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Phylogenetic Relationships of the Partial *G* Gene Sequence of Bovine Ephemeral Fever Virus Isolated from Mainland China, Taiwan, Japan, Australia, Turkey and Israel

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Abstract: Bovine Ephemeral Fever Virus (BEFV) can cause an acute febrile disease in cattle and water buffalo. Since, the first BEFV JB76H strain was isolated in 1976, frequent epidemics of Bovine Ephemeral Fever (BEF) have been observed in Mainland China for over three decades. The phylogenetic relationships of the Glycoprotein (*G*) gene of BEFV derived from Mainland China and other areas and countries have not been analyzed. Therefore, the partial fragments (420 bp) of *BEFV G* gene were amplified and sequenced from JB76H and three field strains obtained from Mainland China. The amplified nucleotide and deduced amino acids sequences were compared with those of other forty seven isolates from Taiwan, Japan, Australia, Turkey and Israel. The homologies of the nucleotide sequences were between 88.1 and 100% and those for the amino acid sequences were between 90.7 and 100%. Based on the corresponding nucleotide sequences, a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5 Software in order to analyze the genetic relationships of fifty one isolates of BEFV. The phylogenetic tree showed that the isolates were grouped into three distinct lineages on the basis of their source.

Key words: Bovine ephemeral fever virus, glycoprotein gene, homology, phylogenetic relationships, Mainland China

INTRODUCTION

Bovine Ephemeral Fever (BEF) caused by Bovine Ephemeral Fever Virus (BEFV) is an acute epidemic infection of cattle and water buffalo in China. It can spread rapidly and have a considerable economic impact. Most infected livestock present with a decrease in the quantity and quality of the milk, abortion and lameness or paralysis. In general, the mortality associated with BEF is low. The infected cattle usually recover 3 days later so, the disease is also named 3 day fever (Hsieh *et al*., 2005; Walker, 2005).

BEFV has a bullet or tapered shape and bears spikes on the surface of the envelope protein. It contains a negative ssRNA genome and five structural proteins comprising a Nucleoprotein (N), a surface Glycoprotein (G), a large RNA-dependent RNA polymerase (L), a polymerase associate Protein (P) and a Matrix protein (M) (Dhillon *et al*., 2000; Walker *et al*., 1991, 1992, 1994). The Protein G is the main protective antigen. There are five antigenic sites (G_1, G_2, G_{3a}, G_{3b} and G_4) on the surface of the G protein. G_1, G_2 and G_4 exist in all strains of BEFV but only a few strains have G_{3a} and G_{3b} (Cybinski *et al*., 1990, 1992; Kongsuwan *et al*., 1998). G_1 reacts only with the sera against BEFV but the other antigenic sites show cross-reactions with the sera against other correlative virus (Hong, 2001). A blocking Enzyme-Linked Immunoadsordent Assay (ELISA) and two indirect ELISAs for detection of the antibodies against the G_1 site of BEFV have been established (Zakrzewski *et al*., 1992; Zheng *et al*., 2009a, 2010).

BEF were recognized for the first time in China in 1955 (Bai *et al*., 1991). The first JB76H strain of BEFV was isolated from the infected dairy cattle during the 1976 epidemic in Mainland China (Bai *et al*., 1987). Currently, BEF occurs in many areas of central, Southern and Southeastern China (Zhao *et al*., 2008). The clinical signs, morbidity and mortality associated with the disease display obviously differences from those of BEF cases reported before 2000 and the morbidity and mortality increased significantly. Luoyang city in the Henan province of Central China is an epidemic area for BEF. The disease was prevalent there in 1983, 1985, 1991, 1997, 2004,

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2005 and 2011. The latest three BEF epizootics in 2011, 2005 and 2004 caused considerable economic loss in dairy cattle farming.

Since, the first BEFV JB76H strain was isolated in 1976, frequent epidemics of BEF have been observed in mainland China for >30 years. The phylogenetic relationships of the *G* gene of BEFV derived from Mainland China and other areas and countries have not been analyzed. In this study, BEF was confirmed in Luoyang city in the Henan province of Mainland China in 2011 by molecular diagnostic methods. The partial DNA fragments of the *G* gene of three BEFV field isolates (LS11, LCY11 and ZT02L) and JB76H vaccine strain were amplified by Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Phylogenetic analysis was performed on the amplified gene fragments of BEFV isolated from Mainland China, Taiwan, Japan, Australia, Turkey and Israel. For the first time, the phylogenetic relationships of the partial *G* gene sequence of BEFV isolated in mainland China and in other countries and areas were analyzed.

MATERIALS AND METHODS

Virus isolation and identification: The BEFV JB76H vaccine strain was isolated in 1976. The ZT02L strain was obtained from an outbreak that occurred in 2002 in Zhejiang province of Mainland China.

In August 2011, an outbreak of BEF occurred in the dairy cattle farming area of Luoyang city in Henan province, Mainland China. The infected dairy cattle showed sudden onset of fever, stiffness and nasal and ocular discharges. Difficulty in breathing and shortness of breath were the most obvious clinical symptoms shown by the infected dairy cattle. Some serious cases died between 6 and 12 h after infection. The morbidity was about 30% and the mortality rate in the infected dairy cattle was 5%. The blood was collected from the infected dairy and mixed with Alsever's solution. BEFV isolation from the blood of infected cattle was carried out in the brains of sucking mice and BHK21 cells as described previously (Zheng *et al*., 2009b).

The presence of BEFV was confirmed with RT-PCR as reported earlier (Zheng *et al*., 2007). BEFV RNAs were extracted from the infected blood and BHK21 cells using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). For RT-PCR, the primers were 420F (AGA GCT TGG TGT GAA TAC) and 420R (CCA ACC TAC AAC AGC AGA TA). The forward primer 420F was used to reverse-transcribe BEFV RNA to cDNA. Subsequently, a partial fragment of *BEFV G* gene was amplified using the primers 420F and 420B. 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 (40 sec), annealing at 46°C (1 min), primer extension at 72°C (40 sec) and the final extension for 10 min at 72°C. The amplified fragments were 420 bp in size.

In addition, BEFV was detected by Reverse-Transcription Loop-mediated isothermal Amplification (RT-LAMP) (Zheng *et al*., 2011). The primers for RT-LAMP were F3 (TGA CAG TAA GAC AAA GGG ATG T), B3 (CAT CCT CGA AAA TGA TGC CAT), FIP (GGA CTT AAC TGT AAT GCA TTC CCA-GGA CAT GGA TAC CAG ATG TGA G) and BIP (ATG AGA CAG AGA GAT TGT GGG A-TCC ACA AAA CGT AGA CAG ACA T). The RT-LAMP reaction was performed in a volume of 25 μL. The amplified products were analyzed by digestion with the EcoRV enzyme (the restriction site was present between B1 and B2) in order to determine the specificity of the RT-LAMP assay. The RT-LAMP products and the products digested with EcoRV were electrophoresed in a 2% agarose gel.

Sequencing and phylogenetic analyses: The amplified partial fragments (420 bp) of *BEFV G* gene were purified with the agarose gel DNA Purification kit (TaKaRa, Dalian, China) and sequenced directly with the primer 420F. The sequences obtained were deposited in the NCBI GenBank database.

Other nucleotide sequences data from the isolates from Taiwan, Japan, Australia, Turkey and Israel were obtained from the GenBank database. The sequences were aligned using the Clustal W program (Thompson *et al*., 1994). The nucleotide and deduced amino acid sequences homologies among the isolates obtained from different areas and countries were analyzed by MegAlign program of DNAstar. The phylogenetic tree of the partial *G* gene was constructed by the Neighbor-Jointing Method (Saitou and Nei, 1987) with the Kimura Two-Parameter Model (Kimura, 1980). The reliability of the branching orders was evaluated by the bootstrap test with 1000 replicates (Felsenstein, 1985). Evolutionary analyses were conducted using MEGA 5 Software (Tamura *et al*., 2011). If the nucleotide sequences of several BEFV strains had the homologies of 100%, a representative isolate was used to construct the phylogenetic tree.

RESULTS AND DISCUSSION

Virus isolation and identification: From the outbreak of BEF in Luoyang in 2011, two BEFV strains were isolated by intracerebral inoculation of suckling mice and in BHK21 cells (Walker *et al*., 1992). The infected suckling mice showed paralysis and stiffness in their hind legs on the 2nd to 3rd day after inoculation and died during 12-24 h post-morbidity. The clinical symptoms were representative for BEFV infection. The infected BHK21 cells showed specific Cytopathic Effect (CPE). The

Table 1: Characteristics of BEFV strains used in this study

Strain	Source	Year collected	Geographical origin	Cluster	Accession No.
JB76H*	Bovine blood	1976	Beijing, Mainland China	I	JQ728557
JT02L*	Bovine blood	2002	Zhejiang, Mainland China	I	JQ728558
LS11*[a]	Bovine blood	2011	Henan, Mainland China	I	JQ728559
LYC11[a]	Bovine blood	2011	Henan, Mainland China	I	JQ728560
YHL*	Bovine blood	1966	Yamaguchi, Japan	I	AB462028
Hirado-6*[b]	Bovine plasma	1988	Nagasaki, Japan	I	AB462029
Hirado-9[b]	Bovine plasma	1988	Nagasaki, Japan	I	AB462030
Amakusa-1[b]	Bovine blood	1988	Kumamoto, Japan	I	AB462031
Amakusa-2[b]	Bovine blood	1988	Kumamoto, Japan	I	AB462032
Azuma[b]	Bovine erythrocyte	1988	Kagoshima, Japan	I	AB462033
ON-BEF-88-1[b]	Bovine white blood cells	1988	Okinawa, Japan	I	AB462034
ON-BEF-88-3[b]	Bovine white blood cells	1988	Okinawa, Japan	I	AB462035
ON-BEF-88-4[b]	Bovine white blood cells	1988	Okinawa, Japan	I	AB462036
ON-BEF-89-1[b]	Bovine white blood cells	1989	Okinawa, Japan	I	AB462037
ON-BEF-89-2[b]	Bovine white blood cells	1989	Okinawa, Japan	I	AB462038
ON-BEF-89-3[b]	Bovine white blood cells	1989	Okinawa, Japan	I	AB462039
Onna3[b]	Bovine erythrocyte	1989	Okinawa, Japan	I	AB462040
ON-BEF-01-1*	Bovine white blood cells	2001	Okinawa, Japan	I	AB462041
ON-BEF-01-2*[c]	Bovine erythrocyte	2001	Okinawa, Japan	I	AB462042
ON-BEF-01-3*	Bovine erythrocyte	2001	Okinawa, Japan	I	AB462043
ON-04-1*	Bovine blood	2004	Okinawa, Japan	I	AB462044
CS1180*	Bovine blood	1982	Queensland, Australia	III	AF058321
CS1647*	*Culicoides brevitarsis*	1984	Queensland, Australia	III	AF058322
CS1619*			Australia	III	AF058323
CS42*[d]	*Anopheles bancrofti*	1975	Northern Territory, Australia	III	AF058324
CS1818[d]	Bovine blood	1970	Queensland, Australia	III	AF058325
BB7721*[e]	Bovine blood	1968	Queensland, Australia	III	AF234533
			Australia	III	NC002526
1984/TW/TN1[b]	Bovine blood	1984	Taiwan	I	AY935239
1996/TW/TN1*	Bovine blood	1996	Taiwan	I	AY935240
TN88128*	Bovine blood	1999	Taiwan	I	AF208840
2001/TW/TN1*[f]	Bovine blood	2001	Taiwan	I	AY935241
2001/TW/TN2[c]	Bovine blood	2001	Taiwan	I	AY954451
2001/TW/TN3*	Bovine blood	2001	Taiwan	I	AY954452
2001/TW/TN4[f]	Bovine blood	2001	Taiwan	I	AY954453
2001/TW/TN5[f]	Bovine blood	2001	Taiwan	I	AY954454
2001/TW/TN6[f]	Bovine blood	2001	Taiwan	I	AY954455
2001/TW/TN7[c]	Bovine blood	2001	Taiwan	I	AY954456
2001/TW/TN8[f]	Bovine blood	2001	Taiwan	I	AY954457
2001/TW/TN9*	Bovine blood	2001	Taiwan	I	AY954458
2001/TW/TN10[f]	Bovine blood	2001	Taiwan	I	AY954459
2001/TW/TN11[f]	Bovine blood	2001	Taiwan	I	AY954460
TN-2004-124*	Bovine blood	2004	Taiwan	I	AY818194
2008/TR/CP62*[g]	Bovine blood	2008	Turkey	II	GQ229451
2008/TR/CP77[g]	Bovine blood	2008	Turkey	II	GQ229452
ISR00*	Bovine blood	2000	Israel	II	JN833630
ISR01*[h]	Bovine blood	2001	Israel	II	JN833631
ISR04[h]	Bovine blood	2004	Israel	II	JN833632
ISR10/1*[i]	Bovine blood	2010	Israel	II	JN833633
ISR10/2[i]	Bovine blood	2010	Israel	II	JN833634
ISR10/3[i]	Bovine blood	2010	Israel	II	JN833635

For the superscripts a-i, the same letter indicates that the isolates' nucleotide sequences used in this report had homology of 100%. The symbol * represents the BEFV isolates used in Fig. 1

infected blood was collected from dairy cattle in the Songxian and Yichuanxian areas of Luoyang city. The two BEFV strains were designated LS11 and LYC11, respectively on the basis of their source. DNA fragments of 420 bp were amplified from the LS11 and LYC11 strains by RT-PCR. It was confirmed that the gene fragments were a partial of BEFV *G* gene by sequence analysis. The same DNA fragments were amplified from the ZT02L isolate and JB76H vaccine strain. The RT-LAMP products from the LS11 and LYC11 strains showed characteristic ladder DNA bands like those of BEFV-positive control as reported earlier (Zakrzewski *et al*., 1992). In addition, the RT-LAMP products digested by EcoRV enzyme yielded three DNA bands which was consistent with the theoretical deduction (Zakrzewski *et al*., 1992). The results above provided supportive evidences that the epidemic disease in the dairy cattle was BEF.

Sequences and phylogenetic analyses: The amplified nucleotide sequences of JB76H, ZT02L, LS11 and LYC11 strains have the accession numbers JQ728557, JQ728558, JQ728559 and JQ728560, respectively in the GenBank database. The corresponding sequences of the other forty seven BEFV isolates were obtained from the GenBank database. A total of fifty one BEFV isolates were used in this study (Table 1). All nucleotide and

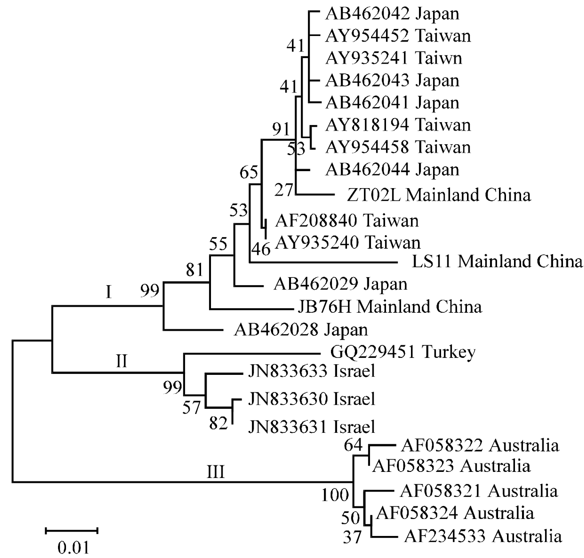

Fig. 1: Phylogenetic profiles of the BEFV isolates on the basis of comparison of the partial *G* gene sequences. The scale represents 1% sequence divergence. A total of twenty four BEFV isolates were used to construct the phylogenetic tree

speculated amino acid sequences were highly conserved among the isolates obtained from Mainland China, Taiwan, Japan, Australia, Turkey and Israel. The homology of the nucleotide sequences was between 88.1 and 100% and that of the amino acid sequences was between 90.7 and 100% for amino acid sequences.

The phylogenetic tree was constructed successfully using a Clustal W Method of multiple sequence alignment. Fifty one BEFV isolates were grouped into three distinct lineages (Fig. 1). Cluster I contained thirty six strains isolated from Mainland China, Taiwan and Japan. The Turkish and Israeli isolates were grouped into Cluster II and the Australian strains were placed in the independent Cluster III (Table 1).

The phylogenetic relationships of the *G* gene sequence of BEFV isolated in Japan, Taiwan and Australia have been analyzed earlier (Tomoko *et al.*, 2009). The genetic relationships of BEFV derived from Mainland China and those from other areas and countries have not been studied so far. In this report, the partial DNA fragments of the *G* gene of four BEFV strains (JB76H, ZT02L, LS11 and LCY11) isolated from Mainland China were amplified and sequenced. JB76H is the oldest Chinese Mainland vaccine strain and ZT02L was isolated in Southern China from an outbreak that occurred in 2002 (Walker *et al.*, 1992). LS11 and LCY11 were isolated from Central China during the 2011 outbreak. To clarify the variation in the *BEFV* genes with time and location, the specific gene fragments of the four isolates were amplified.

The amplified 420 bp DNA fragments included the G_1 antigenic site of BEFV *G* gene. The linear epitope G_1 is specific in BEFV and reacts only with anti-BEFV neutralizing antibody (Kongsuwan *et al.*, 1998; Saitou and Nei, 1987). The protein encoded by the gene fragments is 140 amino acids long and maps to amino acids 390-529 of BEFV G protein. The amino acid sequences corresponding to the epitope G_1 were mapped to amino acids 487-503 of the G protein (Kimura, 1980). The deduced 140 amino acid sequences derived from the fifty one BEFV strains were analyzed using Clustal W Method in the MegAlign program of DNAstar Software. Variations in the amino acids sequences of the epitope G_1 were discovered on the basis of this analysis. The amino acid located at position 499 of the G protein is N among the Australian strains in cluster III and in some Japanese isolates (Accession No. AB462029-AB462040) and a Taiwanese strain (Accession No. AY935239) in Cluster I. Contrary to it, the amino acid at this position is S in the other isolates. T takes the place of K for the amino acid located at position 503 of the G protein among the BEFV isolates in Cluster II. It is necessary to study further the effect of these variants on the antigenicity of BEFV.

The phylogenetic relationships among the partial DNA fragments of the *G* gene of BEFV isolated from mainland China, Taiwan, Japan, Australia, Turkey and Israel were analyzed. Thirty six strains of BEFV obtained from Mainland China, Taiwan and Japan were related closely. Two Turkish strains and six Israeli isolates were grouped into one cluster which had a close relationship with the isolates derived from oriental areas. Seven Australian isolates had a distant relationship with Asian strains of BEFV. The results revealed that the phylogenetic relationships among the BEFV isolates were closely interrelated with geographical location. Close genetic relationships among BEFV strains can be deduced if the isolates originate from adjacent areas. Similarly, the BEFV isolates derived from widely separated regions have distant genetic relationships.

CONCLUSION

The partial fragments of *BEFV G* gene were amplified from four isolates that originated from Mainland China. The DNA fragments included the specific epitope G_1 of BEFV. Two amino acids variants were found among the epitope G_1 sequences from the fifty one isolates of BEFV obtained from mainland China, Taiwan, Japan, Australia, Turkey and Israel. The phylogenetic tree based on the

DNA fragments revealed that the BEFV isolates were grouped into three clusters. The phylogenetic relationships of the fifty one BEFV isolates were closely related to their geographical origin.

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