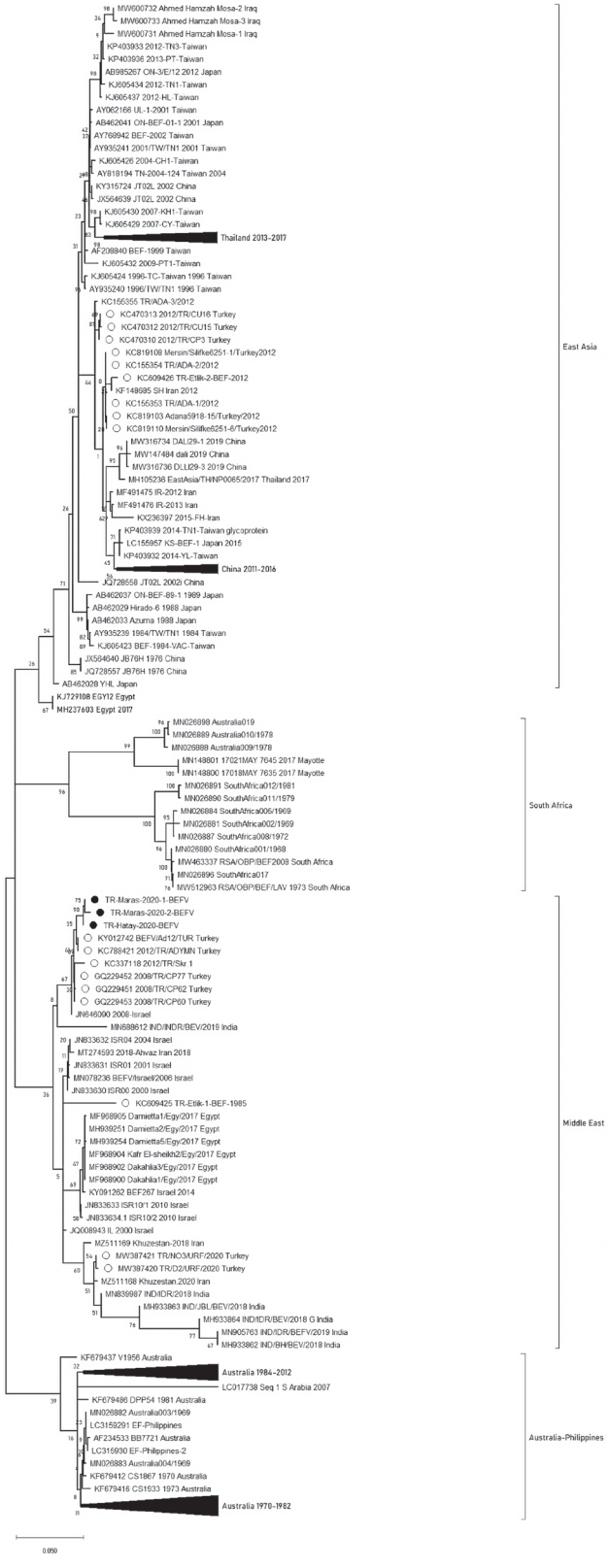

Figure 4. Phylogenetic tree constructed with G gene nucleotide data of 161 BEFV strains/isolates from similar or different geographies. Phylogenetic tree was created with Mega X software in 1000 replicates using Maximum Likelihood method and Tamura-Nei model. Filled circles indicate BEFV strains obtained from this study, and unfilled circles indicate other Turkish strains/isolates.

subtypes have been identified based on variations in the epitopes G3a and G3b (31). Whether substitutions in the G3b region of new BEFVs dominate or cause phenotypic changes should be further investigated, and existing vaccine strains should be updated in case of significant differences.

In conclusion, after eight years, the BEF epidemic again broke out in Turkey, showing that the risk of BEFV continues to be present in Turkey. The fact that the G protein of the BEFVs studied here was frequently the same and that only the G3b epitope was different shows that the virus is still circulating with only minor changes in geography.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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