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REGULAR ARTICLES

Isolation and genetic characterization of bovine ephemeral fever virus from epidemic-2020 in Turkey

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Abstract

Bovine ephemeral fever virus (BEFV) infection occurs seasonally in many tropical and subtropical regions of Africa, Asia (including the Middle East), and Australia while it is exotic in Europe. In this study, the epidemiology of BEFV infection in Turkey that bridges southeastern Europe and Asia, geographically, was investigated according to the comparison of the nucleotide sequences of the virus caused the last epidemic in 2020 with those of the strains previously detected in Turkey as well as BEFV strains from other countries. In the phylogenetic analysis, based on an alignment of full-length G gene sequences, BEFVs from epidemic-2020 were located in Middle Eastern lineage and appear to represent most closely related BEFVs from India-2018 and 2019. The findings will contribute to a better understanding of BEFV epidemiology in Turkey.

Keywords Bovine ephemeral fever · G gene · Isolation · Middle Eastern lineage · Molecular characterization · Turkey

Introduction

Bovine ephemeral fever (BEF, mostly known as 3-day sickness or bovine enzootic fever) is an arthropod-borne viral disease of cattle characterized by rapid onset of fever, anorexia, muscle stiffness, excessive salivation, nasal and ocular discharge, loss of appetite, and sternal recumbency (Lee 2019). While the clinical signs often last 1 to 3 days, the disease causes significant economic losses due to decreased milk production, weight loss, treatment expenses, or sometimes the death of infected animals (Trinidad et al. 2014; Oğuzoğlu et al. 2015; Alkan et al. 2017)

The cause of the disease, bovine ephemeral fever virus (BEFV), is a member of the *Rhabdoviridae* family and genus *Ephemerovirus*. Its genome contains five structural proteins; nucleoprotein (N), surface glycoprotein (G), large RNA-dependent RNA polymerase (L), polymerase-associated protein (P), and matrix protein (M), arranged in the order 3′-N-P-M-G-[GNS-α1-α2- β-γ]-L-5′and length of approximately 14.9 kb (Walker et al. 1991). The BEFV G protein (81 kDa) is responsible for cell entry. It is the target of virus-neutralizing antibodies, with four distinct antigenic sites (G1, G2, G3, and G4) identified on its surface (Walker et al. 1991; Cybinski et al. 1992). BEFV is considered a single serotype worldwide. Strong antigenic cross-reactions have been demonstrated in various neutralization tests (Kato et al. 2009; Trinidad et al. 2014; Walker and Klement 2015), while antigenic variations have been detected in the major neutralization sites of the G protein. BEFVs are grouped into distinct lineages, including Australian, East Asian, Middle Eastern, and South African (Aziz-Boaron et al. 2012; Trinidad et al. 2014; Omar et al. 2020; Pyasi et al. 2020).

BEF occurs seasonally, during the summer and early autumn, in tropical, subtropical, and temperate regions of Africa, Asia, and Australia. The likely mechanism of spread is windborne movement of insect vectors like biting midges (*Culicoides* spp.) and mosquitoes (Doherty et al. 1972; Walker and Klement 2015; Lee 2019).

Since its first description in Turkey (Girgin et al. 1986), BEFV had been reported in 1999, 2003, 2008, and 2012 (Aziz-Boaron et al. 2012; Tonbak et al. 2013; Oğuzoğlu et al. 2015; Alkan et al. 2017). Although most outbreaks emerged in Southern Anatolia, a seroprevalence of 2.5–

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15.3% has also been reported in Turkey's western provinces (Karaoğlu et al. 2007). In 2008, a BEF epidemic was reported in several southern provinces (Adana, Osmaniye, Gaziantep, Hatay, Adiyaman and Sanliurfa) while another epidemic in 2012 started in Adana before suspected BEF cases were announced in many provinces in Southern, Eastern, and Central Anatolia and the Marmara Region (Aziz-Boaron et al. 2012; Tonbak et al. 2013; Oğuzoğlu et al. 2015; Abayli et al. 2017; Alkan et al. 2017). The latest BEFV epidemic-2020 was again observed in Turkey's southern and southeastern regions.

In this study, to understand the characteristics of the BEFVs from epidemic-2020, we analyzed the full-length G gene sequences of isolates from this epidemic and compared them with the BEFVs detected from previous epidemics in Turkey and other countries. The results would contribute understanding of the dynamics of BEF infection in Turkey.

Materials and methods

History of epidemic and samples

The BEFV epidemic-2020 ran from early September to near the end of October in various cities (Hatay, Adiyaman, Kahramanmaras, Gaziantep, Sanliurfa, Adana, Mersin) in the south and southeast of Turkey. Farmers were warned by the authorities and informed through the media. They also had experience from previous epidemics. The blood samples were collected from cattle (n=22), housed in two family farms located in Sanliurfa. They showed the clinical signs of a high fever (41–42 °C), nasal and ocular discharges, muscle fasciculation, etc., which are clinical remarks that make BEF infection suspicious. The samples were immediately tested for BEFV by RT-PCR and subsequently, they were taken to the virus isolation procedure.

Virus isolation

For the virus isolation, the buffy coats from blood samples (n=22) were inoculated onto confluent Green Monkey Kidney (Vero) cell cultures in 25 cm^2 flasks. Following incubation at 37 °C for 1 h, a cell maintenance medium containing MEM (Sigma), fetal calf serum (5%), and $NaHCO_3$ was added into the flask before the cultures were maintained at 37 °C for 5 days and observed every 24 h. The isolates were then identified by RT-PCR.

RNA extraction and RT-PCR for the G protein gene

Total RNA was extracted from samples using the Trizol LS (Invitrogen, Carlsbad, CA, USA) procedure. Reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. To identify BEFV, the primer pairs BEF346F and BEF1155R (Aziz-Boaron et al. 2012), targeting the partial G protein gene (809 bp), were used with the following protocol: denaturation at 94 °C for 3 m, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 7 min. To obtain the full-length G gene sequence (1872 nt), the primer pairs suggested by Dr. Peter Walker and Dr. Kim Blasdell were used (primer sequences are available on request by mentioned researchers). The amplification products were analyzed by 1% agarose gel electrophoresis and visualized under UV light.

Sequencing and phylogenetic analysis

Purified products of the expected size were sequenced in both directions with the same primers used for the amplification. Cognate sequences of reference BEFV representing the G gene were retrieved from GenBank through the BLAST engine. Multiple sequence alignments were prepared by the MUSCLE algorithm as implemented in Aliview Software (Larsson 2014). MEGAX Molecular Evolutionary Genetics Analysis was used to build phylogenetic trees (Kumar et al. 2018), the statistical significance of which was estimated by bootstrap analysis (1000 replicates). Maximum Likelihood methods with the Tamura-3 and Tamura-Nei parameter model plus gamma distribution were performed for the partial and full-length G gene, respectively. The nucleotide (nt) and amino acid (aa) identities were calculated by using the SIAS online tool (http://imed.med.ucm.es/Tools/sias.html).

Results

Virus isolation

By the second serial passage of blood samples (n=22) in the Vero cells, clear CPE was observed within 48 h post infection for six samples and virus isolation was determined by the appearance of a CPE in this passage level. After subsequent passages, CPE was observed within 24 h following the inoculation (Fig. 1). Isolates were confirmed by RT-PCR.

RT-PCR and sequencing

The results showed that all blood samples (n=22) yielded the partial G gene fragment (809 bp). To obtain the full-length G gene sequences, two isolates (TR/D2/URF/2020 and TR/NO3/URF/2020) representing each farm were used. The expected size amplicons of their G gene were sequenced and deposited in GenBank under the following accession numbers: MW387420 and MW387421.

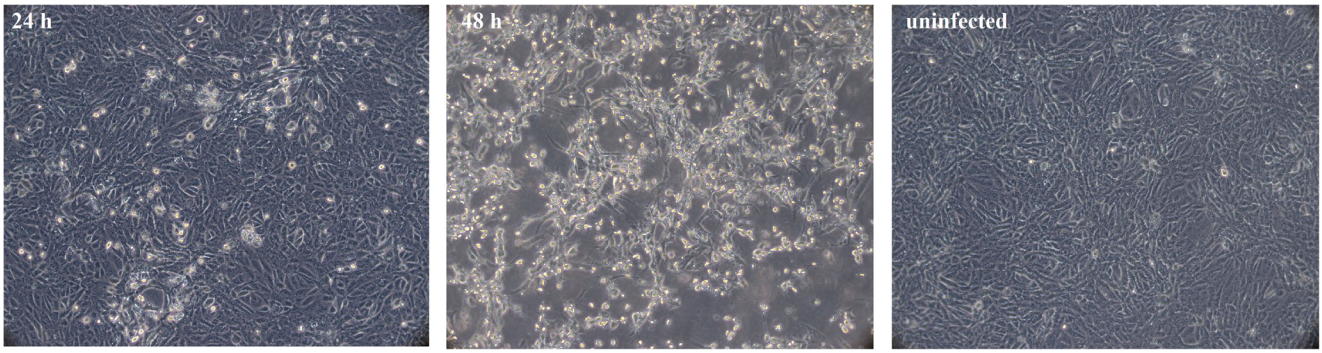

Fig. 1 The cytopathic effect observed in Vero cells infected with Turkish BEFV-2020

Because of the limited number of full-length G gene sequences from previous outbreaks in Turkey, the phylogenetic trees were constructed using both partial and full-length G gene sequences. The phylogenetic trees showed that our BEFVs from epidemic-2020 were clustered in the Middle Eastern lineage (Fig. 2, Fig. S1). Our full-length G genes of BEFV sequence identities calculated by SIAS showed 99.78% nt identity to each other, and 86.75–99.3% nt identity to the G gene sequences from several other countries. The alignment of the full-length G gene sequences of TR/D2/URF/2020 and TR/NO3/URF/2020 isolates, which showed only one different aa substitution at the position A427E, revealed that they share 99.8% aa identity. These two sequences also displayed 93.9–99.5% aa identity with the sequences in other countries. Moreover, our analysis of the G gene showed that both viruses were most closely related genetically with IND/IDR/BEFV/2018 strain (aa identity, 99.35%, and 99.51%) (Table 1).

The alignment of our isolates with the reference strain BB7721 (AF234533) showed 34 aa substitutions in the full-length G gene region. As the G protein is the principal target of neutralizing antibodies, a comparison of the predicted aa sequences of the neutralizing epitopes G1 (487–503), G2 (168–189), G3 (49–63, 215–231, and 262–271), and G4 (455-482) of our BEFVs showed six aa substitutions (K224T, L459I, D465E, V480I, N499S, and K503T) in G1, G3, and G4 epitopes. Comparison of the antigenic sites of BEFVs detected in this study to the other sequences clustered in Middle Eastern lineage showed that they were highly conserved and similar to the Indian, Turkish, and Israeli strains (Fig. 3).

Discussion

BEFV re-emerged in Turkey in September 2020, 8 years after the previous epidemic in 2012. The BEFV epidemic-2020 was reported on farms in provinces in south and southeast Anatolia, specifically in Hatay, Adiyaman, Sanliurfa, Mersin, Gaziantep, Kahramanmaras, and Adana. In contrast, the 2012 outbreak spread across more provinces in Southern, Eastern, and Central Anatolia and the Marmara region (Tonbak et al. 2013; Oğuzoğlu et al. 2015; Alkan et al. 2017). Although many epidemics have been reported since 1985, the knowledge of the molecular characterization of BEFVs is relatively limited in Turkey. Therefore, in this study, we aimed to identify the G protein gene sequences of BEFVs from epidemic-2020 in Turkey and their genetic similarities to BEFV strains detected from previous epidemics in Turkey and other countries.

The phylogenetic analysis based on both the partial and full-length G gene of BEFVs from last epidemic showed that they clustered in the Middle Eastern lineage. More specifically, they grouped with Indian BEFVs detected in 2018–2019 and represented a separate branch both from other Turkish strains from the 1985, 2008, and 2012 epidemics and Israeli strains (Fig. 2 and Fig. S1). Whereas viruses clustered in two different lineages (East Asia and the Middle East) were circulating in the 2012 epidemic, in the latest epidemic, only Middle East strains were detected so far.

Clearly, BEFVs from each epidemic need to be analyzed to determine the origins and/or evolution of the virus. Although BEFV is considered a single serotype (Walker and Klement 2015), comparative analysis of the G gene aa sequences has revealed significant antigenic variations among East Asian, Middle Eastern, Australian, and South African strains (Kato et al. 2009; Zheng and Qiu 2012; Kun et al. 2020; Omar et al. 2020). The aa alignment of the BEFVs from epidemic-2020 with the reference strain BB7721 revealed 34 aa substitutions in the G gene of which only six (K224T, L459I, D465E, V480I, N499S, and K503T) were located in the antigenic epitopes, as shown in Fig. 3. Substitution at residue K224T in epitope G3 has been reported in most of the BEFVs except the Australian one. Kato et al. (2009) suggest this could play an important role in antigenic discrimination of Australian strains. The substitutions at residue L459I, D465E, and V480I were located in the predicted G4 epitope. Of these, substitutions L459I and D465E were detected in sequences from Middle Eastern and other lineages, while substitution V480I was located in all lineages, except for the Australian one (Fig. 3). The remaining two substitutions (N499S and

Table 1 Detailed information of bovine ephemeral fever viruses used in this study and their identity (nt and aa) to Turkish BEFVs-2020

				TR/D2/URF/2020		TR/NO3/URF/2020	
				Identity %			
Strain	Accession No	Year	Country	nt	aa	nt	aa
BB7721	AF234533	1968	Australia	90.27	94.55	90.22	94.71
Australia003/1969	MN026882	1969	Australia	90.54	95.19	90.49	95.35
EastAsia/TH/NMA0071/2017	MH518289	2017	Thailand	91.39	96.63	91.5	96.79
EastAsia/TH/NMA0074/2017	MH105239	2017	Thailand	91.45	96.63	91.55	96.79
Bovine/China/Guangdong/2017	MH756623	2017	China	91.34	96.63	91.45	96.79
EastAsia/TH/LRI0045/2016	MH105245	2016	Thailand	91.55	96.79	91.66	96.95
EastAsia/TH/NMA0012/2015	MH105227	2015	Thailand	91.72	96.95	91.82	97.11
EastAsia/TH/NMA0021/2015	MH518287	2015	Thailand	91.5	96.63	91.61	96.79
EastAsia/TH/NMA0006/2015	MH518286	2015	Thailand	91.5	96.63	91.61	96.79
qy2017	MG729594	2017	China	91.66	96.63	91.72	96.79
2014-HL-Taiwan	KP403938	2014	Taiwan	91.72	97.11	91.77	97.27
2013-PT-Taiwan	KP403936	2013	Taiwan	92.14	96.63	92.14	96.79
TN-2004-124	AY818194	2004	Taiwan	92.2	96.79	92.3	96.95
2004-CH1-Taiwan	KJ605426	2004	Taiwan	92.2	96.95	92.25	97.11
UL-1-2001	AY062166	2001	Taiwan	92.3	96.63	92.41	96.79
2001/TW/TN1	AY935241	2001	Taiwan	92.41	97.11	92.52	97.27
1996/TW/TN1	AY935240	1996	Taiwan	92.41	96.79	92.41	96.95
2012/TR/CU15	KC470312	2012	Turkey	92.04	96.47	92.04	96.63
2012/TR/CU16	KC470313	2012	Turkey	92.09	96.47	92.09	96.63
2012/TR/CP3	KC470310	2012	Turkey	92.14	96.63	92.14	96.79
IR-2013	MF491476	2013	Iran	91.63	96.76	91.69	96.92
IR-2012	MF491475	2012	Iran	91.58	96.6	91.63	96.76
2013-TN-Taiwan	KP403937	2013	Taiwan	91.72	97.11	91.77	97.27
2012-HL-Taiwan	KJ605437	2012	Taiwan	92.2	96.95	92.3	97.11
LYC11	JX564638	2011	China	91.61	96.95	91.61	97.11
2015GX	MN873573	2015	China	91.34	95.99	91.34	96.15
2013ZH	MG729591	2013	China	91.77	96.95	91.77	97.11
11082-2016	MG729593	2016	China	91.55	96.79	91.61	96.95
2014HM	MG729592	2014	China	91.77	96.95	91.77	97.11
1984/TW/TN1	AY935239	1984	Taiwan	92.57	95.83	92.68	95.99
JB76H	JX564640	1976	China	92.73	96.47	92.68	96.63
YHL	AB462028	1966	Japan	93.58	97.03	93.52	97.2
EGY12	KJ729108	2012	Egypt	94.38	97.27	94.49	97.43
2008/TR/CP62	GQ229451	2008	Turkey	96.58	98.39	96.79	98.55
2008/TR/CP77	GQ229452	2008	Turkey	96.63	98.55	96.84	98.71
BEFV/Ad12/TUR	KY012742	2012	Turkey	96.68	98.39	96.9	98.55
2012/TR/ADYMN	KC788421	2012	Turkey	96.68	98.39	96.9	98.55
BEFV/Israel/2006	MN078236	2006	Israel	96.84	99.19	97.06	99.35
IND/IDR/BEFV/2018	MN905763	2018	India	99.09	99.35	99.3	99.51
SouthAfrica011/1979	MN026890	1979	South Africa	86.75	93.91	86.91	94.07
SouthAfrica014/1999	MN026893	1999	South Africa	87.07	93.91	87.23	94.07

K503T) were in the G1 epitope, which is a linear neutralization site. Residue 503 is one of the critical amino acids for virus neutralization because, using monoclonal antibodies, BEFV escape mutants have been identified through substitution at aa 503 (Kongsuwan et al. 1998). However, the conservative substitution K503R did not cause variation in the neutralization titer of BEF viruses (Trinidad et al. 2014). Concerning the aa substitution at residue 503 for strains in the Middle Eastern lineages, the Turkish BEFVs from epidemic-2020 had a semi-conservative K503T substitution like other Middle Eastern strains, whereas the Turkish BEFVs from epidemic-2012 (KY012742 and KC788421) had a non-conservative K503A substitution.

One of the findings is the close similarities of our BEFVs sequences with Indian isolates (MN905763) detected in 2018 (Table 1). It is not possible to explain the certain reason of this similarity. However, it brings to mind Pyasi et al. (2020) who suggested that geographical proximity of Middle Eastern countries enables the disease to spread across continents through vector translocation. Based on the previous reports, this might be the low-level jet streams winds that transport flies between countries in relation to BEFV incursions, such as the BEFV epidemic-2008 in Turkey (Aziz-Boaron et al. 2012; Walker and Klement 2015).

Although BEFV is widely distributed in many tropical and subtropical regions, the vector population is not clearly defined

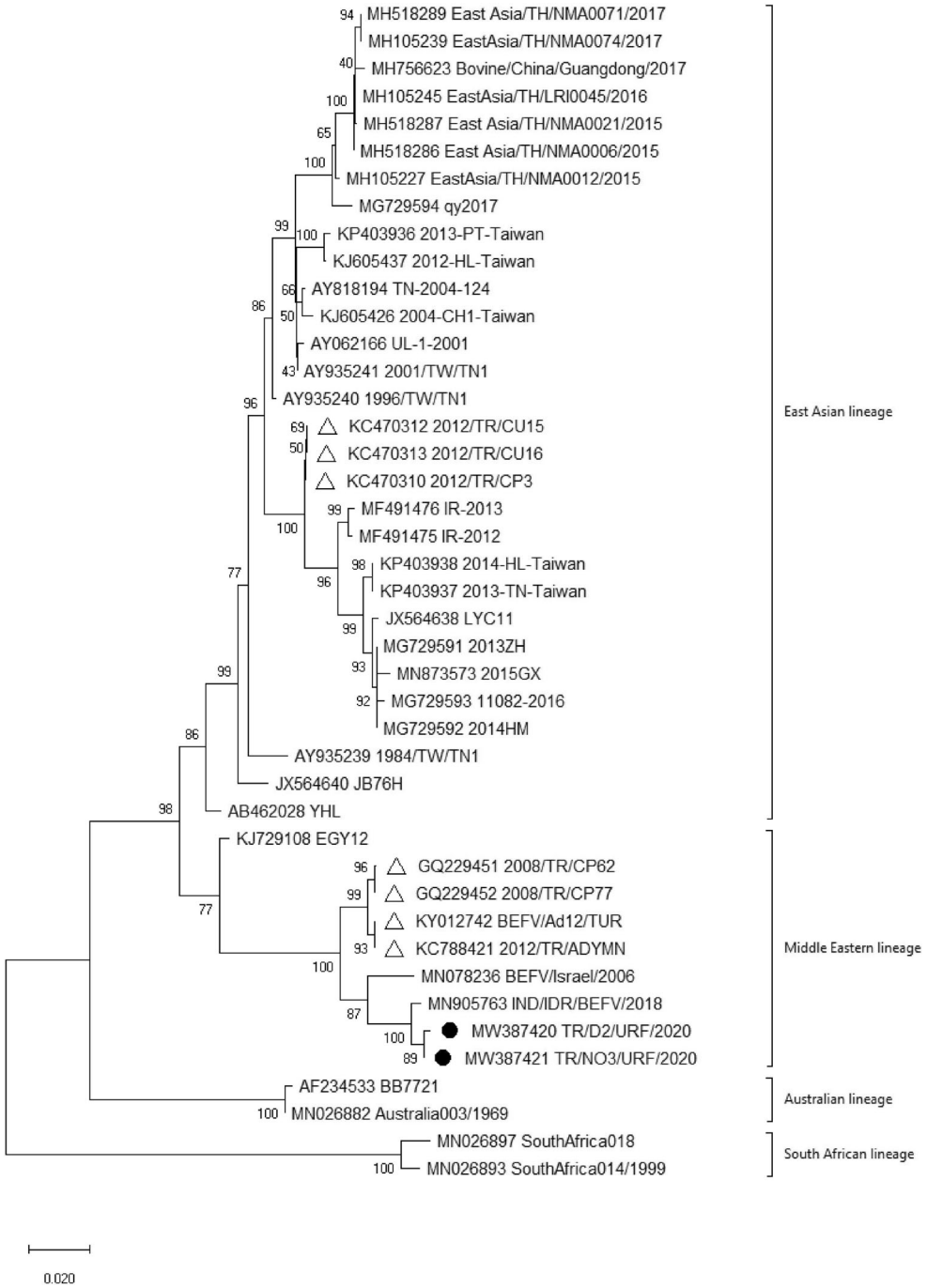

Fig. 2 Maximum likelihood phylogenetic tree based on the full-length nucleotide sequences of the G gene (1872 bp). The strains investigated in this study are indicated by black dots. Turkish isolates from previous epidemics are indicated by triangles

and the global geodynamics of the infection are not fully understood. However, biting midges (*Culicoides*) and mosquitos that traverse the continents through wind-borne movement is most probably a major source of the intercontinental spread of BEFVs (Aziz-Boaron et al. 2012; Chaisirirat et al. 2018; Lee 2019; Pyasi et al. 2020). No scientific article was reported on the detection of BEFV from an invertebrate vector in Turkey; however, there is a BEFV G gene sequence with 387 bp deposited in GenBank (MH299850), which was obtained from *Cl. circumscriptus* collected in 2015. It is an interesting point that a BEFV infection was not reported in Turkey in that year. This situation could be because most animals were still immune after the 2012 epidemic, while those showing clinical signs in the inter-epidemic period were also overlooked.

In conclusion, we isolated the virus on Vero cells besides sequencing the full-length G gene region of BEFVs from epidemic-2020 to increase understanding of the epidemiology of BEFV infection in Turkey. Considering the occurrence of recurrent epidemics since 1985 and the importance of the vaccine to prevent the infection, further studies monitoring

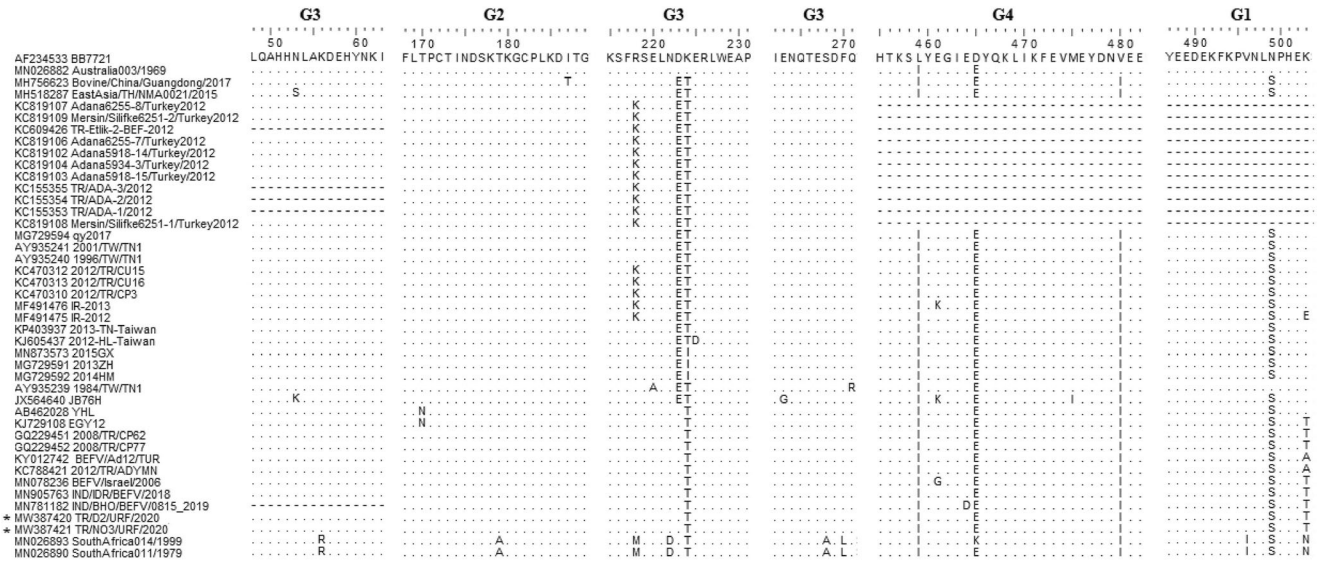

Fig. 3 Alignment of the deduced amino acid sequences corresponding to the antigenic sites of G1, G2, G3, and G4 of BEFV G protein. The two Turkish BEFVs-2020 are indicated by (*)

the epidemiology, vaccine strain acquisition, and production procedures will provide information to help develop more effective prevention and control measures against this infection in Turkey and also other countries.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11250-021-02715-1.

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Author contribution FA was responsible for the supervision of the study. IKH together with SDY performed the molecular biology and bioinformatic analyses (alignments, phylogeny). NU and YV provided the clinical samples and gathered information. IKH and SDY drafted the manuscript. FA reviewed and edited the manuscript. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval Symptomatic cattle were examined for clinical evaluation, and samples were collected by the field veterinarians. All sampling procedures performed on live animals comply with good clinical practice and animal welfare. No ethical approval was required for this study.

Conflict of interest The authors declare no competing interests.

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