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RESEARCH

Detection and genetic analysis of bovine ephemeral fever virus G gene in buffy coat samples from cattle at Qualyubia, Egypt 2017

Ayman S. El-Habbaa[1] and Mervat E. Radwan[2]

ABSTRACT

Background: Bovine Ephemeral Fever (BEF) is endemic in Egypt with repeated periodic outbreaks.
Objective: To investigate strain detection and genetic analysis of BEF virus among suspected cattle in August 2017, Qualyubia, Egypt.
Methods: Partial sequence was generated after detection by reverse transcription-polymerase chain reaction (RT-PCR) and subsequent gel purification of the amplified products of G gene of BEF virus.
Results: BEF virus was circulating in this region. Sequence analysis of G-gene of this Egyptian strain, comparing with sequences of BEF virus circulating globally and regained from GenBank, showed 100% nucleotide homology with BEF virus from Egypt 2014 but nucleotide and deduced amino acid substitution generates were showed with other BEF viruses that reduced this homology. Phylogeny showed that BEF viruses from Egypt 2014 and 2017 had close homology to BEF virus circulated in Israel during the same period suggesting that the virus was circulated in middle east.
Conclusion: These findings demonstrated the recent picture of BEF virus which incriminated for cattle infectivity and responsible for its persistence in the endemic areas. Such epidemiological data could guide the application of efficient control strategies of BEF virus in Egypt.

Keywords: EBF virus; Cattle; sequences; nucleotide, amino acid.

BACKGROUND

Bovine ephemeral fever (BEF) is an arthropod born viral disease, characterized by a disabling febrile infection of cattle and water buffalo. The disease is common in tropical and subtropical regions of Africa, Asia, Australia and the Middle East. It is also an enzootic disease in Egypt with considerable economic impact as reduced milk production in dairy herds, loss of condition in beef cattle (El-Bagoury *et al.*, 2014; Kasem *et al.*, 2014 and Akakpo, 2015). BEF virus is the type species of the genus Ephemerovirus within the family Rhabdoviridae (Yeruham *et al.*, 2010). BEF virus has negative-sense single-stranded RNA that encodes five structural proteins including a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a large RNA-dependent RNA polymerase (L) and a surface glycoprotein (G).

The G protein is the type specific class I transmembrane glycoprotein responsible for cell attachment and entry; also it induces protective immunity including virus-specific neutralizing antibodies (Dhillon, *et al.*, 2000 and Walker et al., 2012). It comprises 4 antigenic sites (G1-G4), the highly conserved G1 site (17aa) is a linear antigenic site mapped to amino acid 487-503 of the 623-amino acid G protein (Yazdani *et al.*, 2017). Reverse transcription-Polymerase Chain reaction (RT-PCR) was found to be a sensitive, specific and rapid method that can be used for detecting BEF virus nucleic acid in clinical samples (Finlayson *et al.*, 2014 and Niwa *et al.*, 2015)

Sequencing of viral genome and phylogenetic analyses are important for understanding molecular nature, epidemiology and control of BEF virus (Abayli *et al.*, 2017). The present work

Correspondence: aymanelhabbaa@gmail.com
Full list of author information is available at the end of the article

aimed for rapid detection of BEF virus from suspected cattle as well as genetic analysis of G-gene predicting antigenicity of the virus.

MATERIALS AND METHODS

Samples

Bovine ephemeral fever (BEF) was suspected among cattle coming from different localities in Qaliubiya governorate, Egypt to the veterinary teaching hospital, faculty of veterinary medicine, Benha university during summer 2017. Blood samples on sodium citrate as anticoagulant were collected from suspected cattle at early febrile stage of the disease for separation of buffy coat used as source for suspected viral samples (Van Der Westhuizen, 1967). The animals had no history of vaccination. These samples (No.= 8) were used for direct detection using conventional reverse transcription polymerase chain reaction (RT-PCR).

Direct detection of viral RNA in suspected samples using RT-PCR

Extraction of viral RNA in suspected buffy coat samples was done using a Gene Jet viral RNA Purification kit (Thermo Scientific). The primers sequences were designed based on the sequence of BEF virus G gene as described by Zheng *et al.*, (2007). The specific primers were manufactured in the laboratories of the Midland Certified Reagent company Inc. of Midland, Texas. The sequence of oligonucleotides primers was 420F (5' AGAGCT TGG TGT GAA TAC 3') and 420R (5' CCA ACCTAC AAC AGC AGA TA 3'). The forward primer 420F was used to reverse-transcribe BEFV RNA to cDNA. For RT-PCR, a partial fragment of the BEFV G gene was amplified using the primers 420F and 420R. After the initial denaturation at 94°C for 5 min, the amplification proceeded through a total of 35 cycles consisting of denaturation at 94°C for 40s, annealing at 48°C for 1 min, primer extension at 72°C for 40s and a final extension for 10 min at 72°C. The expected DNA fragments were 420 base pairs (bp) in length detected using 1.5 % agarose gel in TAE buffer stained with ethidium bromide.

Determining and analyzing partial sequence of G-gene of a recent strain of BEF virus from cattle, Egypt 2017

Then these amplified products were purified and analyzed by gel documentation system for sequencing process that showed sharp clear bands at the specific size of 420 bp.

Sequencing of the purified product of the RT-PCR reaction of the amplified gene of BEF virus (G gene) was carried out (Big Dye® Terminator v3.1 Cycle Sequencing Kit, Perkin-Elmer) and DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). Computer assisted sequence alignment and phylogeny was performed using BLAST search program (Altschul *et al.*, 1990) *www.blast.ncbi.nlm.nih.gov/Blast.cgi*, *Clustal* W (Thompson *et al.*, 1994) and DNA star mega (Tamura *et al.*, 2011) with aid of nucleotide and amino acid sequences of published BEF virus sequences in Gene Bank data base at National Center for Biotechnology Information (NCBI), (Table, 1).

Table 1: Reference BEF virus sequences published in Gene Bank data base at National Center for Biotechnology Information (NCBI).

No.	Accession Number on gene bank	Locality of the BEF virus	Year of gene bank publication
1	KJ605437.1	Taiwan	2016
2	KJ729108.1	Egypt	2015
3	MH237603.1	Egypt	2018
4	JX564639.1	Japan	2012
5	AB462044.1	Taiwan	2016
6	AY768942.1	Taiwan	2005
7	AY062166.1	Taiwan	2001
8	AY935240.1	Taiwan	2009
9	AF208840.1	Taiwan	2000
10	AB462039.1	Japan	2016
11	AB462037.1	Japan	2016
12	AB462033.1	Japan	2016
13	KC470313.1	Turkey	2013
14	KC470312.1	Turkey	2013
15	KC470310.1	Turkey	2013
16	JX564640.1	Japan	2012
17	JX564638.1	China	2012
18	JX564637.1	China	2012
19	JN833635.1	Israel	2012
20	JN833634.1	Israel	2012
21	GQ229452.1	Turkey	2009
22	GQ229451.1	Turkey	2009

RESULTS

Molecular detection of BEF virus in samples from suspected cattle using RT-PCR

BEF virus was detected in buffy coat samples from cattle at Qalyubia governorate, Egypt targeting glycoprotein coding (G) gene. Positive result was obtained with amplification of a specific products 420 bp from the extracted nucleic acid using RT-PCR (Figure-1).

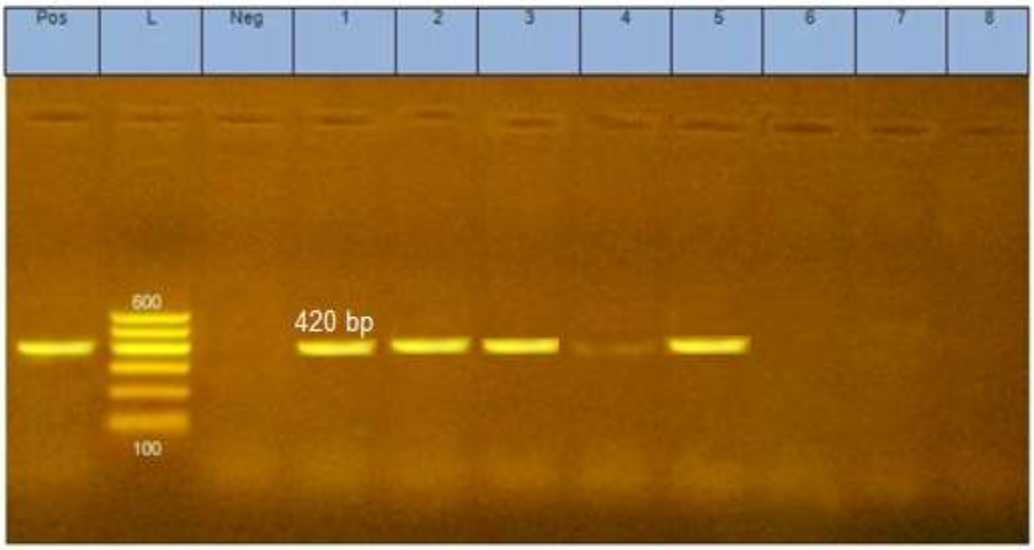


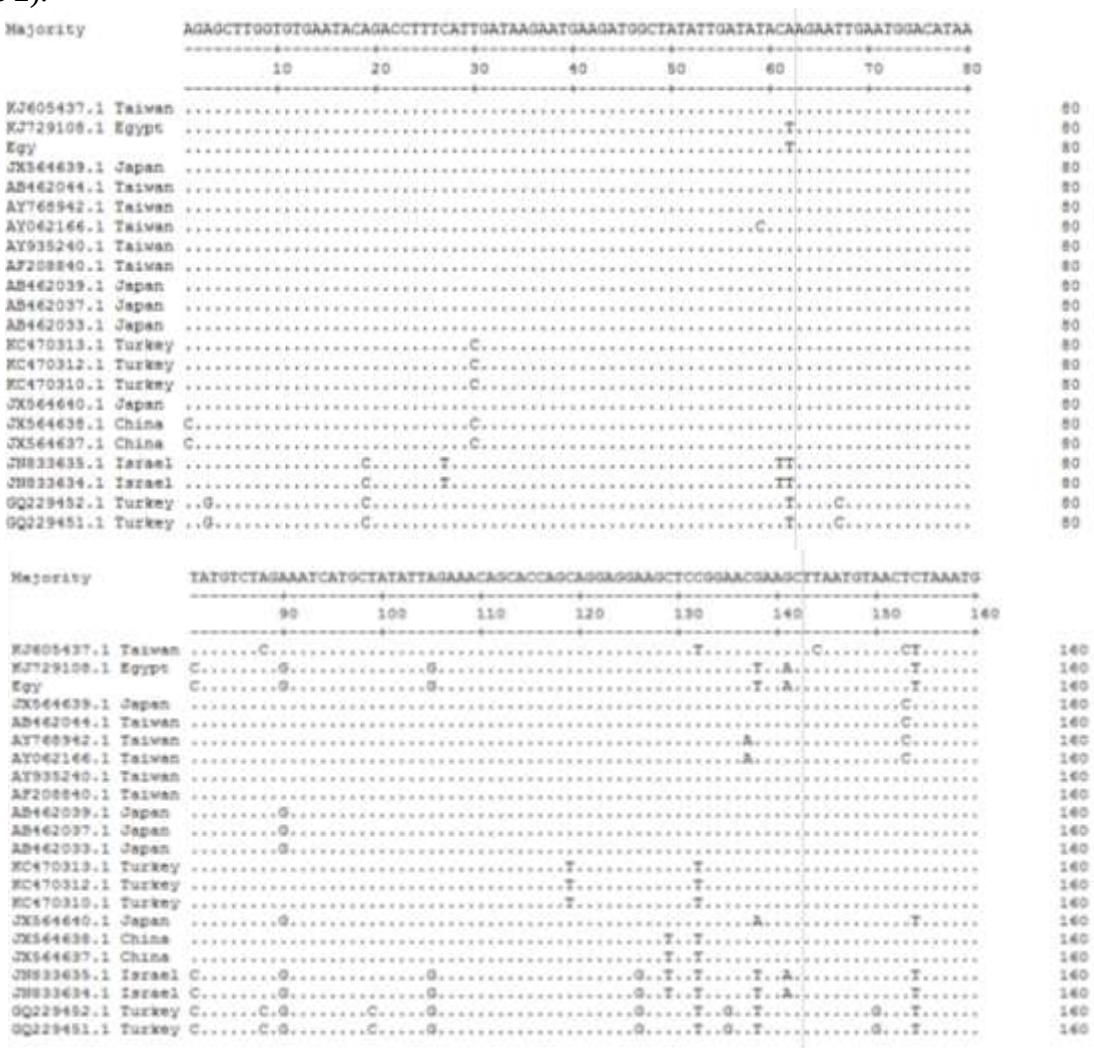
Fig. 1: Electrophoretic pattern of the amplified products 420 bp of the G gene for BEF virus from cattle. Pos: Reference BEF virus. L: High molecular weight nucleic acid marker (100bp), Lanes 1 – 8: Buffy coat samples from suspected cattle.

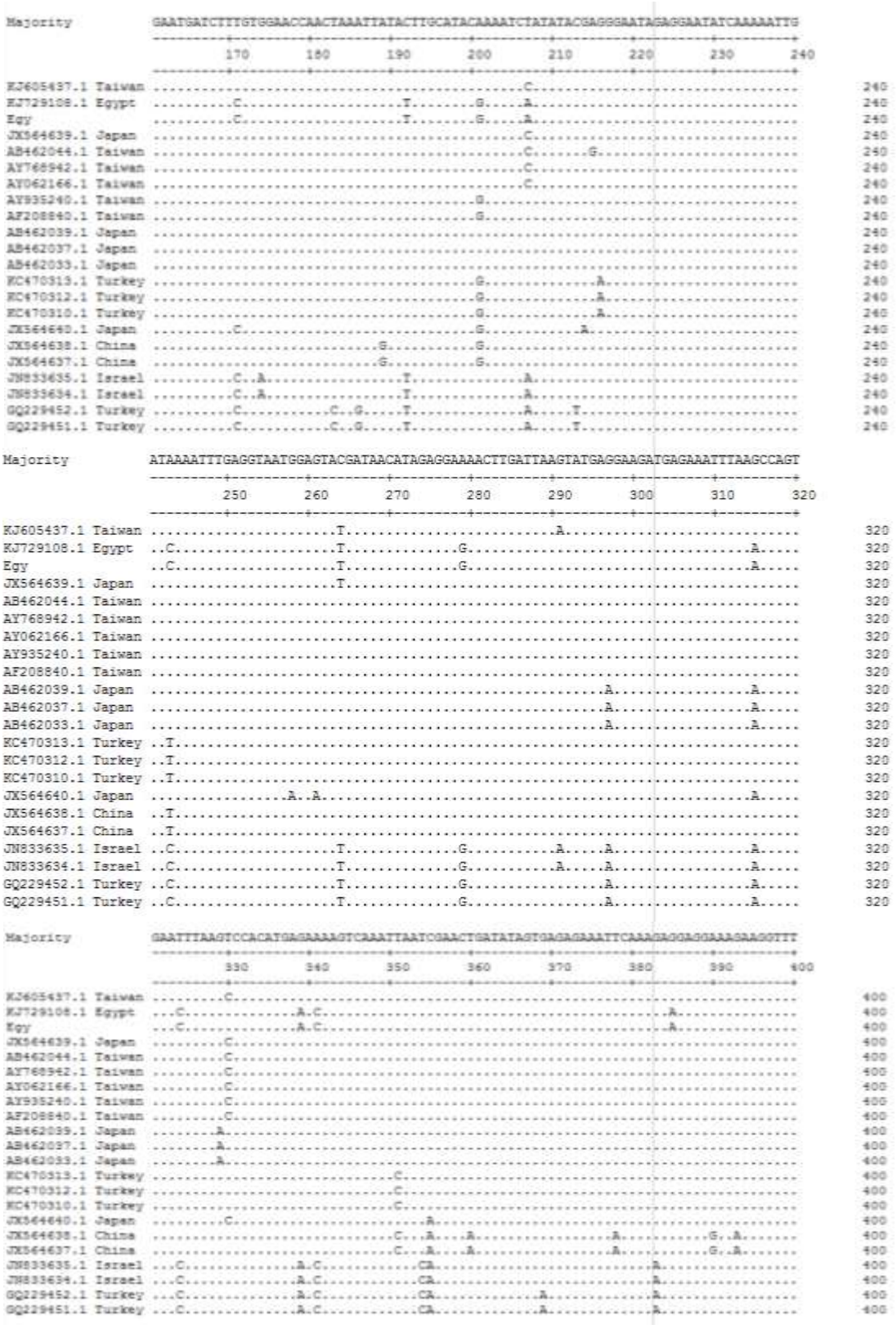
Partial Sequencing of the G gene for BEF virus from cattle at Qalyubia governorate, Egypt:

Partial sequencing of the G- gene from the purified products of RT-PCR for BEF virus produced a sequence for approximately 420 bp that was published on the Gene Bank database (**acc. No.: MH237603**).

Alignment of partial sequence of G gene for BEF virus Qalyubia, Egypt 2017 and other reference BEF viruses

Alignment and the pair-wise table showed high nucleotide similarity between BEF virus Qalyubia, Egypt 2017 and some reference BEF viruses (table 1). BEF virus Qalyubia, Egypt 2017 (MH237603.1) showed percent homology of 100% with BEF virus, Egypt 2014 (KJ729108.1,); 96.6% with BEF virus, Israel (JN833635.1 and JN833634.1); 95.4% with BEF viruses, Taiwan (AY935240.1, and AF208840.1) and Japan (AB462039.1, AB462037.1 and AB462033.1); 95.2 % with BEF viruses, Japan (JX564639.1 and JX564640.1); 94.9 % with BEF viruses, Turkey (GQ229452.1 and GQ229451.1); 94.7 % with BEF viruses, Taiwan (AB462044.1 and AY768942.1); 94.4 % with BEF viruses, Taiwan (AY062166.1), Turkey (KC470313.1, KC470312.1, and KC470310.1) and 93 % with BEF viruses, China (JX564638.1 and JX564637.1), (Figure 2 and Table 2).




Majority GAATGATCTTTGTGGAACCAACTAAATTATACTTGCATACAAAATCTATATACGAGGGAATAGAGGAATATCAAAAATTG
170 180 190 200 210 220 230 240
KJ605437.1 Taiwan 240
KJ729108.1 Egypt 240
Egy 240
JX564639.1 Japan 240
AB462044.1 Taiwan 240
AY768942.1 Taiwan 240
AY062166.1 Taiwan 240
AY935240.1 Taiwan 240
AF208840.1 Taiwan 240
AB462039.1 Japan 240
AB462037.1 Japan 240
AB462033.1 Japan 240
KC470313.1 Turkey 240
KC470312.1 Turkey 240
KC470310.1 Turkey 240
JX564640.1 Japan 240
JX564638.1 China 240
JX564637.1 China 240
JN833635.1 Israel 240
JN833634.1 Israel 240
GQ229452.1 Turkey 240
GQ229451.1 Turkey 240
Majority ATAAAATTTGAGGTAATGGAGTACGATAACATAGAGGAAAACTTGATTAAGTATGAGGAAGATGAGAAATTTAAGCCAGT
250 260 270 280 290 300 310 320
KJ605437.1 Taiwan 320
KJ729108.1 Egypt 320
Egy 320
JX564639.1 Japan 320
AB462044.1 Taiwan 320
AY768942.1 Taiwan 320
AY062166.1 Taiwan 320
AY935240.1 Taiwan 320
AF208840.1 Taiwan 320
AB462039.1 Japan 320
AB462037.1 Japan 320
AB462033.1 Japan 320
KC470313.1 Turkey 320
KC470312.1 Turkey 320
KC470310.1 Turkey 320
JX564640.1 Japan 320
JX564638.1 China 320
JX564637.1 China 320
JN833635.1 Israel 320
JN833634.1 Israel 320
GQ229452.1 Turkey 320
GQ229451.1 Turkey 320
Majority GAATTTAAGTCCACATGAGAAAAGTCAAATTAATCGAACTGATATAGTGAGAGAAATTCAAAGAGGAGGAAAGAAGGTTT
330 340 350 360 370 380 390 400
KJ605437.1 Taiwan 400
KJ729108.1 Egypt 400
Egy 400
JX564639.1 Japan 400
AB462044.1 Taiwan 400
AY768942.1 Taiwan 400
AY062166.1 Taiwan 400
AY935240.1 Taiwan 400
AF208840.1 Taiwan 400
AB462039.1 Japan 400
AB462037.1 Japan 400
AB462033.1 Japan 400
KC470313.1 Turkey 400
KC470312.1 Turkey 400
KC470310.1 Turkey 400
JX564640.1 Japan 400
JX564638.1 China 400
JX564637.1 China 400
JN833635.1 Israel 400
JN833634.1 Israel 400
GQ229452.1 Turkey 400
GQ229451.1 Turkey 400

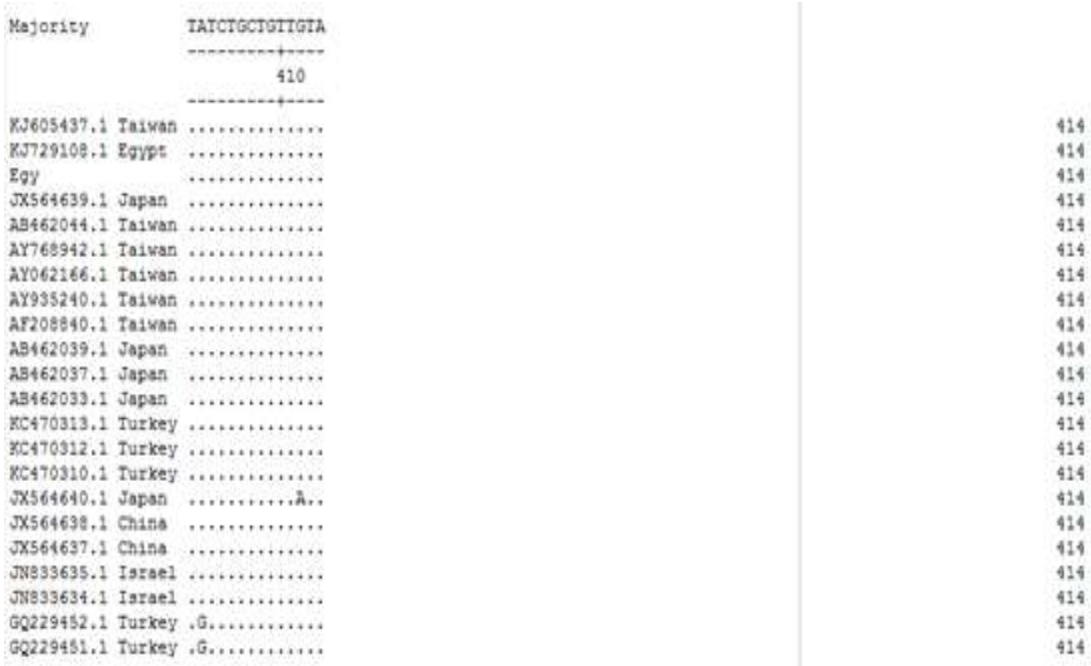


Fig. 2: Alignment of partial nucleotide sequences for G-gene of BEF viruses from Egypt and reference viruses.

Table 2: Pair wise table showing sequence distance between nucleotide sequences for G-gene of BEF viruses, Egypt and reference viruses.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1		94.4	94.4	98.8	98.3	98.3	98.1	97.8	97.8	96.9	96.9	96.9	96.6	96.6	96.6	95.9	95.2	95.2	93.0	93.0	91.8	91.8	1	KJ605437.1 Taiwan
2	5.8		100.0	95.2	94.7	94.7	94.4	95.4	95.4	95.4	95.4	95.4	94.4	94.4	94.4	95.2	93.0	93.0	96.6	96.6	94.9	94.9	2	KJ729108.1 Egypt
3	5.8	0.0		95.2	94.7	94.7	94.4	95.4	95.4	95.4	95.4	95.4	94.4	94.4	94.4	95.2	93.0	93.0	96.6	96.6	94.9	94.9	3	Egy
4	1.2	5.0	5.0		99.5	99.5	99.3	99.0	99.0	98.1	98.1	98.1	97.3	97.3	97.3	96.6	95.9	95.9	92.8	92.8	91.5	91.5	4	JX564639.1 Japan
5	1.7	5.6	5.6	0.5		99.5	99.3	99.0	99.0	98.1	98.1	98.1	97.3	97.3	97.3	96.6	95.9	95.9	92.3	92.3	91.1	91.1	5	AB462044.1 Taiwan
6	1.7	5.5	5.5	0.5	0.5		99.8	99.0	99.0	98.1	98.1	98.1	97.3	97.3	97.3	96.6	95.9	95.9	92.3	92.3	91.1	91.1	6	AY768942.1 Taiwan
7	2.0	5.8	5.8	0.7	0.7	0.2		98.8	98.8	97.8	97.8	97.8	97.1	97.1	97.1	96.4	95.7	95.7	92.0	92.0	90.8	90.8	7	AY062166.1 Taiwan
8	2.2	4.8	4.8	1.0	1.0	1.0	1.2		100.0	98.6	98.6	98.6	98.3	98.3	98.3	97.6	96.9	96.9	92.5	92.5	91.3	91.3	8	AY935240.1 Taiwan
9	2.2	4.8	4.8	1.0	1.0	1.0	1.2	0.0		98.6	98.6	98.6	98.3	98.3	98.3	97.6	96.9	96.9	92.5	92.5	91.3	91.3	9	AF208840.1 Taiwan
10	3.2	4.8	4.8	2.0	2.0	2.0	2.2	1.5	1.5		100.0	100.0	97.3	97.3	97.3	97.1	95.9	95.9	93.5	93.5	92.3	92.3	10	AB462039.1 Japan
11	3.2	4.8	4.8	2.0	2.0	2.0	2.2	1.5	1.5	0.0		100.0	97.3	97.3	97.3	97.1	95.9	95.9	93.5	93.5	92.3	92.3	11	AB462037.1 Japan
12	3.2	4.8	4.8	2.0	2.0	2.0	2.2	1.5	1.5	0.0	0.0		97.3	97.3	97.3	97.1	95.9	95.9	93.5	93.5	92.3	92.3	12	AB462033.1 Japan
13	3.5	5.8	5.8	2.7	2.7	2.7	3.0	1.7	1.7	2.7	2.7	2.7		100.0	100.0	95.9	97.6	97.6	92.0	92.0	90.8	90.8	13	KC470313.1 Turkey
14	3.5	5.8	5.8	2.7	2.7	2.7	3.0	1.7	1.7	2.7	2.7	2.7	0.0		100.0	95.9	97.6	97.6	92.0	92.0	90.8	90.8	14	KC470312.1 Turkey
15	3.5	5.8	5.8	2.7	2.7	2.7	3.0	1.7	1.7	2.7	2.7	2.7	0.0	0.0		95.9	97.6	97.6	92.0	92.0	90.8	90.8	15	KC470310.1 Turkey
16	4.3	5.0	5.0	3.5	3.5	3.5	3.7	2.5	2.5	3.0	3.0	3.0	4.3	4.3	4.3		94.9	94.9	92.8	92.8	91.5	91.5	16	JX564640.1 Japan
17	5.0	7.4	7.4	4.2	4.2	4.2	4.5	3.2	3.2	4.2	4.2	4.2	2.5	2.5	2.5	5.3		100.0	91.5	91.5	89.9	89.9	17	JX564638.1 China
18	5.0	7.4	7.4	4.2	4.2	4.2	4.5	3.2	3.2	4.2	4.2	4.2	2.5	2.5	2.5	5.3	0.0		91.5	91.5	89.9	89.9	18	JX564637.1 China
19	7.4	3.5	3.5	7.7	8.3	8.3	8.5	8.0	8.0	6.9	6.9	6.9	8.6	8.6	8.6	7.7	9.1	9.1		100.0	95.9	95.9	19	JN833635.1 Israel
20	7.4	3.5	3.5	7.7	8.3	8.3	8.5	8.0	8.0	6.9	6.9	6.9	8.6	8.6	8.6	7.7	9.1	9.1	0.0		95.9	95.9	20	JN833634.1 Israel
21	8.8	5.3	5.3	9.1	9.7	9.7	9.9	9.4	9.4	8.3	8.3	8.3	10.0	10.0	10.0	9.1	11.1	11.1	4.3	4.3		100.0	21	GQ229452.1 Turkey
22	8.8	5.3	5.3	9.1	9.7	9.7	9.9	9.4	9.4	8.3	8.3	8.3	10.0	10.0	10.0	9.1	11.1	11.1	4.3	4.3	0.0		22	GQ229451.1 Turkey
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

Sequence alignment also revealed the occurrence of nucleotide substitution in BEF virus, Egypt 2014 and 2017 generating a single nucleotide polymorphism at 19 positions as shown in table (3).

Table 3: Position of substitution of partial nucleotide sequences for G-gene of BEF viruses published on gene bank data base.

Position of substitution	Nucleotide at majority	Nucleotide substituted
62	A	T
81	T	C
90	A	G
105	A	G
138	G	T
141	G	A
154	C	T
171	T	C
192	C	T
201	A	G
207	T	A
243	A	C
264	C	T
279	A	G
315	G	A
324	T	C
339	G	A
341	A	C
385	G	A

Alignment of the deduced amino acid sequence for the G gene of BEF virus Qalyubia, Egypt, 2017 with other reference BEF viruses

Sequence alignment of deduced amino acids of G-gene of BEF virus, Egypt (MH237603.1 and KJ729108.1,) with other reference BEF virus revealed the occurrence of amino acid substitution generating a single amino acid at 3 positions (31 where L instead of Q, 114 where T instead of K and 129 where R instead of G (Figure 3).

Majority	RAWCEYRPFIDKNEDGYIDIQELNGHNMSRNHAILETAPAGGSSGTKLNVTLNGMIFVEPTKLYLHTKSIYEGIEEYQKL	
	10 20 30 40 50 60 70 80	
KJ605437.1 Taiwan	.....	238
KJ729108.1 Egypt	.....L.....	238
Egy	.....L.....	238
JX564639.1 Japan	.....	238
AB462044.1 Taiwan	.....G.....	238
AY768942.1 Taiwan	.....K.....	238
AY062166.1 Taiwan	.....T.....K.....	238
AY935240.1 Taiwan	.....	238
AF208840.1 Taiwan	.....	238
AB462039.1 Japan	.....	238
AB462037.1 Japan	.....	238
AB462033.1 Japan	.....	238
KC470313.1 Turkey	.....V.....	238
KC470312.1 Turkey	.....V.....	238
KC470310.1 Turkey	.....V.....	238
JX564640.1 Japan	.....K.....	238
JX564638.1 China	.....	238
JX564637.1 China	.....	238
JN833635.1 Israel	.....L.....	238
JN833634.1 Israel	.....L.....	238
GQ229452.1 Turkey	.....L.....	238
GQ229451.1 Turkey	.....L.....	238

Majority	IKFEVMEYDNIEENLIKYEEDEKFKPVNLSPHEKSQINRTDIVREIQRGGKKVLSAVV 90 100 110 120 130	
KJ605437.1 Taiwan	.....	412
KJ729108.1 Egypt	.....T.....R.....	412
Egy	.....T.....R.....	412
JX564639.1 Japan	.....	412
AB462044.1 Taiwan	.....	412
AY768942.1 Taiwan	.....	412
AY062166.1 Taiwan	.....	412
AY935240.1 Taiwan	.....	412
AF208840.1 Taiwan	.....	412
AB462039.1 Japan	.....N.....	412
AB462037.1 Japan	.....N.....	412
AB462033.1 Japan	.....N.....	412
KC470313.1 Turkey	.....	412
KC470312.1 Turkey	.....	412
KC470310.1 Turkey	.....	412
JX564640.1 Japan	.....I.....I	412
JX564638.1 China	.....	412
JX564637.1 China	.....	412
JN833635.1 Israel	.....T.....K.....	412
JN833634.1 Israel	.....T.....K.....	412
GQ229452.1 Turkey	.....T.....K.....	412
GQ229451.1 Turkey	.....T.....K.....	412

Fig. 3: Alignment of amino acids deduced from the partial nucleotide sequences for G-gene of BEF viruses published on gene bank data base.

Phylogenetic analysis of the BEF virus, Qalyubia, Egypt, 2017, and other reference BEF viruses

Phylogenetic analysis showed that BEF viruses from Egypt were clustered with strong bootstrap values at relevant nodes on the phylogenetic tree (Figure 4). The very short length of the horizontal line between the two BEF viruses from Egypt revealed that they were identical. Also, it showed that BEF viruses, Egypt 2014 and 2017 were of close homology to BEF virus circulated in Israel during the same period suggesting that the virus was circulated in Middle East.

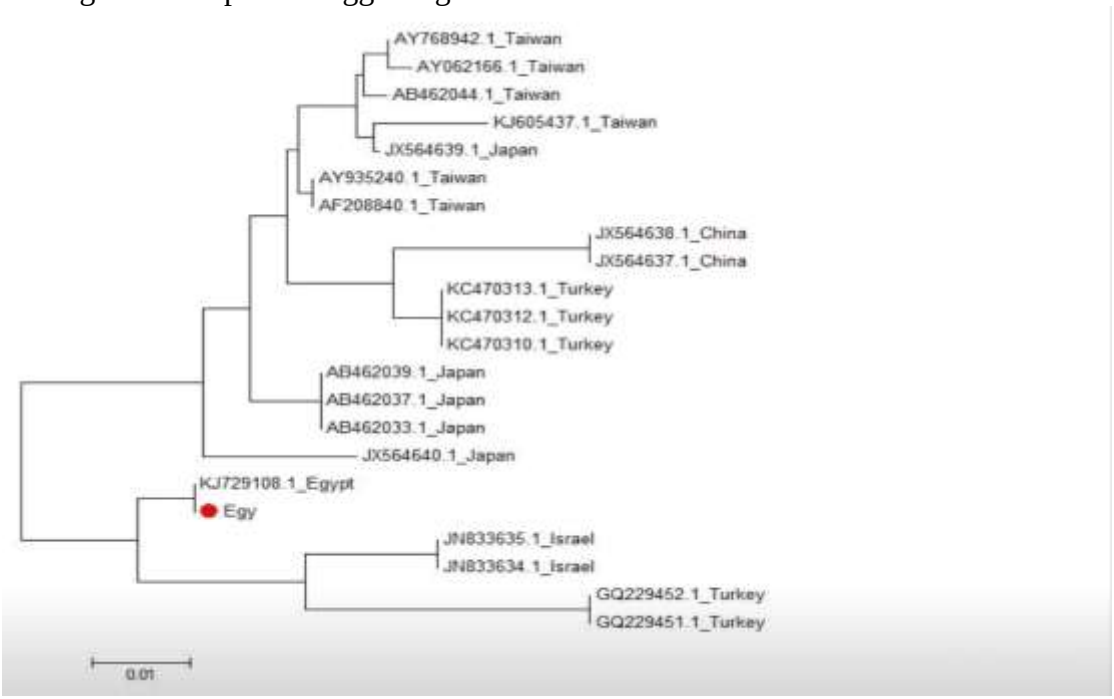


Fig. 4: Phylogenetic tree based on partial nucleotide sequences for G-gene of BEF viruses published on gene bank data base.

DISCUSSION

In August 2017, several cows from different localities in Qaliubiya governorate, Egypt came to the veterinary teaching hospital, faculty of veterinary medicine, Benha university. They showed fever, anorexia and locomotory disturbance and the cases were put under suspesion as BEF, this result agreed with Niwa *et al.,* (2015) who concluded that BEF in cattle and water buffalo is characterized by fever, anorexia, muscle stiffness, ocular and nasal discharge, salivation, depression and lameness.

Rapid detection of BEF virus in buffy coat samples from suspected cattle was tried using RT-PCR. Positive result was obtained with amplification of a specific products 420 bp from the extracted nucleic acid (Figure-1). This result agreed with Finlayson *et al.,* (2014) and Niwa *et al.,* (2015) who concluded that RT-PCR was a sensitive, specific and rapid method for detection of BEF virus nucleic acid in clinical samples especially from bovine peripheral blood mononuclear cells. Also, this result come in agreement with Kasem *et al.,* (2014) who reported BEF outbreak struck cattle in Delta Governorates (Kafrelsheikh, Al Gharbia, Al Dakahlyia, Al Behaira and Damietta) also with El-Bagoury *et al.,* (2014) who reported BEF outbreak among cattle in Qaliubiya governorate, Egypt.

Partial sequencing of the G- gene from the purified products of RT-PCR produced a sequence for approximately 420 bp then published on the GeneBank database (Acc. No. MH237603) that was analyzed and the pair-wise table showed 100% homology with BEF virus Egypt 2014 and 96.6% homology with BEF virus Israel 2010 (Figure 2 and Table 2).

Nucleotide sequencing revealed the occurrence of nucleotide substitution generating a single nucleotide polymorphism at 19 positions. Also, sequence alignment of the deduced amino acids showed substitution at a single amino acid at 3 positions, 31 (where L instead of Q), 114 (T instead of K) and 129 (R instead of G), (Figure 3). This result agreed with that of Hirashima *et al.,* (2017) who found amino acid substitutions between the YHL isolate and the currently circulating BEF viruses in East Asia as BEF virus Japan 2015.

Phylogenetic analysis showed that BEF virus from Egypt 2017 was sorted into the same cluster as other BEF virus isolated in Egypt 2014 and was most closely related to a 2010 Israel isolate. This result agreed with those of Kato *et al.,* (2009) and Bakhshesh and Abdollahi (2015) who mentioned that G protein coding gene is commonly used for phylogenetic analysis of BEF virus because it contains type-specific and neutralizing antigenic sites. Phylogenetic analysis of the G gene grouped BEF virus into three main clusters (Far-East Asia, Middle East and Australia lineages).

Our study demonstrated the recent picture of BEF virus that can circulate within the cattle populations at different localities of Egypt through unrestricted animal movements

Monitoring the emergence and evolution of BEF virus in Egypt through studying nucleotide substitution translated in the protein sequence, these substitutions could associate with change in the virus infectivity and antigenicity. Such epidemiological data could guide the application of efficient control strategies of BEF virus and important to enable appropriate vaccine selection for control measurement as rapidly as possible.

AUTHOR DETAILS

[1]Department of Virology, Faculty of Veterinary Medicine, Benha University, Qualyubia, Egypt.
[2]Department of Infectious Diseases, Faculty of Veterinary Medicine, Benha University, Qualyubia, Egypt.

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