ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (9): 529 - 535
doi: 10.30466/vrf.2025.2040560.4446
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Molecular epizootiology of bovine ephemeral fever virus in Iran during 2015 to 2022

Ali Naderian[1], Mehran Bakhshesh[1]*, Mohammad Hasan Ebrahimi-Jam[2]

[1] Department of Animal Virology, Research and Diagnosis, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Organization (AREEO), Karaj, Iran; [2] Department of Animal Viral Vaccine Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Organization (AREEO), Karaj, Iran.

Article Info

Article history:

Received: 16 September 2024
Accepted: 18 February 2025
Available online: 15 September 2025

Keywords:

BEFV
Iran
Lineage change
Outbreak

Abstract

Bovine ephemeral fever (BEF) is a debilitating disease of cattle and water buffaloes. Bovine ephemeral fever viruses (BEFVs) form four phylogenetic lineages including the Middle East, East of Asia, Australia and Africa, while the exotic viral strains have also been detected in different geographic areas. We characterized eight BEFVs from different regions of Iran during a period of seven years from 2015 to 2022. Sequencing the entire length of the *G* gene, the BEFVs were classified in the Middle Eastern lineage with the maximum of 99.73% and minimum of 97.30% nucleotide identity. The all Iranian and Turkish BEFVs detected during the large epizootic in 2020 were clustered phylogenetically together. However, no amino acid variation was observed between the Iranian viruses detected in 2020 and those identified before 2020 in the Middle Eastern lineage suggesting that host, environmental and other genetic factor (s) might have involved in occurrence of the epizootic in 2020. Two BEFVs detected during 2022 outbreak from Kermanshah and Narmashir in the west and east of Iran, respectively, were clustered in two distinct groups as a novel amino acid substitution H51Y in the epitope G3 was also identified in Kermanshah 2022 sequence. These results imply that the Middle Eastern lineage replaced the previously circulated East Asian BEFVs in Iran during 2012 to 2013 and also signify the emergence of new BEFVs due to the intra-lineage evolution. Continuous monitoring of the circulating viruses and identifying the potential vector (s) and its biology help better understand epizootiology of BEFV in the high-risk region.

Introduction

Bovine ephemeral fever (BEF) is an acute febrile disease of cattle and water buffaloes caused by BEF virus (BEFV), a member of the genus *Ephemerovirus* in the family of *Rhabdoviridae*.[1] The virus is transmitted by several species of arthropod vectors including biting midges and mosquitoes and is distributed in tropical, subtropical and temperate regions of Australia, Middle East, Asia and Africa.[2]

Bovine ephemeral fever is represented by sudden onset of fever, anorexia, depression, rapid breathing, sternal recumbency, lacrimation, frothy salivation, arthritis, muscle stiffness, lameness and anastasis. Significant economic losses due to the reduction in milk production, abortion, infertility, delayed estrus, culling, loss of body condition in beef cattle and restriction on animal trade have been documented.[3,4]

The enveloped, cone-shaped BEF virion contains a helical nucleocapsid with single-stranded negative sense RNA genome of approximately 14,900 nucleotides in length that encode five structural proteins: Nucleoprotein, phosphoprotein, matrix protein, glycoprotein (G) and RNA-dependent RNA polymerase.[5] The envelope glycoprotein G projects from the virion, attaches to the host receptor, mediates fusion and viral entry, and is also the main inducer of type-specific neutralizing antibodies.[6,7] Four independent antigenic sites G1, G2, G3 and G4 have been identified in the G protein.[7,8] Epitope G1 is linear and the most conserved antigenic site and epitope G3 which is mapped at the distal end of the spike is the most conformational and variable antigenic site and comprises two discontinuous epitopes, G3a and G3b.[8-10]

Moreover, cytotoxic T lymphocyte epitopes have also been predicted for the BEFV glycoprotein G.[11] Although BEFV isolates are serologically believed to be as single

***Correspondence:**

Mehran Bakhshesh. DVM, PhD
Department of Animal Virology, Research and Diagnosis, Razi Vaccine and Serum Research Institute (AREEO), Karaj, Iran.
E-mail: m.bakhshesh@rvsri.ac.ir

serotype worldwide, wide range of cross neutralizations have been reported.[9,12] Several variations have been continuously observed in glycoprotein G in the enzootic areas of BEFV worldwide, and these changes particularly in the antigenic sites (G1 - G4) have largely influenced the levels of cross neutralizations between heterologous viruses and are likely responsible for the outbreaks of the disease frequently observed.[9] Consistently, phylogenetic analyses based on the *virus glycoprotein G-encoding* gene also suggest that BEFVs can be globally clustered into the four main lineages including Australia, East Asia, Middle East and Africa which have been supposed to be distinctively evolved in a geographic area of the world.[9,13,14] However, emerging the exotic BEFVs in the geographic areas, particularly in the Middle East, has also been reported.[12,15,16]

Bovine ephemeral fever virus is enzootic in Iran as large outbreaks have also been observed throughout the country.[12,17] Iran, a big country with a strategic geographic situation, located between the Middle East and East Asia, longitude 44° to 63° E and latitude 25° to 39° N, that contain over five million cattle and water buffaloes, is potentially at risk of viral transmission from the BEFV Middle Eastern and East Asian lineages through both the insect dispersal and animal transport.[2,16] Characterization of BEFVs in the Middle East revealed that beside the Middle Eastern lineage, East Asian lineage of the virus may also circulate in the Middle East.[12,16] Our previous investigation showed that BEFVs that caused severe outbreaks in Iran during 2012 and 2013 clustered in the East Asian lineage.[12,14] However, not unexpectedly, since then only the Middle Eastern lineage of BEFV was detected as enzootic in local areas and also in a large epizootic throughout the country in 2020. In order to realize the molecular epizootiology of the viral agent circulating in Iran in recent years, eight BEFV samples from different infected zones of the country were characterized based on the entire sequence of their *G* gene and subjected to phylogenetic study.

Materials and Methods

Sample collection. From 2015 to 2022, unclotted whole blood samples were taken from cattle representing BEF clinical symptoms in various geographic regions of Iran. The samples detected positive in diagnostic polymerase chain reaction (PCR) test,[17] from each infected zone were selected for molecular characterization. The geographic locations and times of sampling comprised: Gerash in Fars province in 2015, Shaft in Gilan province in 2017, Larestan in Fars province in 2019, Nur in Mazandaran province in 2020, Dashtestan in Bushehr province in 2020, Baneh in Kurdistan province in 2020, Narmashir in Kerman province in 2022 and Kermanshah in Kermanshah province in 2022.

RNA extraction and reverse transcription (RT)-PCR for amplification of the BEFV *G* gene. The RNA extraction procedure was conducted on whole blood taken from affected cattle, except for Gerash 2015 and Nur 2020 which were cell culture supernatant including propagated BEFVs. Gerash 2015 BEFV isolate was three times serially passaged in suckling mouse brain followed by three times serially passage in Vero cell line. Nur 2020 BEFV isolate was passaged in cattle followed by three times serially passage in Vero cell line. Total RNA was extracted from the whole blood samples containing anticoagulant or cell culture supernatant using RNX Plus Solution (SinaClon, Tehran, Iran) according to the manufacturers' instructions. One and two-step RT-PCR were both exploited to amplify the full length of the *G* gene. The one-step RT-PCR was performed using the One-Step PrimeScript RT-PCR kit (TaKaRa, Kusatsu, Japan) as previously explained in detail.[12] The two-step RT-PCR reactions were also included a RT and two PCR reactions to amplify two overlapping PCR fragments spanning the full length of the BEFV *G* gene. The RT reaction was performed using Random hexamer primer and Thermo Scientific™ RevertAid First Strand cDNA Synthesis Kit according to the manufactures' protocol (Waltham, USA). The PCR reactions contained 25.00 µL Super PCR Master Mix 2.00 X with proofreading ability (Takapouzist, Tehran, Iran), 4.00 µL cDNA, 2.00 µL (10.00 pmol) of each forward and reverse primers and 17.00 µL dionized water in a total volume of 50.00 µL. The first PCR reaction amplified a 1,159 bps using forward primer: GACTAGTCATATGGGAG AACCAG and revers primer: TCTGTTCTATCTGTGTGC ATCC which targeted nucleotides 2,780 – 2,802 and nucleotides 3,914 - 3,935 on BEFV genomes, respectively. Thermal cycling program was 94.00 ˚C for 2 min as initial denaturation, followed by 35 cycles of 94.00 ˚C for 30 sec, 53.00 ˚C for 30 sec, 72.00 ˚C for 40 sec and final extension of 72.00 ˚C for 2 min. The second PCR reaction amplified a 1,179 bps using forward primer: TGGTGGAGTATAGAAA ACCAAAC and revers primer: AGGAACATGATTGCCCTGT TG which targeted nucleotides 3,832 – 3,854 and nucleotides 4,993 – 5,013 on BEFV genomes, respectively. Thermal cycling program was 94.00 ˚C for 2 min as initial denaturation, followed by 35 cycles of 94.00 ˚C for 30 sec, 52.00 ˚C for 30 sec, 72.00 ˚C for 20 sec and final extension of 72.00 ˚C for 2 min. The PCR products were loaded on 1.00% agarose gel, stained with SYBR® Safe (Invitrogen, Carlsbad, USA) and were visualized under ultraviolet light.

Sequencing and phylogenetic analysis. The correct size PCR products were sequenced directly in both directions using the desired primers (Topaz Gene Research Co., Karaj, Iran). The obtained sequences were assembled using Geneious software (version 2020.2; Biomatters Ltd., Auckland, New Zealand) submitted in the NCBI GenBank® database, compared to each other and the selected sequences from the world and aligned using

Clustal W Multiple Sequence Alignment Program (UCD, Dublin, Ireland).[18] Phylogenetic trees were constructed for the all *G* gene sequences of Iran and selected sequences from the world with Neighbor-joining method provided in MEGA Software (version 7.0; Biodesign Institute, Tempe, USA).[19,20] The substitution model was estimated with Kimura 2-parameter and the robustness of the branches was tested using bootstrap analysis with 100 replications.

Statistical analysis. The nucleotide identity and the deduced amino acid among the Iranian BEFV G sequences were examined with Bioedit (version 7.2; Ibis Therapeutics, Carlsbad, USA)as amino acids variations in the G1 - G4 antigenic sites were also identified and compared.

Results

The nucleotide sequence of the all (eight) BEFV *G* genes were successfully sequenced and deposited in the GenBank®. They were assigned accession numbers: OR509487- OR509494 as depicted in Table 1.

As previously reported, BEFVs constituted four distinct phylogenetic lineages included the Middle East, East Asia, Australia, and Africa.[9,16,21,22] Bovine ephemeral fever viruses collected from different geographic regions of Iran during our study from 2015 to 2022, along with the two BEFVs previously reported and identified from Khuzestan province (southwest of Iran) in 2018 and 2020,[23] were all categorized as the Middle Eastern lineage of the virus. The sequences obtained from Iran (Baneh, Dashtestan, Nur and Khuzestan) and Türkiye during the 2020 BEFV epizootic, together with Kermanshah 2022, constructed a distinct cluster in the lineage. The two sequences of Larestan 2019 and Narmashir 2022 with the Indian sequence, India 2019, were also clustered together. The other BEFV sequences of Shaft 2017 and Khuzestan 2018 also constructed a cluster while Gerash 2015 was observed as a separate branch.

However, as it was also previously described, the viruses identified in Iran from 2012 to 2013 were classified in the BEFV East Asian lineage (Fig. 1).[12]

The relationship between all BEFVs characterized in Iran since 2012 was also specifically investigated and depicted in a phylogenetic tree (Fig. 2).

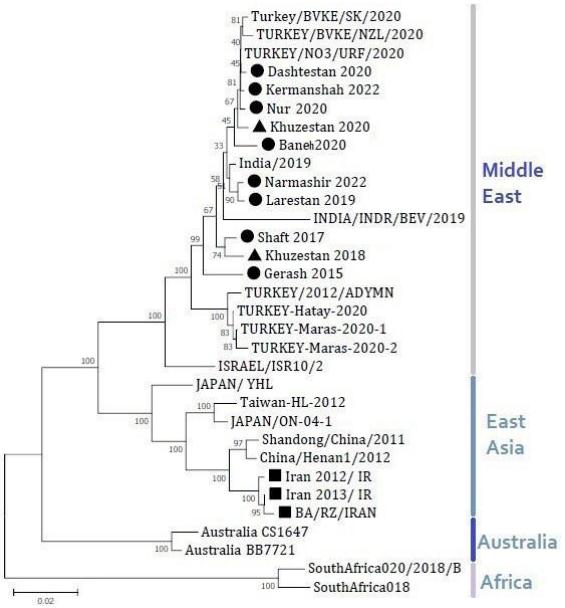

Fig. 1. Phylogenetic tree shows the genetic relationship of the Iranian and 19 bovine ephemeral fever viruses (BEFVs) from the world. The tree was constructed based on the alignment of the *G* gene using Neighbor-joining method and Kimura 2-parameter substitution model applying bootstrap values of 100 replicates. Black circles represent the eight BEFVs sequenced at the present study. Black triangles and squares represent Middle Eastern and East Asian BEFV viruses, respectively, characterized in previous studies. The scale bar represents 0.02 substitutions *per* site.

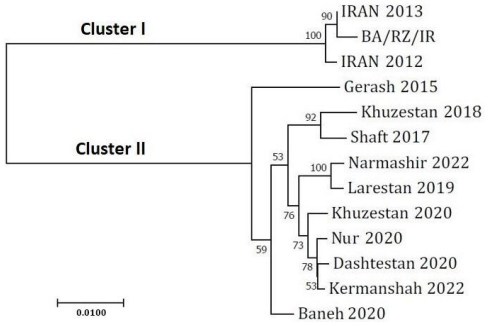

Fig. 2. The phylogenetic tree shows the genetic relationship of the all Iranian eight bovine ephemeral fever viruses characterized since 2012 based on the *G* gene sequence. The tree was constructed using the Neighbor-joining method with Kimura 2-parameter substitution model and bootstrap values of 100 replicates. The scale bar indicates 0.01 substitutions *per* site.

Table 1. Nucleotide sequence identity among the eight Iranian bovine ephemeral fever viruses characterized in this study.

No.	Sequences	Nucleotide identity (%)							
1	**Kermanshah.2022 (OR509493)**	100							
2	**Larestan.2019 (OR509489)**	98.98	100						
3	**Narmashir.2022 (OR509494)**	98.93	99.62	100					
4	**Nur.2020 (OR509490)**	99.73	98.98	98.93	100				
5	**Dashtestan.2020 (OR509492)**	99.73	98.93	98.87	99.67	100			
6	**Baneh.2020 (OR509491)**	99.09	98.45	98.50	99.03	98.93	100		
7	**Shaft.2017 (OR509488)**	98.61	98.55	98.61	98.50	98.45	98.29	100	
8	**Gerash.2015 (OR509487)**	97.54	97.38	97.43	97.43	97.38	98.39	97.54	100
No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**

As expected, the BEFV sequences characterized in Iran from 2012 to 2022 constructed two distinct clusters. Cluster I, Iranian East Asian lineage, includes the viruses isolated and characterized during 2012 and 2013.[12] Cluster II, Middle Eastern lineage, comprises the sequences characterized from 2015 to 2022 containing two main subclusters. Subcluster I include the closely related sequences Dashtestan 2020, Nur 2020, Khuzestan 2020 and Kermanshah 2022 which grouped together, and the two highly identical sequences Larestan 2019 and Narmashir 2022 build another group. Subcluster II comprises BEFV Shaft 2017 and Khuzestan 2018 sequences. Baneh 2020 which exhibited the highest sequence identity with Kermanshah 2022 (99.09%) and Nur 2020 (99.03%) in subcluster I, was seen as a separate branch. Gerash 2015 sequence also constructed a distinct branch in the Iranian Middle Eastern clusters (Fig. 3). Among the Iranian Middle Eastern sequences, Nur 2020, Dashtestan 2020 and Kermanshah 2022 exhibited the highest nucleotide identity (99.73%) while the lowest sequence identity was estimated between Gerash 2015 and both Larestan 2019 and Dashtestan 2020 as 97.38% (Table 1).

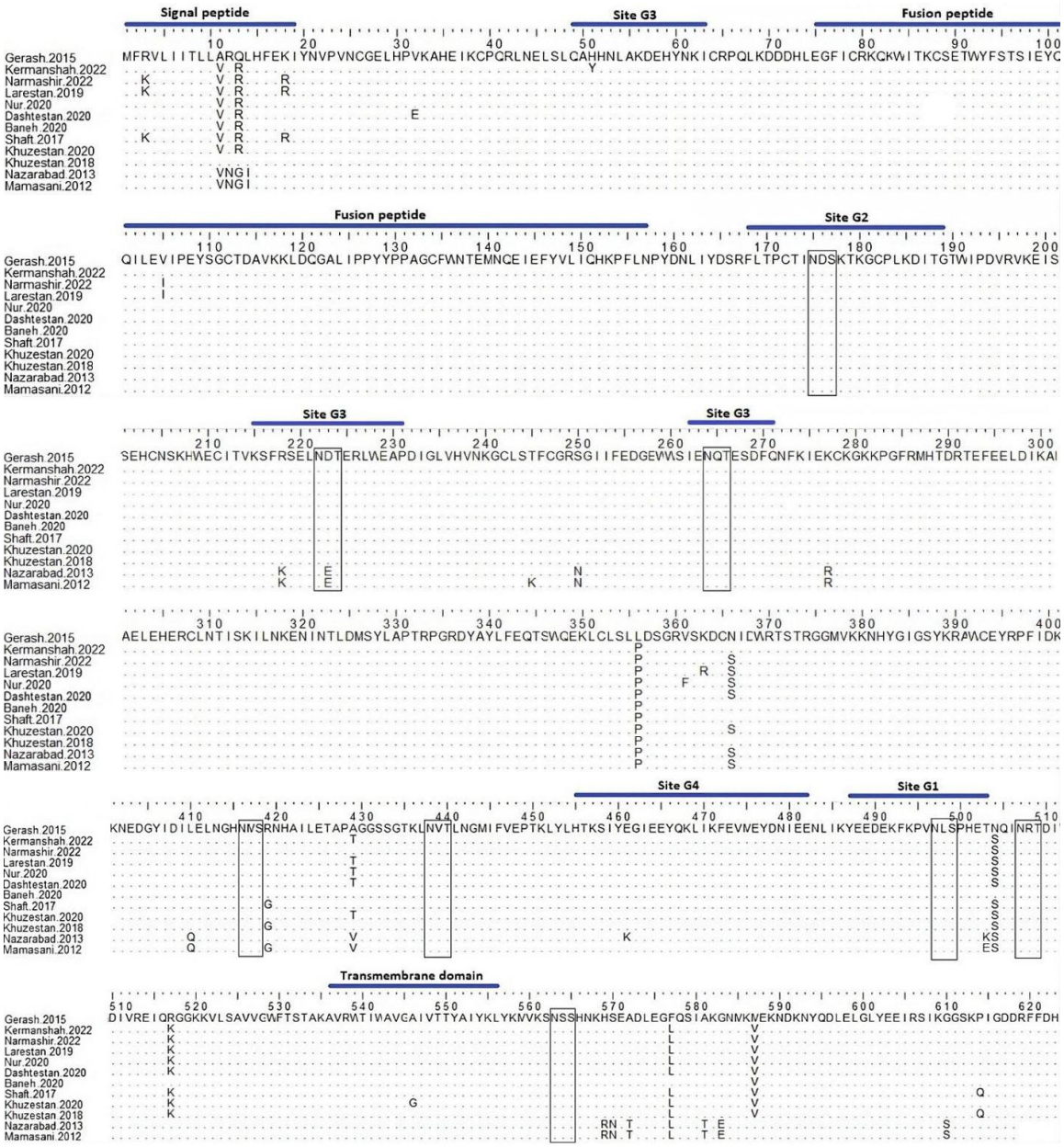

Fig. 3. Multiple alignment of the deduced amino acid sequences corresponding to the G protein of Bovine ephemeral fever viruses was registered from Iran (from 2012 to 2022). The location of neutralizing epitopes (G1 - G4), signal peptide, fusion peptid, and transmembrane domain are indicated by a marker above the amino acid sequences. The amino acid residues differing from the sequences of the majority are exhibited. Predicted glycosylation sites are boxed.

Amino acid variations among the Iranian BEFV *G* gene sequences from 2015 to 2022 were investigated (Fig. 3). A total number of 19 substitutions among the Iranian Middle Eastern lineage of BEFV were observed. Amino acid Histidine at residue 51 in the G3 antigen site was substituted with Tyrosine in the Kermanshah 2022 sequence. This was a unique variation reported so far in the literatures for the BEFV glycoprotein G.

In the signal peptide site, three substitutions at positions three, 11, and 13 were observed: Arginine was substituted by Lysine at residue three in Shaft 2017, Narmashir 2022 and Larestan 2019 sequences. Valin and Arginine at residues 11 and 13 were substituted by Alanine and Glutamine in Gerash 2015 and Khuzestan 2018 sequences, respectively.

In the Fusion peptide, Valine at residue 105 was substituted by Isoleucine in Larestan 2019 and Narmashir 2022 sequences. Amino acid Alanine at position 546 in transmembrane domain was also replaced by Glycine in Khuzestan 2020 sequence.

Comparison of antigenic epitopes between the two BEFV Iranian clusters, cluster I (viruses isolated during 2012 - 2013) and cluster II (viruses isolated during 2015 - 2022) revealed that they had four amino acids substitution including R218K and D223E in the G3 site, T503K in the G1 site and E461K in the G4 site (Fig. 3).

Using NetNGlyc program (version 1.0; DTU Health Tech, Kongens Lengby, Denmark), eight identical N-glycosylation sites at positions 175 - 177 (NDS: Asparagine-Aspartic acid-Serine), 222 - 224 (NDT: Asparagine-Aspartic acid-Threonine), 264 - 266 (NQT: Asparagine-Glutamine-Threonine), 416 - 418 (NMS: Asparagine-Methionine-Serine), 438 - 440 (NVT: Asparagine-Valine-Threonine), 497 - 499 (NLS: Asparagine-Leucine-Serine), 507 - 509 (NRT: Asparagine-Arginine-Threonine), 563 - 565 (NSS: Asparagine-Serine-Serine) of the glycoprotein G were predicted for the all Iranian BEFV sequences from 2012 to 2022 (Fig. 3).

Our evaluation also revealed that all East Asian and Middle Eastern BEFVs identically possessed these eight N-glycosylation sites. However, the two N-glycosylation sites 222 - 224 (NDT: Asparagine-Aspartic acid-Threonine) and 497 - 499 (NLS: Asparagine-Leucine-Serine) were not found in the Australian BEFV lineage. Also, the African lineage displayed no N-glycosylation site 222 - 224 (NDT: Asparagine-Aspartic acid-Threonine).

Discussion

A large country with a unique geographic location in the enzootic zone of BEFV, Iran, faces frequent outbreaks of the virus with considerable economic consequences. Molecular characterization of the circulating viruses during the past decade revealed that Iran could be potentially threatened by both the Middle Eastern as well as East Asia lineages of the BEFVs. Our previous analyses revealed that the circulating BEFVs in Iran during 2012 to 2013 were classified as the East Asian lineage.[12,14] Further characterization of BEFVs detected since 2015 from different areas of Iran revealed that they all were classified in the Middle Eastern lineage suggesting that these viruses replaced the East Asian viruses detected in 2012 and 2013.[23] However, within the BEFV, Iranian Middle Eastern lineage variations were observed and they could also be phylogenetically grouped in different clusters.

The BEFVs detected during the large epizootic of BEF throughout Iran in 2020 together with the viruses identified in Türkiye in 2020 were all closely related, and grouped together. This result, in line with the data available from Türkiye, suggested re-emergence of BEFV circulated before 2020 in the Middle East that caused epizootic in a vast geographic area of the Middle East.[24,25] However, among all BEFVs characterized in the Middle East, only TR-Maras-2020-2 exhibited amino acid variation at positions 223 and 224 in the G3b epitope.[25] Therefore, the large BEFV epizootic observed in 2020 might have not been associated with amino acid variation in the four putative epitopes. It remained to be elucidated whether polymorphism in other BEFV genes was involved in the viral virulence or escaped from pre-existing immunity caused the viral spread or other epizootiological factors such as waning the herd immunity or change in the vector (s) competence have contributed to the large epizootic.[26] Regarding to genetic variations, our previous analyses on 11 BEFV full genomes from the world revealed that amino acid diversities occurred throughout the BEFV genome, particularly L and accessory proteins β, α2 and Gns, suggesting that they might be involved in viral replication efficiency or regulation of the innate immune response.[14]

Outbreak of BEFV also occurred in Iran during the summer of 2022. The two viruses detected in 2022 from Kermanshah in the west and Narmashir in southeast of Iran displayed 98.93% sequence identity and were categorized in two distinct groups. Kermanshah 2022 virus was clustered with the BEFVs detected from the epizootic in 2020 and showed the highest nucleotide identity (99.73%) to Nur and Dashtestan 2020 viruses. It displayed a unique substitution at position H51Y in the epitope G3 that was likely responsible for the outbreak in the West of Iran. Narmashir 2022 sequence, however, exhibited the highest nucleotide identity (99.62%) to Larestan 2019 with no amino acid substitution in the four putative antigenic sites and constructed another group with the highest similarity to BEFVs identified in India in 2019. Occurrence of BEFV outbreak in India during 2018 to 2019 is believed to be associated with wind-borne transmission of the arthropod-vector from Israel or Türkiye to India.[27] However, our analyses showed

that the Indian viruses identified in 2019 were closer to the Iranian BEFVs sequenced in 2019 and 2022 suggesting that the virus have transmitted and evolved in the area between east of Iran, Pakistan and India during 2019 to 2022.[28] However, the minor difference in the *G* gene observed could not be solely considered for the occurrence of BEF outbreak in this geographic area and, therefore, other epizootiological factor (s) might have been involved in the spread of the virus. These results also suggested that BEFVs could evolve simultaneously in local geographic areas, although at lower evolutionary rates than was estimated in Australian episystem.[9]

Gerash 2015 BEFV isolate constructed a quite distinct branch in the lineage, therefore, exhibited the highest difference with the other the Iranian Middle Eastern BEFVs. It also exhibited a unique amino acid substitution at position P356Y which has identically been observed in African-like MN148800.1 Mayotte virus.[29] This virus, however, was serially passaged in baby mouse brain and cell culture which might have increased viral fitness and *in vitro* adaptability.[30-32]

Our bioinformatics analyses revealed that eight N-glycosylation sites could be predicted throughout the BEFV glycoprotein G which were conserved among the all Middle Eastern and east Asian viruses suggesting that they might have evolved from one origin. The 563 - 565 (NSS: Asparagine-Serine-Serine) was an additional N-glycosylation site to those previously reported for the East Asian viruses.[23]

The BEFV continues to evolve through point mutation and recombination in both vertebrate and invertebrate hosts resulting in emergence of new viruses with epizootic potential.[14,33] Therefore, it is important to discover unknown possible factors that can influence the emergence, pathogenicity and spread of BEF, particularly in the Middle East. These results added our knowledge about the intra-lineage evolution of the arbovirus BEFV in the enzootic and high-risk zone during a period of 7 years. Further studies that comprise full genome characterization of BEFVs circulating in the Middle East and South Asia, identification of the competent arthropod vector (s) and its biology can provide more comprehensive information about the epizootiology of the highly economic disease BEF, thus, implementing appropriate preventive strategies.

Acknowledgments

The authors gratefully thank to Razi Vaccine and Serum Research Institute, Karaj, Iran, for financial support of the present study.

Conflict of interest

The authors declare no conflict of interests.

References

1. Whelan S, Bloyet LM. Rhabdoviridae. In: Howley PM, Knipe DM, Whelan S (Eds). Fields virology. 7th ed. Philadelphia, USA: Lippincott Williams & Wilkins 2020; 494-620.
2. Walker PJ, Klement E. Epidemiology and control of bovine ephemeral fever. Vet Res 2015; 46:124. doi: 10.1186/s13567-015-0262-4.
3. Lavon Y, Ezra E, Friedgut O, et al. Economic aspects of bovine ephemeral fever (BEF) outbreaks in dairy cattle herds. Vet Sci 2023; 10(11): 645. doi: 10.3390/ vetsci10110645.
4. Constable PD, Hinchcliff KW, Done SH, et al. Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats. 11th ed. Philadelphia, USA: Elsevier Health Sciences 2017; 2081-2084.
5. Walker PJ, Tesh RB. Vesicular stomatitis virus and bovine ephemeral fever virus (Rhabdoviridae). In: Bamford D, Zuckerman M (Eds). Encyclopedia of virology. 4th ed. Amsterdam, Netherlands: Elsevier 2021; 875-883.
6. Walker PJ, Byrne KA, Cybinski DH, et al. Proteins of bovine ephemeral fever virus. J Gen Virol 1991; 72(Pt 1): 67-74.
7. Cybinski D, Walker P, Byrne K, et al. Mapping of antigenic sites on the bovine ephemeral fever virus glycoprotein using monoclonal antibodies. J Gen Virol1990; 71(Pt 9): 2065-2072.
8. Kongsuwan K, Cybinski DH, Cooper J, et al. Location of neutralizing epitopes on the G protein of bovine ephemeral fever rhabdovirus. J Gen Virol 1998; 79(Pt 11): 2573-2581.
9. Trinidad L, Blasdell KR, Joubert DA, et al. Evolution of bovine ephemeral fever virus in the Australian episystem. J Virol 2014; 88(3): 1525-1535.
10. Yazdani F, Bakhshesh M, Esmaelizad M, et al. Expression of G1-epitope of bovine ephemeral fever virus in *E. coli*: a novel candidate to develop ELISA kit. Vet Res Forum 2017; 8(3): 209-213.
11. Mollazadeh S, Bakhshesh M, Keyvanfar H, et al. Identification of cytotoxic T lymphocyte (CTL) epitope and design of an immunogenic multi-epitope of bovine ephemeral fever virus (BEFV) glycoprotein G for vaccine development. Res Vet Sci 2022; 144: 18-26.
12. Almasi S, Bakhshesh M. Antigenic variation of bovine ephemeral fever viruses isolated in Iran, 2012-2013. Virus Genes 2019; 55(5): 654-659.
13. He CQ, Liu YX, Wang HM, et al. New genetic mechanism, origin and population dynamic of bovine ephemeral fever virus. Vet Microbiol 2016; 182: 50-56.
14. Bakhshesh M, Mollazadeh S, Almasi S, et al. Whole genome characterization and evolutionary analysis of bovine ephemeral fever virus isolated in Iran. Arch Microbiol 2023; 205(5): 196. doi: 10.1007/s00203-023-03527-7.

15. Golender N, Hoffmann B, Kenigswald G, et al. Bovine ephemeral fever viruses in Israel 2014-2023: genetic characterization of local and emerging strains. Pathogens 2024; 13(8): 636. doi: 10.3390/ pathogens13080636.
16. Aziz-Boaron O, Klausner Z, Hasoksuz M, et al. Circulation of bovine ephemeral fever in the Middle East - - strong evidence for transmission by winds and animal transport. Vet microbiol 2012; 158(3-4): 300-307.
17. Bakhshesh M, Abdollahi D. Bovine ephemeral fever in Iran: diagnosis, isolation and molecular characterization. J Arthropod Borne Dis 2015; 9(2): 195-203.
18. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22(22): 4673-4680.
19. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4(4): 406-425.
20. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33(7): 1870-1874.
21. Walker PJ. Bovine ephemeral fever in Australia and the world. Curr Top Microbiol Immunol 2005; 292: 57-80.
22. Kato T, Aizawa M, Takayoshi K, et al. Phylogenetic relationships of the G gene sequence of bovine ephemeral fever virus isolated in Japan, Taiwan and Australia.Vet Microbiol 2009; 137(3-4): 217-223.
23. Rezatofighi SE, Mirzadeh K, Mahmoodi F. Molecular characterization and phylogenetic analysis of bovine ephemeral fever viruses in Khuzestan province of Iran in 2018 and 2020. BMC Vet Res 2022; 18(1): 19. doi: 10.1186/s12917-021-03119-x.
24. Tokgoz BS, Tokgoz EA, Söezmen MA, et al. Prevalence, isolation and molecular characterization of Bovine Ephemeral Fever Virus in south and southeast regions of Turkey in the outbreak of 2020. J Hellenic Vet Med Soc 2023; 74(4): 6543-6552.
25. Abayli H, Tonbak S, Azkur AK. Re-emergence of bovine ephemeral fever in Turkey in 2020 after an 8-Year Absence: a molecular analysis study. Isr J Vet Med 2023; 78(2): 37-46.
26. Ting LJ, Lee MS, Lin YL, et al. Invasion of exotic bovine ephemeral fever virus into Taiwan in 2013-2014. Vet Microbiol 2016; 182: 15-17.
27. Pyasi S, Sahu BP, Sahoo P, et al. Identification and phylogenetic characterization of bovine ephemeral fever virus (BEFV) of Middle Eastern lineage associated with 2018-2019 outbreaks in India. Transbound Emerg Dis 2020; 67(5): 2226-2232.
28. Nadeem S, Aslam R, Sajjad-Ur-Rahman, et al. Risk analysis and seroprevalence of bovine ephemeral fever virus in Punjab, Pakistan. Vet Med (Praha) 2024; 69(3): 67-76.
29. Mlingo TAM, Nthangeni BM, Mokoena NB. Genome sequence of bovine ephemeral fever virus vaccine strain of South African origin. Vet Med Sci 2021; 7(5): 1611-1615.
30. Villa TG, Abril AG, Sánchez S, et al. Animal and human RNA viruses: genetic variability and ability to overcome vaccines. Arch Microbiol 2021; 203(2): 443-464.
31. Sun B, Ni M, Liu H, et al. Viral intra-host evolutionary dynamics revealed via serial passage of Japanese encephalitis virus *in vitro*. Virus Evol 2023; 9(1): veac103. doi: 10.1093/ve/veac103.
32. Domingo E, de Ávila AI, Gallego I, et al. Viral fitness: history and relevance for viral pathogenesis and antiviral interventions. Pathog Dis 2019; 77(2): ftz021. doi: 10.1093/femspd/ftz021.
33. Lee F. Bovine ephemeral fever in Asia: recent status and research gaps. Viruses 2019; 11(5): 412. doi: 10.3390/v11050412.