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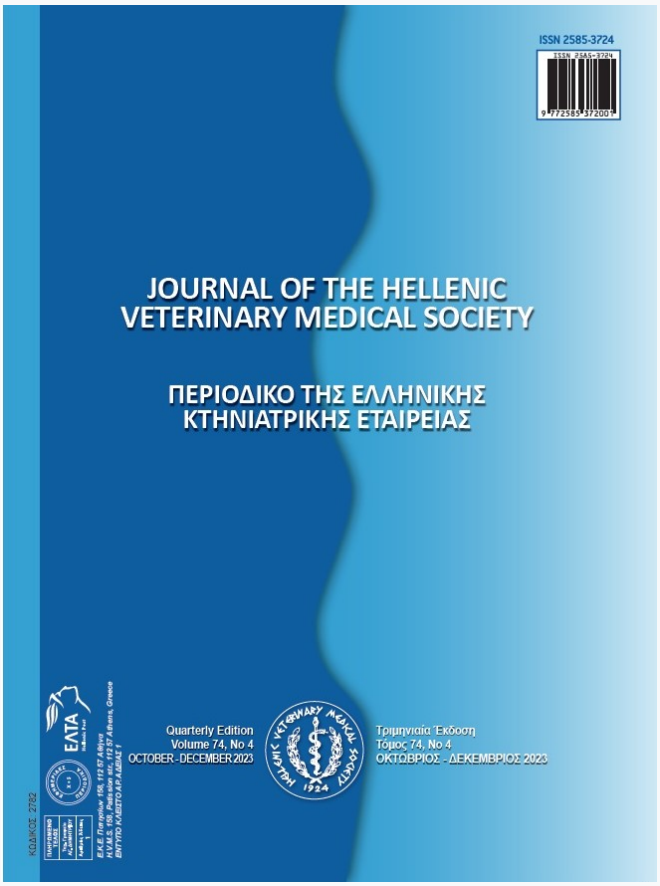


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# Prevalence, isolation and molecular characterization of Bovine Ephemeral Fever Virus in south and southeast regions of Turkey in the outbreak of 2020

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**ABSTRACT:** The aim of this study is to investigate the presence of Bovine Ephemeral Fever (BEF) virus in EDTA-treated blood, sera, and spleen samples of 228 suspected animals originated from 5 different provinces of Turkey in 2020, as well as to perform molecular characterization and phylogenetic analysis of positive cases. For this purpose, 79 whole blood and 2 spleen samples as well as 168 sera samples from cattle varying in breed, age, and sex,were examined by using Real Time RT-PCR and Blocking ELISA, respectively. Two new degenerate primers amplifying 956 bp of the protein G (AVKEF_AATGTTCCNGTGAATTGTGGAG and AVKER_TGCATAATCYCTTCCTGGTCT) were designed for RT-PCR testing and the phylogenetic analysis of positive samples was performed. 64.20% (52/81) of the defibrinated blood (63.29%, 50/79) and spleen (100%, 2/2) samples, and 69.04% (116/168) of sera samples resulted positive by RT-PCR. By using VERO cell culture, BEF virus was isolated from blood (n=1) and spleen(n=2) tissues in Adana and Şanliurfa provinces. The phylogenetic analysis revealed that the BEF virus circulated in Turkey during the 2020 regional epidemic belongs to the Middle East lineage, which has its significance for the selection of proper vaccine and the control of the disease.

**Keywords:** Bovine Ephemeral Fever; phylogenetics; real time RT-PCR;Turkey;virus isolation.

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## INTRODUCTION

Bovine Ephemeral Fever (BEF) is an acute, vector-born viral disease, which causes significant economic losses in cattle and water buffalo (Kirkland, 2002; Zheng and Qiu, 2012; OIE, 2016). The disease is caused by the Bovine Ephemeral Fever Virus (BEFV) of the family *Rhabdoviridae*. Bovine ephemeral fever virus has a 14.9-kb negative-sense single-stranded RNA (ssRNA) genome (Omar et al., 2020;Pyasiet al., 2021). Its virion structure consists of 5structural proteins (N, P, M, G, and L), and a non-structural glycoprotein,as well as several small accessory proteins of unknown function (Trinidad et al., 2014). According to recent phylogenetic analysis, the virus belongs to four different lineages, namely Eastern Asia, Middle East, South Africa, and Australia (Dorey- Robinson et al., 2019;Omar et al., 2020).

The gene G, which is significant in the molecular detection and classification of the virus, is 1872 bp in size (Zheng and Qiu, 2012; Alkan et al., 2017). This gene encodes glycoprotein G, which is responsible for the virus attachment and entrance into cells(-Bakhsheshand Abdollahi, 2015). Neutralizing antibodies are produced against protein G (G1, G2, G3, G4), which is encoded by this domain and known to be the main protective antigen. G1 only reacts with anti-BEFV antibodies, whereas other antigenic sites (G2, G3 and G4) (Zheng and Qui, 2012) can cross-react withviruses such as Berrimah (BRMV), Kimberley (KIMV), Malakal and Puchong virus. Therefore, the serological diagnosis relies on the detection of antibodies against G1 region with Blocking ELISA (Enzyme-Linked Immunosorbent Assay) and Indirect ELISA (I-ELISA), while protein G is preferred as vaccine antigen within the framework of the disease control (Walker et al., 1991;1992).

Even though the Bovine Ephemeral Fever is isolated from different arthropods, the primary vectors of the agent are the biting flies of the *Culicoides* genus (Kirkland, 2002; Trinidad et al., 2014).The disease has been endemic and occasionally causing epidemics in Australia and some countries located in Africa, Asia and the Middle East between the latitudes of 38°North and 36° South(Walker, 2005; Trinidad et al., 2014). In Turkey, the first case was reported in 1985 (Girgin et al., 1986). Every 4 to 5 years, outbreaks of BEFV are being reported and the disease is endemic in Turkey, especially in several provinces with subtropical climate characteristics (Albayrak and Ozan, 2010, Oguzogluet al., 2015).

The morbidity rate of the disease is over 80%, while its mortality rate is 1-2% on average. However, in the recent outbreaks, mortality rates reaching up to 30% have been reported (Walker and Klement, 2015; OIE, 2016).The incidence of the disease significantly increases in warmer seasons (Zahid et al., 2019; Rezatofighi et al., 2022). Infected animals show biphasic fever, inappetence, stiffness, nasal and ocular discharges, cessation of rumination and lameness (Walker, 2005).

The economic losses caused by 3-day stiff-sickness are associated with reduced milk production, deconditioning in cattle, temporary infertility in bulls, abortion, and workforce loss in animals (Nandi and Negi, 1999). The cost of the BEF outbreaks in 1970s for the livestock industry in Australia amounted to 200 million dollars in total, while the estimated cost of the BEF outbreak in Israel in 1999 is reported to be $280 per lactating cow and $112 per non lactating cow on average (Walker, 2008). The BEF outbreaks occurred from 1949 to 1951 in the middle and southern Japan, on the other hand, are reported to have resulted in an economic loss of 5.5 million dollars(Lee, 2019).

For BEFV diagnosis epidemiological data, clinical symptoms, serological(Blocking ELISA, I-ELISA, neutralization test, complement fixation test) and molecular (Real Time RT-PCR, RT- PCR) methods are used (Walker, 2005). For the isolationof the virus, cell cultures from mouse brain, bovine kidney, hamster lungs, African Green Monkey Kidney (VERO), Baby Hamster Kidney (BHK) and *Aedes albopictus* cells are used (Nandi and Negi, 1999). Vaccination is acknowledged to be the most effective control method for the disease (Bakhshesh and Abdollahi, 2015). Live, inactivated, and recombinant vaccines have been used for protection (Walker, 2005).

The aim of this study is to identify the seroprevalence of BEFV, which caused the 2020 outbreak among cattle in Turkey, as well as to perform molecular characterization and phylogenetic analysis of the circulating virus in Turkey. Subsequently, we intend to identify the lineage of the virus as well as,to isolate it in order to use in future vaccine studies.

## MATERIAL AND METHODS

In the present study, we examined 79 EDTA-treated blood, 168 sera and 2 spleen samples, 249 samples in total obtained from cattle varying in breed, age (from 7 months to 5 years), and sex, and delivered

from different provinces of Turkey (Adana, Adıyaman, Hatay, Şanlıurfa and Siirt) to the Viral Diagnosis Laboratory of Adana Veterinary Control Institute with suspected BEF from September to December of 2020.

The RNA extraction from the blood and tissue samples was performed with Roche MagNa Pure Compact Nucleic Acid Isolation Kit (Roche, Germany) as described by the manufacturer. Sera was inactivated for 30 minutes at 56°C. Obtained RNA was stored at -80°C and the sera at-20°C until further use.

Blocking ELISA was performed based on the protocol recommended by the kit manufacturer(Virology Laboratory, EMAI, Camden NSW Australia). Plates were read on ELISA reader (Biochrom Ezread400, the UK)using a wavelength of 450 nm. Samples with >60% inhibition value were regarded as positive, while those with 40-59% were regarded as suspicious and those with <40% as negative.

Real Time Reverse Transcription-Polymerase Chain Reaction (Real Time RT-PCR) was performed based on the recommended kit protocol(Qiagen Quantinova Probe Kit, Qiagen, Germany). For this purpose, we used 10 µl RT PCR Master Mix, 0.2 µl Reverse Transcriptase, 0.5 µl from each primer [(BEF-F: 5'-GAGATCAAATGTCCACAACGTTTAA-3' BEF-R: 5'- AATGTTCATCCTTTGCAAGATTATGA-3'), 1 µl Prob (5'-AATTATCACTTCAAGCCC-3') (Stram et al., 2005)], 4.8 µl water and 3 µl template for each (blood and spleen)sample, with a total volume of 20 µl. We performed PCR under the following conditions: one cycle of cDNA synthesis at 45°C for 10 minutes, one cycle of preliminary denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 5 minutes,annealing andextension at 60°C for 60 seconds (Roche Lightcycler 480).We used samples confirmed positive with ring and comparative tests as positive control, and DNase and RNase free water (Qiagen, Germany) as negative control.

To isolate virus, we used the blood (n =1) and spleen (n =2) samples that were collected and tested positive. Tissue samples were mashed in a mortar and homogenized with cell culture media (Gibco, Minimum Essential Medium, MEM) with 1% antibiotics (Penicillin-Streptomycin-Amphotericin B Solution (Biological industrial, Israel-Beit HaEmek). To obtain inoculum, the homogenate centrifuged at +4°C for 5 minutes at 3000 rpm (NüveNF400R, Turkey) was filtered through a filter of 0,45µm. Vero continuous cell line was prepared by using 1% HEPES buffer solution (Gibco,1M) in 25 $cm^2$ flasks, 10% Fetal Bovine Serum (FBS) (Gibco) and MEM containing antibiotic. Inoculum from the spleen tissues were incubated in the 25 $cm^2$ flasks covered with Vero cell line at 37°C for one hour and then, added with 1% HEPES and MEM containing antibiotic. Cell culture was examined under invert microscope (Olympus X71, Japan) for CPE at 24 hours intervals for 5 days.

A new degenerate primer pair, AVKEF_AATGTTCCNGTGAATTGTGGAG and AVKER_TGCATAATCYCTTCCTGGTCT, which amplifies the 956 bp region were designed by using the full sequence of G protein. Multiple sequence alignment of Africa (MN026882, MN026883), Australia (MN026888, KF679480, AF058325, NC002526, MN026883), China (KY315724), India (MN905763, MN839987), Israel (JN833635, JN833633, MN078236, JN833632, JN833631, JN833630), Thailand (MH105239), Taiwan (KJ605434, AY935239, KJ605423), Japan (AB985267, AB462030) and Turkey (KC788421, KY012742, KC470310, GQ229452, GQ229451) isolates of BEF virus was done by using the CLC MainworkBench Software. Conserved regions were detected by visual comparison. Primer's evaluation was performed with Oligo Calc software (http://biotools.nubic.northwestern.edu/OligoCalc.html) and BLAST search.

The isolates from Adana (n:1) and Şanlıurfa (n:2) tested positive with Real Time RT-PCR were amplified through using AVKEF and AVKER primer pair according to the recommended kit protocol (Megafi One Step RT-PCR kit). For RT-PCR, we used 12 µl water (Multicell Dnase-Rnase-proteinase free sterile water), 25 µl 2X One-Step RT-PCR Buffer, 2µl (20pmol) (sentebiolab, Turkey-Ankara) from each primer, 5µL target RNA and 4 µl RT-PCR enzyme with a final volume of 50 µl. e performed RT-PCR as such: one cycle of cDNA synthesis at 60°C for 15 minutes, one cycle of preliminary denaturation at 94°C for 1 minute followed by 30 cycles of denaturation at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, one cycle of final extension at 72°C for 5 minutes (Roche Lightcycler 480). Obtained amplicons were dyed with ethidium bromide after 1.5% agarose gel electrophoresis and visualized under UV light.

Obtained amplicons were sequenced bidirectionally (Medsantek, Istanbul, Turkey). Sequences were edited with Geneious Prime 2021.0.3 (https://www.

geneious.com) software and aligned with the Clustal Omega Program (http://www.clustal.org/omega/). Sequence analysis was undertaken by BLAST algorithms and databases from the National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov). Before the phylogenetic analysis, best substitution models were determined by ModelFinder (Kalyaanamoorthy et al., 2017). IQ-TREE algorithm (Nguyen et al., 2015)was used for the construction of Maximum Likelihood phylogenetic tree and then, phylogenetic tree in Newick format was graphed using the FigTree v1.4.4 program (Institute of Evolutionary Biology, University of Edinburgh). Bootstrap analysis (1000 replications) was done to determine the confidence of tree topology (Felsenstein, 1985).

The results of the study were statistically compared with Chi-square test (SPSS 22.0). $P<0.05$ value was regarded as statistically significant.

## RESULTS

After the serological examinations, we detected that the positivity rates were 62.99% (80/127) in female, 87.80% (36/41) in male and 69.04% (116/168) on average; while in respect of breed, 63.64% (70/110) in Holstein, 63.16% (12/19) in Simental and 87.18% (34/39) in Brown Swiss (Table 1, 3). Our molecular examinations, on the other hand, revealed that the positivity rates were 70.31% (45/64) in female, 41.17% (7/17) in male and 64.20% (52/81) on average; while in respect of breed, 60.78% (31/51) in Holstein, 73.91% (17/23) in Simental and 57.14 % (4/7) in Brown Swiss (Table 2, 4).

Twenty-four hours after 3 consecutive passages of the inoculum prepared in Vero cell lines from one

**Table 1:** Serologic (Blocking ELISA) positivity rates of BEFV in tested samples according to provinces.

| Province | Number of animals (n) | Positive Animals (n/+) | Positivity rate (%) |
|---|---|---|---|
| **Adana** | 113 | 69 | 61.06 |
| **Adıyaman** | 8 | 6 | 75 |
| **Hatay** | - | - | - |
| **Şanlıurfa** | 42 | 38 | 90.47 |
| **Siirt** | 5 | 3 | 60 |
| **Total** | 168 | 116 | 69.04 |

**Table 2:** Molecular (Real Time RT-PCR) positivity rates of BEFV in tested samples according to provinces.

| Province | Number of animals (n) | Positive Animals (n/+) | Positivity rate (%) |
|---|---|---|---|
| **Adana** | 32 | 21 | 65.62 |
| **Adıyaman** | 18 | 7 | 38.88 |
| **Hatay** | 2 | 2 | 100 |
| **Şanlıurfa** | 28 | 21 | 75 |
| **Siirt** | 1 | 1 | 100 |
| **Total** | 81 | 52 | 64.20 |

**Table 3:** Serologic positivity rates of BEFV in different breeds and sex in tested animals.

| Blocking | Holstein | | | Simmental | | | Brown Swiss | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ELISA** | n | n(+) | % (+) | n | n (+) | % (+) | n | n (+) | % (+) | n | n (+) | % (+) |
| **Male** | - | - | - | 2 | 2 | 100 | 39 | 34 | 87.18 | 41 | 36 | 87.80 |
| **Female** | 110 | 70 | 63.64 | 17 | 10 | 58.82 | - | - | - | 127 | 80 | 62.99* |
| **Total** | 110 | 70 | 63.64 | 19 | 12 | 63.16 | 39 | 34 | 87.18 | 168 | 116 | 69.04 |

*: The values in the same column are statistically different ($p< 0.05$).

**Table 4:** Molecular positivity rates of BEFV in different breeds and sex in tested animals.

| Real time | Holstein | | | Simmental | | | Brown Swiss | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RT-PCR** | n | n (+) | % (+) | n | n (+) | % (+) | n | n (+) | % (+) | n | n (+) | % (+) |
| **Male** | 6 | 2 | 33.33 | 7 | 4 | 57.14 | 4 | 1 | 25 | 17 | 7 | 41.17 |
| **Female** | 45 | 29 | 64.44 | 16 | 13 | 81.25 | 3 | 3 | 100 | 64 | 45 | 70.31* |
| **Total** | 51 | 31 | 60.78 | 23 | 17 | 73.91 | 7 | 4 | 57.14 | 81 | 52 | 64.20 |

*: The values in the same column are statistically different ($p< 0.05$).

blood and two spleen samples collected from 2 different animals, we discovered cytopathic effect (CPE) (Fig. 1). We confirmed that the isolates were identified as BEFV through Real Time RT- PCR.

After the RT-PCR performed with AVKEF and AVKER primer pair, we obtained 956 bp product to use in the phylogenetic analysis (Fig.2). The multiple sequence analysis performed indicated that the isolates from Adana (n:1) and Şanlıurfa (n:2) were 100% identical to each other. We then compared these sequences with the data in GenBank (BEFV Reference Sequence: NC_002526.1) and identified that they were 92.87-100% compatible with BEFV. The phylogenetical analysis revealed that Adana and Şanlıurfa isolates obtained during the 2020 outbreak in Turkey belong to the Middle East lineage with the encoding MW680304, MW680305, MW680306 (Fig. 3).

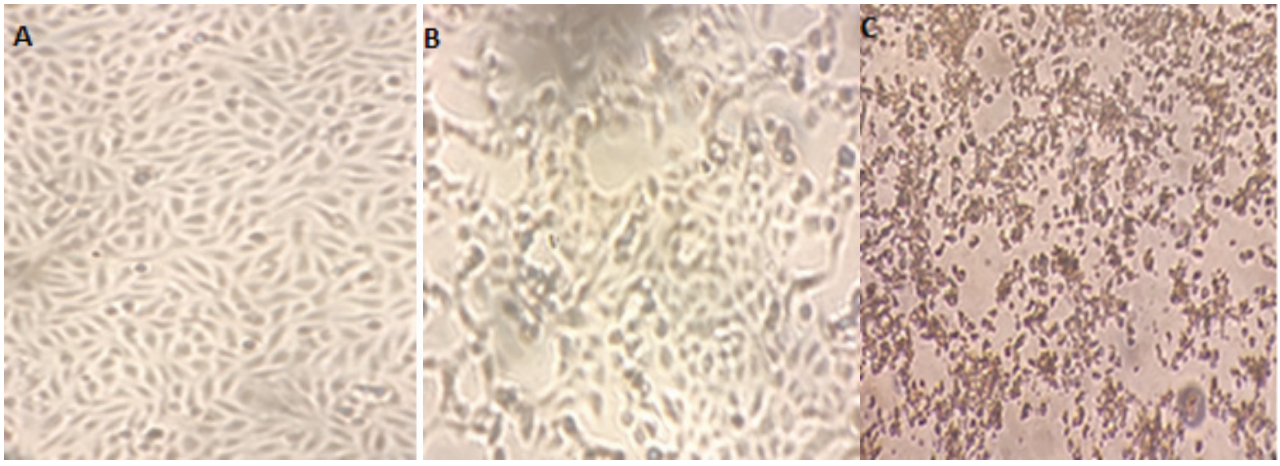


**Figure 1.** A) Vero cell control B) CPE of BEFV (24th hour) C) CPE of BEFV (48th hour).

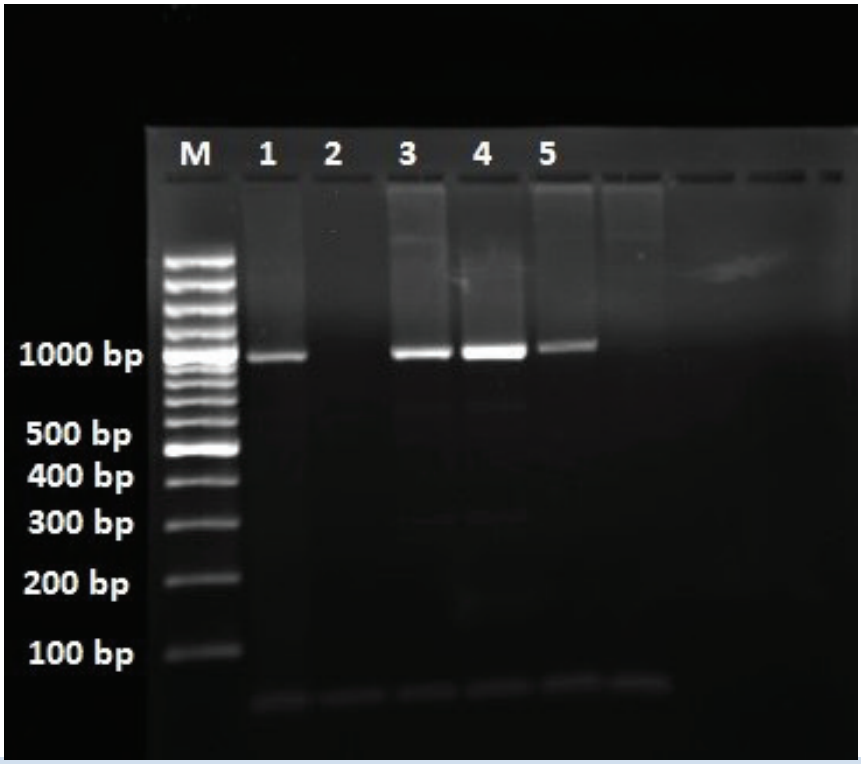


**Figure 2:** Agarose gel image of the amplicons of isolates of BEFV in Turkey.

M: 100 bp ladder, 1: Positive control, 2: Negative control, 3 and 4:Şanlıurfaisolates 5: Adana isolate

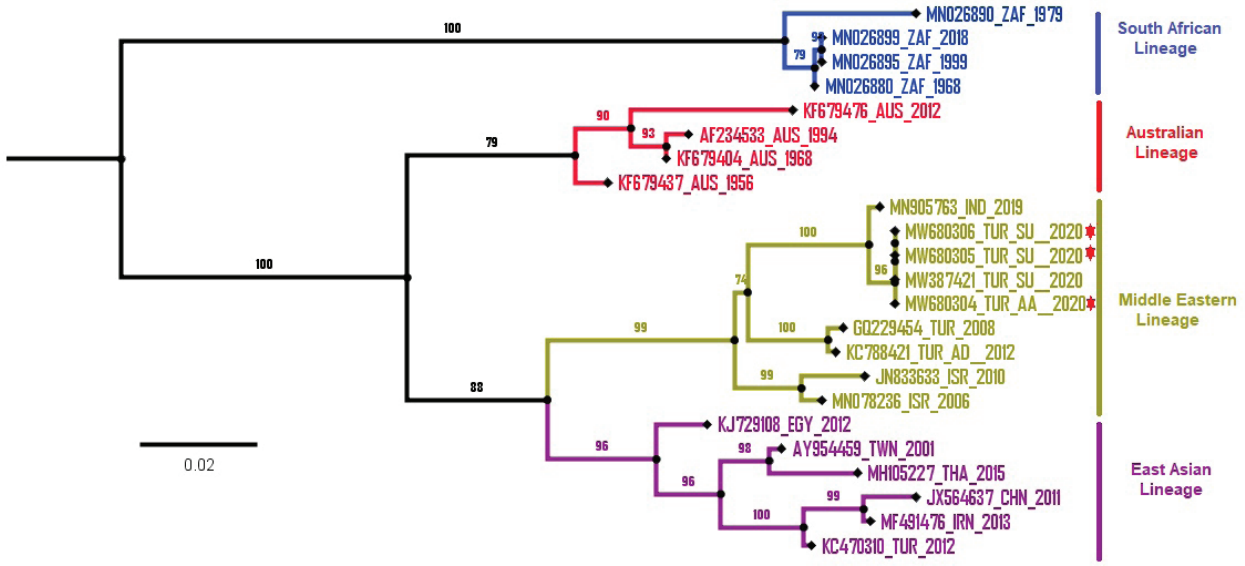


**Figure 3:** Maximum Likelihood phylogenetic tree based on the partial sequences of G protein gene, showed the relationships between the Turkish isolates (MW680306, MW680305, MW680304) and the other lineages of BEFV. GenBank accession numbers are listed in parenthesis near the taxon names. Bootstrap support values (1000 replications) were represented on the branches.

## DISCUSSION

Serological and molecular methods were used to diagnose BEF and to investigate its prevalence. In this study, we used commercial Blocking ELISA among the serological methods considering its high specificity and sensitivity rates and Real Time RT-PCR as molecular method (Walker, 2005; Zheng et al.,2009; 2010).

In some studies conducted, the seroprevalence of the BEF with ELISA and RT-PCR assays were found in Israel (0.68-100%) (Yeruham et al., 2010; Aziz-Boaron et al., 2013), Egypt (20-60%) (Degheidy et al., 2011; Zaghawa et al., 2016; Abed Elnaby and Rateb, 2019), Tibet (34-40.4%) (Liu et al., 2017), Pakistan (4.39-66.53%) (Zahid et al., 2019), and Saudi Arabia (28.7-70%) (Zaghawa et al., 2017). Researchers (Zaghawa et al., 2016) indicated that breed, age, and sex are factors affecting the seroprevalence; in other words, the disease is more prevalent in dairy breeds than non-dairy, in males than females and in animals of 1-3 years old compared to other age groups.

The number of studies conducted for the investigation of seroprevalence of BEF in Turkey is limited. In a study conducted to investigate the existence and prevalence of the disease in Aegean region, 125 sera sample from Aydın and 100 from Mugla province, 225 sera samples in total, collected randomly from family-run dairy farms were examined with Blocking ELISA, and none was tested positive for the presence of anti-BEFV antibodies (Erol et al., 2015). The seroprevalence rates in respect of provinces were 2.5% (1/40) in Samsun, 27.5% (11/40) in Amasya and 37.5% (15/40) in Sinop, while no BEFV antibody was found in the samples collected from Tokat and Ordu (Albayrak and Ozan, 2010). Çanakkale, Tekirdağ, Edirne, Istanbul and Kırklareli provinces of Thrace region were tested with Blocking ELISA method and the seroprevalence rates were reported to be 8.04% on average and in respect of provinces, 2.5%, 6.6%, 15.3%, 2.8% and 13%, respectively (Karaoglu et al., 2007).

In the phylogenetic analysis based on G protein, it was reported that the isolate of the outbreak belongs to Australia lineage and BEFV was reported for the first time in Saudi Arabia (Zaghawa et al., 2017). In Menoufia (Eg-Menoufia) region of Egypt, the phylogenetic analysis of a bovine isolate of 2004 outbreak indicated that the virus circulating during the outbreak was in the same group with the Japanese isolates, not in the group of Turkish isolates (Mahmoud, 2012). In Australia, in a study conducted to understand the epidemiology and molecular evolution of BEFV, G gene of 97 virus isolates obtained from different arthropods in Northern and Eastern Australia between the years 1956 and 2012 were molecularly examined

and as a result of the phylogenetic analysis, the virus circulated in Australia differed from other lineages and in fact, was the source of the three other lineages (Eastern Asia, the Middle East, South Africa) which had been causing outbreaks in various geographical regions of the world (Trinidad et al., 2014).

The phylogenetic analysis of G proteins from three isolates from the 2012 outbreak obtained from Southern, Central and North-western parts of the Iran indicated that these three isolates were 100% identical to each other and belonging to the Eastern Asia lineage together with some Turkish isolates (Bakhshesh and Abdollahi, 2015). In another study in Iran, protein G based phylogenetic analysis of 90 isolates from the years 2018 (n:50) and 2020 (n:40) was performed and it was seen that these isolates belong to the Middle East lineage together with Indian, Turkish and Israeli isolates (Rezatofighi et al., 2022). The phylogenetic analysis of 2000 and 2008 isolates in Israel indicted that 2008 Israeli and Turkish isolates were 99% identical, and together with Turkish isolate, Israeli isolate from 2000 formed a new and distinct group (Aziz- Boaron et al., 2013). In another study in Israel, a full genome analysis (MN078236) of bovine isolate of 2006 was performed and compared with the full genome of Turkish isolate of 2012 (KY012742). As a result of this analysis, the authors reported that the genome showed 95.3% similarity with the Turkish genome; both belonged to the Middle East lineage and showed similarity between 90.0% and 91.6% to Australian and Eastern Asia isolates (Dorey-Robinson et al., 2019).

Sera samples collected from cattle in Thailand displaying symptoms of BEF between 2013 and 2017 were analyzed (Chaisiririrat et al., 2018)and phylogenetic analysis was performed in the PCR-positive samples. The outcome data indicated that the isolates belonged to the Eastern Asia lineage (Chaisiririrat et al., 2018). In a study conducted in China, phylogenetic analysis of 51 virus isolates was conducted and it was indicated that the China isolates belonged to the same lineage with Taiwanese and Japanese isolates, whereas the Turkish and Israel isolates together constituted one cluster and the Australian isolates another (Zheng and Qiu, 2012). Sera samples collected from cattle with suspected BEF between 2018 and 2019 in India, were analyzed and based on the phylogenetic analysis of positive samples, it was detected that the virus circulating belonged to the Middle East lineage (Pyasi et al., 2020; 2021). The phylogenetic analysis of 14 virus strains circulating between 1968 and 1999 in South Africa, it was indicated that these isolates belonged to the South Africa lineage; the authors reported that this lineage distinguished from the other clusters (Omar et al., 2020). In another up-to-date study in South Africa, strains used in vaccines and those circulating were examined, which confirmed that the virus circulating in this region belonged to the South Africa lineage (Mlingo et al., 2021).

Serological studies of BEFV in Turkey, the number of studies regarding the molecular identification and typing of BEFV is very limited (Tonbak et al., 2013; Oguzoglu et al., 2015; Abayli et al., 2017; Alkan et al., 2017; Karayel-Hacioglu et al., 2021). Sera samples collected from 10 cattle from Sakarya, 20 from Adıyaman and 26 from Adana, 56 cattle in total, during the 2012 BEF outbreak in Turkey were examined with RT-PCR, and 85.71% (48/56) positivity was detected. The phylogenetic analysis of three samples selected randomly showed that the sequences of the isolates from 2012 outbreak were clustered in the Middle East lineage (Tonbak et al., 2013). In another study conducted in Turkey on the outbreaks in 1985 and 2012, the authors concluded that 1985 and 2012 isolates belonged to the Middle East and Eastern Asia lineages, respectively (Oguzoglu et al., 2015). Twenty-seven sera samples in total, 23 collected from Adana province and 4 from Diyarbakir, during the 2012 outbreak in Turkey were analyzed through RT-PCR; 10 samples from Adana and 3 samples from Diyarbakir tested positive. The phylogenetic analysis conducted on the four positive samples indicated that the virus circulating during 2012 outbreak belonged to the Eastern Asia lineage (Alkan et al., 2017).

The full genome analysis performed on the high pathogenic BEFV isolated during the 2012 outbreak in Turkey showed that this isolate clustered under the Middle East lineage (Abayli et al., 2017). The phylogenetic analysis of the virus isolated during the 2012 outbreak in Turkey revealed that both the Middle East and Eastern Asia lineages simultaneously triggered an outbreak (Erganiş et al., 2013, Tonbak et al., 2013, Oguzoglu et al., 2015). The researchers (Karayel-Hacioglu et al., 2021) reported that 2020 isolates belonged to the Middle East lineage based on the phylogenetic analysis of two positive samples.

In this study, as a result of our serological analysis, we detected that the positivity rates were 62.99 % (80/127) in females, 87.80% (36/41) in males and 69.04% (116/168) on average; while in respect of

breed, 63.64% (70/110) in Holstein, 63.16% (12/19) in Simental and 87.18% (34/39) in Brown Swiss. The variance between the seropositivity rates of male and females is statistically significant ($P<0.05$), while there is no difference among breeds. The highest rate of seropositivity was found in Şanlıurfa with 90.47% (38/42) whereas the lowest in Siirt with 60% (3/5), which was not statistically assessed due to the big difference in the number of samples sent from these provinces (Table 1, 3).

Molecular assays of this study revealed that the positivity rates were 70.31% (45/64) in female, 41.17% (7/17) in male and 64.20% (52/81) in general; while in respect of breeds, 60.78% (31/51) in Holstein, 73.91% (17/23) in Simental and 57.14 % (4/7) in Brown Swiss breeds (Table 2, 4). The variance between the seropositivity rates of male and females is statistically significant, while there is no difference among breeds. We did not evaluate the statistical significance of the variance in respect of provinces due to the big difference in the number of samples sent from provinces to our laboratory (Table 2, 4). Additionally, 24 hours after 3 consecutive passages of the inoculum prepared in Vero cell culture from one blood and two spleen samples collected from 2 different animals, CPE was detected (Fig. 1). It confirmed that the isolates were BEFV through RT-PCR (Fig.2) and Real Time RT- PCR.

It was determined that the BEF viruses circulating during the 2020 outbreak in Turkey belonged to the Middle East lineage (Fig. 3), which is also in line with the results of another study regarding the same outbreak (Karayel-Hacioglu et al., 2021).

In conclusion, we examined the samples sent to our laboratory from five provinces located in the South and Southeast of Turkey (Adana Adıyaman, Hatay, Şanlıurfa and Siirt) during the 2020 BEF outbreak and investigated the serological and molecular prevalence of the agent as well as its phylogenetic relations, also including the virus isolation. The number of studies investigating BEF in Turkey and of samples used is rather limited. We concluded that future studies of BEF should be conducted in a more planned manner with appropriate sample size and variance in breed, age, breed and sex, covering regions with different geographical features, which could contribute to better understanding of the epidemiological drivers of BEF in Turkey and in the world, and ultimately, form the basis of future emergency action plans. \

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declared no conflicts of interest.

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