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ORIGINAL PAPER

Antigenic variation of bovine ephemeral fever viruses isolated in Iran, 2012–2013

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Abstract

Bovine ephemeral fever virus (BEFV) is an economic arthropod-borne virus distributed in Africa, Asia, and Australia. Based on the sequence of the gene encoding the surface glycoprotein G, the viral antigenic determinant, BEFV has been phylogenetically classified into three clusters, including Australia, East Asia, and the Middle East. Here, we provide evidence for antigenic variations among the BEFV isolates in Iran during the period of 2012 to 2013 and also the exotic YHL strain, which are all classified into the East Asian cluster of the virus. For this propose, the entire length of the G gene of the viruses were sequenced and phylogenetically compared. The corresponding antigenic sites (G1–G4) were analyzed and antigenic relatedness among these viruses was measured. The two Iranian viruses, which displayed substitutions at residues E503K in the site G1 and E461K in the predicted site G4, were partially neutralized by each other's antisera (*R* value = 63.23%); however, these two viruses exhibited much lower cross-neutralization that measured by *R* value as 28.28% and 22.82%, respectively. The crucial substitution at amino acid R218K in the site G3a is believed to be the foremost cause of these declines. The data emphasize the frequent evolution of BEFV in different time periods and geographic regions, in which the new variants can emerge and likely escape from the pre-existing immunities. Thus, continuous monitoring of the circulating viruses is necessary for understanding the viral evolution and evaluation of protective immunity induced by the heterologous viruses.

Keywords BEFV · Genetic variation · Iran · Protectiveness

Introduction

Bovine ephemeral fever (BEF) is an arthropod-borne disease of cattle and water buffaloes in tropical and subtropical areas of Africa, Australia, Asia, and Middle East. The disease is mainly identified by acute fever, lameness, and disabling condition with low mortality but severe economic consequences [1]. BEF is also well known throughout the Middle East, including Iran, as its periodic outbreaks have been reported over the past century [2].

The viral agent is a member of the genus *Ephemerovirus* within the *Rhabdoviridae* family. Its genome is ~ 15 kb negative sense RNA flanking 10 open reading frames in the order of 3′-N-P-M-G-(G_{NS}-α1/α2/α3-β-γ)-L-5′ in that the accessory genes (G_{NS}-α1/α2/α3-β-γ) are only specific to the genus *Ephemerovirus* [3, 4].

The G gene encodes a transmembrane 623 amino acid glycoprotein (G), the only viral surface protein that protrudes from the virion, is responsible for cell attachment, fusion, and entry, and also elicits type-specific neutralizing antibodies [3, 5], capable of protecting cattle to experimental infection [6]. The class I transmembrane glycoprotein G includes four independent antigenic sites (G1, G2, G3, and G4), which are located in the G protein ectodomain [5, 7]. The site G1 is the linear and the most conserved epitope, while the site G3 is the major conformational and the most variable epitope located at the distal end of the spike comprising two discontinuous epitopes (G3a and G3b) [7, 8].

Despite of being a single serotype around the world, heterologous BEF viruses from distinct geographical regions or different times exhibited a wide range of partial cross-neutralization [9–13]. Consistently, the glycoprotein G of these heterologous viruses also has been reported to have

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variations, remarkably in the three major antigenic epitopes (G1–G3) [8, 12, 14, 15]. Phylogenetic studies proposed that a variety of bovine ephemeral fever virus (BEFV) strains from the East and West Asia are likely to circulate in the Middle East, through winds and animal transportation [2, 16–18]. These diverse viruses can potentially induce different levels of immunity against each other [17] and, particularly, escape from the protective immunity induced by a specific vaccinal strain [11, 13, 14, 19]. Therefore, continuous monitoring of the circulating viruses is essential for understanding epizootiological pattern, genetic evolution, and antigenic relationships among these viruses.

In the present study, the two Iranian BEFV isolated in 2012 and 2013 and an exotic (Japanese YHL-1966) strain (also available as inactivated vaccine in Iran) were compared phylogenetically and serologically. For this aim, the complete G gene of the three viruses was sequenced; the corresponding antigenic epitopes were compared and the degrees of antigenic relatedness (*R* value) were evaluated.

Materials and methods

Cell culture

African green monkey (Vero) cells were grown in DMEM high glucose (4.5 g/l) medium (Gibco, Carlsbad, CA, USA) supplemented with 2% fetal calf serum (Gibco), 2.2 g/l NaHCO3 (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml Penicillin, and 50 µg/ml Streptomycin (Sigma-Aldrich) on 25 cm^2 filter cap flasks (SPL, Pocheon, South Korea) and incubated at 37 °C in a humidified incubator (Binder, Tuttlingen, Germany) with 5% CO_2 until they became confluent. The monolayer cells were inoculated with lysed buffy coats or mouse brain containing BEFV, and the infected cell culture supernatants were then frozen and thawed and inoculated into a new culture [18]. The serial passages of the infected cell cultures were repeated until cytopathic effect (CPE) was observed and the viruses were adapted to the cells with the optimum titer.

Viruses

BEFV that was isolated from infected cattle during 2012 from Mamasani (30°N, 46°E) in Fars province was passaged 3 times in the suckling mouse brain and 5 times on Vero cell, named IR-2012. BEFV that was isolated from infected cattle during 2013 from Nazar Abad (35°N, 50°E) in Alborz province was passaged in cattle and 5 times on Vero cell named IR-2013. The Japanese Yamaguchi strain (YHL) that was isolated from cattle in 1966 and then serially passaged in calf, suckling hamster, mouse, rat [20], and unknown passage number in cell culture (kindly provided by Kyoto Biken Laboratories, Japan).

Hyperimmune serum production

The hyperimmune sera against the two Iranian (IR-2012 and IR-2013) and Japanese (YHL) BEFV were raised specifically in rabbits as described in Almasi and Bakhshesh (2019) (accepted for publication by journal of Animal Researches/Iranian Journal of Biology). Briefly, 2 ml of the cell culture supernatant containing at least 10^6 $TCID_{50}$/ml of each of the viruses was injected into the marginal ear vein on days 0, 7, and 28. Two weeks later blood samples were collected from the heart; the sera were separated by centrifugation at 3000 rpm for 5 min, heated at 56 °C for 30 min, and stored at − 20 °C until tested.

Amplification of G protein encoding gene

Total RNA sample extracted from virus infected Vero cell culture supernatant using viral RNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The full-length G gene was amplified by using One-Step PrimeScript RT-PCR kit (TaKaRa, Kusatsu, Japan) as follows: 25 µl of 2× buffer, 10 pmol of each forward: 5′-ATGTTCAAGGTCCTCATAATTACC-3′ (nt 1–24) and reverse: 5′-TAATGATCAAAGAACCTATCATCAC-3′ (nt 1874–1871) primers as described by Wang et al. [14]. The RT-PCR reactions (50 µl total volume) comprised 4 µl of sample RNA, 2 µl PrimeScript RT-PCR enzyme mixture, and 44 µl DEPC-treated water. Thermal cycling program was carried out as 50 °C for 30 min, 94 °C for 2 min as initial denaturation, followed by 27 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min, and final extension of 72 °C for 10 min. The PCR products were run on 1% agarose gel, stained with SYBR® Safe (Invitrogen, Carlsbad, CA, USA), and visualized under ultraviolet light.

Sequencing and phylogenetic analysis

The PCR products (1871 bp) spanning the entire length of the G gene were sequenced directly in the both directions by using external (PCR primers) and also internal primers including forward 5′-CTTGCCAACTTGTTTGCTC-3′ (nt 1045–1065) and reverse 5′-AGGTCTGTATTCGCA CCAAGCTCT-3′ (nt 1131–1155) (Bioneer, Daejeo, South Korea). The obtained sequences were assembled using Geneious (4.8.5) software, compared with the sequences in the GenBank, and aligned by using Clustal W program [21]. The nucleotide and deduced amino acid (aa) sequence homologies among the sequences were analyzed with BioEdit 7.0.5 software, and the amino acids in the G1–G3 sites were identified. Phylogenetic analyses was carried out

by using MEGA 5.2 software [22] and the phylogenetic tree based on nucleotide sequences was constructed for the sequences of this study and 28 sequences from the GenBank by using neighbor-joining method [23]. The reliability of the branching was evaluated by the bootstrap test with 500 replicates [24].

Virus neutralization (VN) test

VN tests were carried out in 96-well microplate with the above-mentioned hyperimmune sera and the constant titer (100 $TCID_{50}$) of each BEFV. In quadruplicate wells, 50 µl of the virus was mixed with the equal volume of serum in twofold serially dilutions from 1/2 to 1/2048. The mixture was incubated at room temperature (RT) for 60 min, then cell suspension (30,000 Vero cell/150 µl per well) was added, and the microplate was incubated at 37 °C in a moist atmosphere with 5% CO_2. After 5 days of incubation, the microplate was observed for the presence of CPE. The antibody titer was calculated based on reciprocal of the highest serum dilution that inhibits CPE in 50% of wells according to Reed and Muench formula [25]. All tests were repeated three times and the mean was calculated for each test.

Antigenic relatedness

The antigenic relatedness of the three BEFV strains was evaluated by pairwise comparison based on Archetti and Horsfall formula [26]. The final result expresses antigenic relatedness between the two viral strains also known as *R* value and calculated by the following formula:

$$R = \sqrt{r1 \times r2},$$

where $r1$ is heterologous titer virusB/homologous titer virusA, and $r2$ is heterologous titer virusA/homologous titer virusB.

If the *R* value falls between 0 and 5%, the two viruses are considered as two different serotypes. Two subtypes exist when the *R* value is calculated between 5.1 and 70%, and little or no differences present when it is evaluated >70%.

Results

The full length of the G gene sequences of IR-2012, IR-2013, and YHL BEF viruses were successfully amplified and sequenced, and the Iranian sequences IR-2012 and IR-2013 have been assigned the Accession number of MF491475 and MF491476, respectively, in the GenBank. Alignment of the complete G gene sequences of IR-2012 and IR-2013 isolates revealed that they share 99.6% nucleotide sequence identity, while these two sequences exhibit 95.5% and 95.4% identity with that of YHL strain, respectively. The phylogenetic tree was constructed for a total of 31 sequences from the world. The tree comprised three clusters including East Asia, Middle East, and Australia. The two identical Iranian sequences with the Turkish TR-ETLIK-2/2012, TRCP3/2012, TR-CU15/2012, and those from East Asia detected since 2011 were all grouped into a sub-cluster of the East Asian cluster. As expected, YHL strain was placed into a distinct sub-cluster more closely related to the old East Asian viruses (Fig. 1).

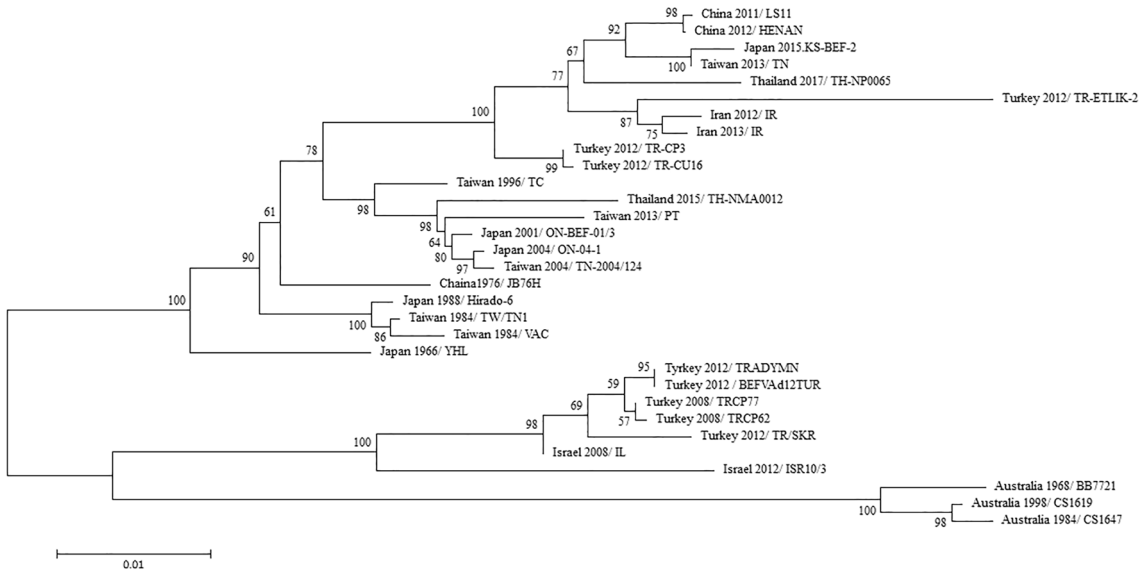

Fig. 1 Phylogenetic analysis of G gene sequences of the IR-2012 (MF491475), IR-2013 (MF491476), and 28 BEF viruses from Australia, East Asia, and the Middle East. The scale represents 1% sequence divergence

Amino acid sequence alignment of the G protein (Fig. 2) revealed that totally four substitutions including K245T, E419R, E461K, and E503K (in the site G1) between IR-2012 and IR-2013 isolates occurred. The Japanese YHL strain differed 10 amino acids from both the Iranian viruses including Q75E, V106I, R218K, D223E, K277R, R518Q, Q534A, H569R, and V587M; of these, the residues 218, 223, and 277 were located in the site G3 and the residue 106 is located in the fusion peptide. YHL strain also differed from the IR-2012 isolate at residues T245K, R419E, and K503E (in the site G1), and from the IR-2013 isolate at residue E461K (in the predicted site G4), respectively.

Viral neutralization assay was carried out between the IR-2012, IR-2013, and YHL BEF viruses and their homologous and heterologous antisera. All sera were capable of neutralizing homologous and, with lower titers, heterologous viruses (Table 1). Consistent with the phylogenetic data, the two Iranian isolates exhibited partial cross-neutralization calculated by the *R* value as 63.23%, indicating that these viruses could be classified into subtypes. Much lower cross-neutralization was observed between each of these viruses and YHL strain, and the *R* value was evaluated between the IR-2012 and IR-2013 isolates and YHL strain as 28.28% and 22.82%, respectively.

Table 1 Cross-neutralization tests of IR-2012, IR-2013, and YHL BEF viruses

Antisera	Viruses		
	IR-2012	IR-2013	YHL
IR-2012	1024	465	255
IR-2013	940	1065	195
YHL	280	248	871

Discussion

Previous studies have identified that all BEFV strains exhibit degrees of cross-neutralization, although homologous titers have always been reported to be higher than heterologous titers. The diversity of cross-neutralization has been found to be associated with times and areas that the viruses were circulated and detected [8–12, 27]. In the present study, we assessed the phylogenetic and antigenic relationship of the two BEF viruses isolated in Iran during the outbreaks of 2012 and 2013, and the exotic YHL strain isolated from Japan in 1966.

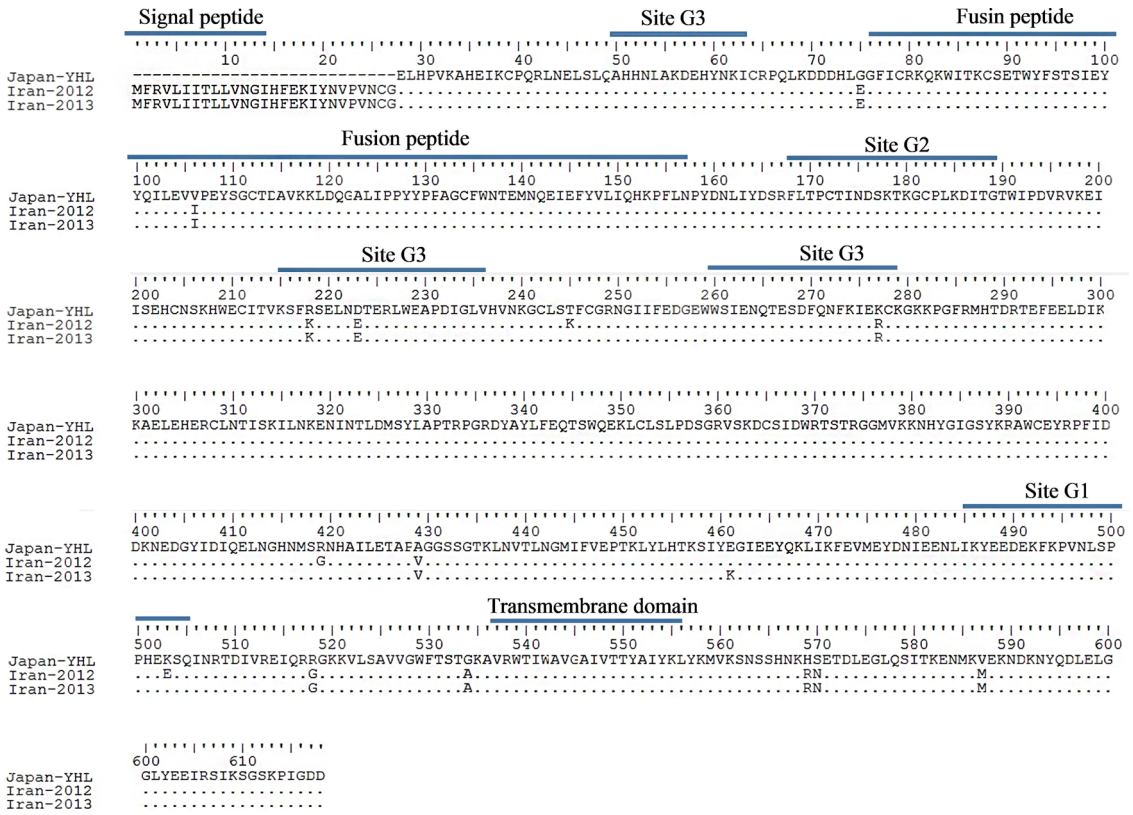

Fig. 2 Alignment of the deduced amino acid sequences of G protein of IR-2012, IR-2013, and YHL viruses. The antigenic sites (G1–G3), signal and fusion peptide, and transmembrane domain are identified with a line above the sequences. The residue differences are denoted

The two Iranian IR-2012 and IR-2013 viruses, which were isolated in a period of less than a year but from distinct geographic regions, displayed high nucleotide sequence identity (99.6%) with a total of 4 aa substitutions. However, their antisera exhibited partial cross-neutralization as the *R* value between these viruses estimated at 63.23%, implying that they may be classified into subtypes. Comparison of the antigenic epitopes (G1–G4) of these viruses shows only 1 aa substitutions at position E503K in the G1 epitope; to what extent this unique substitution caused an approximately 1 log decline in neutralization titer of IR-2013 serum against the IR-2012 virus remains debatable. Using monoclonal antibodies, BEFV escape mutants have been determined as a result of substitution at amino acid 503 [7], whereas the conservative substitution K503R did not cause variation in neutralization titer of BEF viruses circulated in Australia since 1981 [8]. The role of substitution at residue E461R, in the predicted epitope G4 [8], on antigenic variation of these closely related viruses also needs further elucidation.

Consistent with the phylogenetic tree, representing YHL and Iranian viruses as two distinct sub-clusters, the Japanese YHL strain exhibits low antigenic relatedness with the IR-2012 and IR-2013 isolates, which expressed by *R* value as 28.28% and 22.82%, respectively. YHL strain differed from both the Iranian BEFV isolates 4 aa in the major conformational epitope G3 and 2 aa in the fusion peptide area. The substitution R218K at the site G3a is supposed to be the most critical change occurred as approved by monoclonal antibodies [7, 8]. Strikingly, the natural single substitution R218K resulted in antigenic shift and changed the epidemiological feature of the virus in Australia since 1973 [28]. Adjacent to this substitution, the residue 223, which has been proposed as an additional N-glycosylation site in East Asian cluster of the virus, changed (D to E) [12]; however, the effect of this single substitution on neutralization phenotype of the East Asian viruses remains to be identified [12, 29]. Using monoclonal antibodies, the substitution in the antigenic site G3b at residue 215, together with change at amino acids 220 and 223, has also been partially associated with antigenic shift in Australian isolates [8].

The substitutions K277R in the site G3, V106I in the fusion peptide, and G75E adjacent to the fusion peptide may have also influenced the degree of cross-neutralization between these viruses. The IR-2012 and IR-2013 also varied at amino acids E503K in the site G1 and E461K in the predicted site G4 from YHL strain, respectively, may also affect the neutralization phenotype of these viruses.

These results, in accordance with the previous studies, provide convincing evidence that heterologous BEF viruses characterized by phylogenetic classification can fail to induce full protective immunity against each other; however, quite distinct viruses still exhibit a degree of cross-neutralization.

The vector-borne *Rhabdovirus*, BEFV, possesses high capacity to evolve in both vertebrate and invertebrate hosts creating the new genetically adaptive viruses which can escape from the existing immunity. The recently new East Asian sub-cluster of the virus, which originated from China in 2011 [15] and spread dominantly in both directions to the Middle East and Far East with a higher case fatality rate, is an example of this adaptive evolution [17, 18, 27, 29]. Therefore, a combination of molecular epidemiologic studies along with evaluation of potential immunogenicity of the circulating viruses against each other is recommended especially if prophylactic programs using vaccine are undertaken in BEFV enzootic areas.

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Author contributions SA carried out the experiment and analyzed the data. MB designed the study and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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