



EXPRESSION OF THE G1 EPITOPE OF BOVINE EPHEMERAL FEVER VIRUS G GLYCOPROTEIN IN EUKARYOTIC CELLS

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Summary

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The envelope glycoprotein (protein G) of bovine ephemeral fever virus (BEFV) has been identified as a plausible vaccine candidate against the BEF disease. In the present study, G1 epitope of the G glycoprotein gene was cloned in an eukaryotic expression vector, pcDNA3.1(+), under the control of the human cytomegalovirus (CMV) promoter. The pcDNA3.1-G1 construct was transfected into human embryonic kidney 293 (HEK 293) cell line and the expression efficiency was verified by immunofluorescence staining of transfected cells and Western blot analysis. The results indicated that G1 protein was expressed by the recombinant pcDNA3.1-G1 construct in the transfected cells. The recombinant plasmid constructed in this study can be used as a DNA vaccine to evaluate its potential for immunogenicity and protection against BEF virus in animal models.

Key words: bovine ephemeral fever virus, DNA vaccine, eukaryotic expression, HEK 293 cell line, recombinant G1 protein

INTRODUCTION

Bovine ephemeral fever (BEF) is an insect-transmitted, non-contagious and viral disease of cattle and water buffalo. It occurs in many tropical and subtropical regions around the world from the southern tip of Africa to the Nile River Delta,

across the Middle East through South and southeastern Asia, into northern and eastern Australia, and throughout most of China, extending into Taiwan, the Korean Peninsula and southern Japan (Walker & Klement, 2015). Signs, which occur sud-

denly and vary in severity, can include a sudden onset of fever (41–42 °C), stiffness, lameness, depression, cessation of rumination and constipation (Walker, 2005; Zheng *et al.*, 2009). The BEF virus (BEFV) is an important economically pathogen, classified as a member of the genus *Ephemerovirus* in the family Rhabdoviridae. BEFV single-stranded, negative sense RNA genome 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 glycoprotein (G) spanning the viral envelope and a nonstructural glycoprotein (GNS). The G protein is a class I transmembrane surface glycoprotein containing five distinct neutralising antigenic sites (G1, G2, G3a, G3b, and G4) on its surface (Cybinski *et al.*, 1992; Kongsuwan *et al.*, 1998; Dhillon *et al.*, 2000). Epitope-G1 is a linear site (Y⁴⁸⁷–K⁵⁰³) in the C-terminal region of the ectodomain (Trinidad *et al.*, 2014) that reacts only with the sera against BEFV; but other antigenic sites have cross-reactions with the sera against the related viruses besides BEFV (Yin & Liu, 1997). The G1 site is predicted to face the viral membrane in both the pre-fusion and post-fusion forms of the trimer and may be accessible to antibodies only during a transitional monomeric phase (Trinidad *et al.*, 2014).

BEF can be spread rapidly and leads to considerable economic losses in the cattle industry (Walker & Klement, 2015). Prevention and control of BEF infection can be made through vaccination and treatment of affected cattle (Wallace & Viljoen, 2005; Aziz-Boaron *et al.*, 2013). Several researches have been directed towards the development of an efficient vaccine for BEF including live attenuated, inactivated, subunit G protein- based,

and recombinant vaccines (Walker & Klement, 2015). The BEFV G glycoprotein is the target of virus neutralising antibodies (Cybinski *et al.*, 1992; Uren *et al.*, 1994). In addition, the highly protective characteristics of the native BEFV G protein suggest that its recombinant expressed product may be a useful vaccine antigen (Johal *et al.*, 2008). Accordingly, recombinant or DNA vaccine based on G protein may be an appropriate alternative for current vaccines (Uren *et al.*, 1994). Because of a number of distinct advantages such as safety, stability, lower cost than some traditional vaccine types and long-term persistence of immunogenicity (Gurunathan *et al.*, 2000), DNA vaccination can be an attractive approach to BEF vaccine development. In addition, DNA-based immunisation induces protective humoral and significant cellular immune responses to the expressed antigens (Khan, 2013).

Although various experimental and commercial vaccines have been developed for BEF, it appears that protective immunity for most of them is for a limited period. Therefore, the influence of these vaccines in the field may be poor unless further booster doses are administered at the regular intervals. More researches based on advanced technologies are needed to reduce the required number of doses and extend the duration of protection (Walker & Klement, 2015). Hence, the aim of the present study was to construct a eukaryotic plasmid, expressing G1 epitope of BEFV G glycoprotein gene, for application as a possible DNA vaccine for immunisation of animal models and evaluation of its efficacy in future studies. This is the first study to design a eukaryotic expression construct for G1 epitope of BEFV G glycoprotein gene in order to use as a DNA vaccine.

MATERIALS AND METHODS

Virus and cell lines

The strain of BEF virus used in this study was procured from Razi Vaccine and Serum Research Institute (Hesarak, Karaj, Iran). BLAST analysis based on *G* gene sequence showed that this strain had the closest identity with the YHL strain isolated in Japan's Yamaguchi prefecture in 1966. Hamster lung (HmLu-1) cells were used to propagate the BEFV using Roswell Park Memorial Institute (RPMI) medium (Bio Idea, Iran) supplemented with 5% foetal bovine serum (Gibco, UK). Human embryonic kidney 293 (HEK 293) cells were used for plasmid transfection and expression experiments. HmLu-1 and HEK 293 cell lines were received from the National Cell Bank of Iran (NCBI) affiliated with the Pasteur Institute of Iran.

Bacterial strain and plasmid

The *Escherichia coli* (*E. coli*) DH5 α (CinnaGen, Iran) was used as host during the cloning experiments and for propagation of plasmids. The eukaryotic expression vector, pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) was used for cloning of G1 epitope of G glycoprotein gene.

Design of G1 epitope primer sets

The sequence of the G glycoprotein gene with the accession number of AB462028 was obtained from the Gene Bank and used for designing G1 specific primers in order to amplify the G1 encoding sequence (nucleotides 1168 to 1588). Suitable restriction sites were also added to the 5' ends of primers to enable directional cloning of the amplicon into the pcDNA3.1(+) vector. The final sequences of G1F and G1R primers designed using Oligo Analyzer software 3.0 (Integrated DNA Technologies, Coralville, IA) were:

G1F (5'-GTGGGTACCGCCACCATGGTGAGAGCTTGGTGTGAATACA-3' – *KpnI* site underlined) and G1R (5'-CATTGGATCCTCACCAACCTACAACAGCAGATA-3' – *BamHI* site underlined).

G1 gene amplification

Briefly, total RNA was extracted from the supernatant of BEFV-infected cells using the RNX-Plus Solution (CinnaGen, Iran). Extracted RNAs were reverse-transcribed into cDNA using AccuPower[®] Rocket-Script[™] RT PreMix kit (Bioneer, South Korea). The cDNA template was amplified by polymerase chain reaction (PCR) using the appropriate primer sets (Wang *et al.*, 2001) in order to amplify the full length of *G* gene. This generated an amplified 1800 bp fragment, which was cloned into pTZ57R/T vector (Fermentas, USA) and then transferred into *E. coli* DH5 α . Insert-positive clones were isolated by screening bacterial colonies on ampicillin-X-gal-IPTG-plates. This recombinant plasmid containing the G glycoprotein gene (pTZ57R-G) was used as the template for PCR amplification of the sequence encoding G1 epitope. PCR was performed by *Pfu* DNA polymerase in a final volume of 25 μ L including 1 μ L of the pTZ57R-G plasmid, 0.4 mM of dNTPs mix, 0.2 units of *Pfu* DNA polymerase, 2.5 μ L of 10 \times *Pfu* PCR buffer (without MgSO₄), 5 mM of MgCl₂, 0.32 pmol of G1F and G1R primers and 16.2 μ L of DEPC-treated water. The PCR thermal programme was optimised under these conditions: after an initial denaturation at 95 °C for 3 min, 30 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s) followed by a 10 min final extension at 72 °C were carried out. The PCR product was purified by GF-1 AmbiClean Kit (Gel & PCR) according to

the manufacturer's instructions (Vivantis, Malaysia).

Construction of expression vector and plasmid preparation

Purified *G1* fragment was subsequently digested with *KpnI* and *BamHI* restriction enzymes (Fermentas, USA), through the sites created by the primers, electrophoresed in 1.5% agarose gel, and purified by GF-1 AmbiClean Kit (Gel & PCR). Then, the digested fragment was cloned into the corresponding sites of the eukaryotic expression vector, pcDNA3.1(+), under the control of the human cytomegalovirus (CMV) promoter. Ligation of the insert and vector was done by T4 DNA ligase (Fermentas, Lithuania). The recombinant plasmid (pcDNA3.1-G1) was amplified in *E.coli* DH5 α and purified with the Endo-free plasmid purification kit (Qiagen, Germany). Finally, the recombinant plasmid was sequenced (Takapozist, Iran) to confirm that the insert was intact and in the correct orientation.

Transfection of eukaryotic cells

HEK 293 cells were transfected by pcDNA3.1-G1 to verify protein expression. For this purpose, the cells were cultured in 96-well plate at 37 °C in a humidified incubator with an atmosphere of 5% CO₂ to give a density of approximately 90% at the time of transfection. The cells were transferred using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) with 0.3 μ g of recombinant pcDNA3.1-G1 vector, containing the *G1* gene, according to manufacturer's instructions. The pEGFP plasmid (encoding enhanced green fluorescent protein) was used for monitoring transfection efficiency of Lipofectamine 2000. Hence, it was added to the cells in equal amounts as a positive control. Expression of *G1* pro-

tein was examined by immunofluorescence staining of transfected cells and Western blot analysis, 48 h post transfection.

Production of mouse monospecific polyclonal antibody against G1 protein

Recombinant MBP-G1 protein expressed in *E. coli* Rosetta strain using pMalc2x expression vector, under the control of the lac promoter, had been previously produced in our laboratory (Beygi Nassiri *et al.*, 2016). It was emulsified in Freund's incomplete adjuvant, to enhance the immune response, and used for production of mouse monospecific polyclonal serum. Three 6-week-old female mice of Balb/c strain were immunised with the prepared antigen intraperitoneally. Immunisation was repeated three more times with two weeks interval. One week after the fourth immunisation, animals were bled and the sera were examined by an in house indirect ELISA using the recombinant MBP-G1 protein as the antigen. The serum sample with the highest optical density in ELISA was used in immunofluorescence staining and Western blot analysis.

Verification of G1 protein expression by immunofluorescence staining

To examine expression of *G1* protein, after removing of the culture supernatant, cells were fixed with methanol at room temperature for 20 min. Then, the cells were permeabilised with 0.5% Triton X-100 in PBS for 15 min and incubated with 10% normal goat serum at room temperature for 20 min to block nonspecific binding. Samples were incubated with mouse monospecific polyclonal antibody against *G1* protein (1/20 in PBS containing 1% bovine serum albumin and 1.5% normal goat serum) at 37 °C for 30 min. Sheep Anti Mouse IgG conjugated with FITC (SINA BIOTECH) (IgG polyclonal; 1/20)

was used as the secondary antibody at 37 °C for 30 min. Finally, cells were washed 3× with PBS, mounted using 50% glycerol in PBS, and viewed under an Olympus IX71 inverted microscope.

Verification of G1 protein expression by Western blot

After fixing the cells with methanol, cells were scraped with 2× SDS-PAGE sample buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, and 0.01% bromophenol blue), boiled for 10 min, and electrophoresed on a 12% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane by electroblotting at 60 V for 3 h and the membrane was blocked overnight in TBS (15 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing 5% skim milk (Merck, Germany) at 4 °C. After 3× washing with TBS containing 0.1% Tween-20 (TBST), membrane was incubated for 2 h with mouse monospecific polyclonal antibody against G1 protein (1:100 in TBS containing 0.2% Triton X-100, 10% normal horse serum, and 2% bovine serum albumin) at room temperature. After being washed three times, the membrane was submerged into Anti-mouse IgG (H+L)-HRP (Bio-Rad, USA) solution (1:3000 in TBS containing 0.2% Triton X-100, 5% normal horse serum, and 2% bovine serum albumin) and remained for 1 h at room temperature. The membrane was washed again and finally specific protein bands were detected using the ECL Western Blotting Detection Kit (Najm Biotech, Iran).

RESULTS

Construction of expression vector

The 420 bp fragment, *G1* coding region of G glycoprotein gene, was successfully

amplified in PCR using the designed specific primers. The amplicon was subsequently cloned into the pcDNA3.1 vector. Sequencing of recombinant plasmid with universal primers confirmed that the gene was correctly inserted in the plasmid and there was no PCR induced mutation in the gene. Therefore, the plasmid containing *G1* gene (designated pcDNA3.1-G1) was correctly constructed.

Expression of G1 protein from the recombinant plasmid

The expression of G1 protein was monitored by immunofluorescence staining and Western blot analysis. As shown by immunofluorescence staining, HEK 293 cells were successfully transfected with pcDNA3.1-G1 and the G1 protein was expressed in the host cells (Fig. 1). It seems that the G1 protein was expressed into the cytoplasm of the transfected cells.

Using Western blot analysis, the proteins of transfected HEK 293 cells were transferred onto nitrocellulose membrane. As demonstrated in Fig. 2, the transfected cells with pcDNA3.1-G1 compared to that of transfected cells with pcDNA3.1, reacted positively with the monospecific polyclonal antibody against G1 protein. In the transfected cells, an obvious band was appeared at an approximate molecular weight of 26 kDa. This result confirmed again that G1 protein was expressed by recombinant pcDNA3.1-G1 construct in transfected cells.

DISCUSSION

In the present study the G1 epitope of BEFV G glycoprotein gene was cloned and transfected into eukaryotic cells with the recombinant pcDNA3.1-G1 construct. Transfection was performed using Lipofectamine 2000, which is a cationic li-

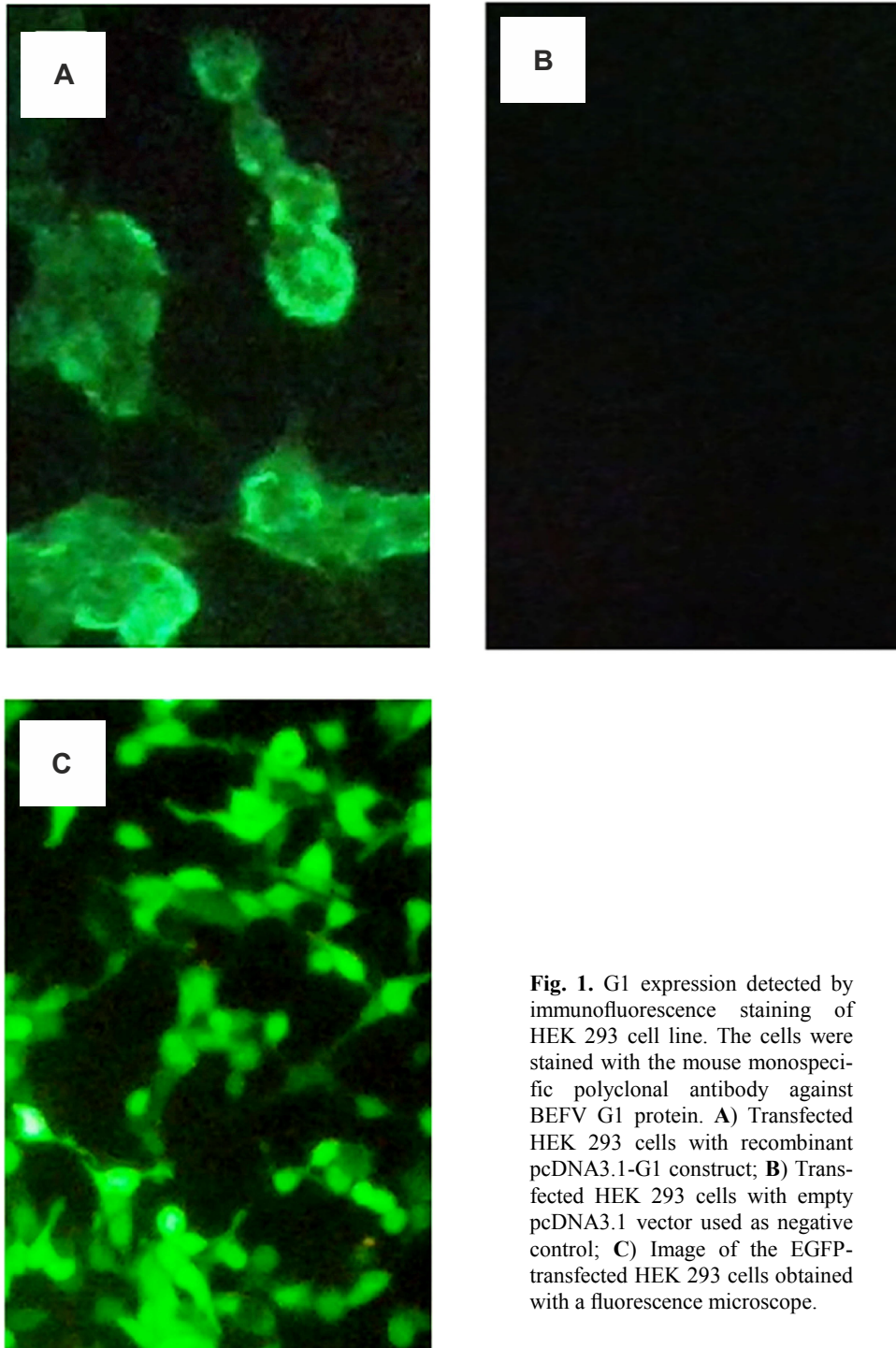


Fig. 1. G1 expression detected by immunofluorescence staining of HEK 293 cell line. The cells were stained with the mouse monospecific polyclonal antibody against BEFV G1 protein. **A)** Transfected HEK 293 cells with recombinant pcDNA3.1-G1 construct; **B)** Transfected HEK 293 cells with empty pcDNA3.1 vector used as negative control; **C)** Image of the EGFP-transfected HEK 293 cells obtained with a fluorescence microscope.

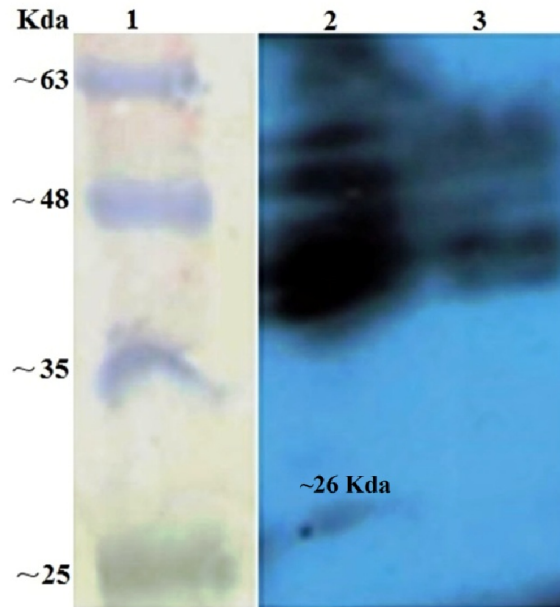


Fig. 2. Western blot analysis of G1 protein expression. Lane 1: marker polypeptides; lane 2: extract of transfected HEK 293 cells with recombinant pcDNA3.1-G1 construct; lane 3: extract of transfected HEK 293 cells with empty pcDNA3.1 vector used as negative control.

posome based reagent that provides a simple and efficient transfection for foreign gene into eukaryotic cells (Dalby *et al.*, 2004). The HEK 293 cell line was used for transfection and as the expression host. The major properties which have made the HEK 293 cells appropriate for transfection include its quick and easy reproduction and maintenance, high efficiency of transfection and protein production, and performing most of the post-translational folding and processing required to generate functional and mature protein from (Thomas & Smart, 2005).

The expression of the G1 protein was monitored using immunological methods such as immunofluorescence staining and Western blot analysis. Moderate intracellular fluorescence was seen in transfected HEK 293 cells using mouse monospecific polyclonal antibody against G1 protein.

The localisation of fluorescence seemed to be in the cytoplasm of the transfected cells. Using Western blot analysis a distinct band at an approximate molecular weight of 26 kDa was visible in the material extracted from transfected HEK 293 cells. This observed size was consistent accurately to the molecular weight of the expressed G1 protein into *Pichia pastoris* GS115 (Zheng *et al.*, 2007b). These findings indicate that the expression of the G1 epitope was successfully performed in transfected cells by the recombinant pcDNA3.1-G1 vector.

The G glycoprotein is one of the five structural proteins of BEFV that has five independent neutralising antigenic sites (G1, G2, G3a, G3b, and G4) located on its surface (Cybinski *et al.*, 1992; Trinidad *et al.*, 2014). It has been confirmed that the G1 protein is specific to BEFV and

only reacts with the anti-BEFV neutralising antibodies (Yin & Liu, 1997; Hsieh *et al.*, 2006). The expressed G1 protein in a prokaryotic system, *E. coli*, had appropriate reaction activity and specificity with an anti-BEFV serum in immunoblot analysis (Zheng *et al.*, 2007a; Beygi Nasiri *et al.*, 2016). Johal *et al.* (2008) showed that the expressed BEFV glycoprotein G by recombinant baculoviruses reacted with BEFV-neutralising monoclonal antibodies (MAbs) to all continuous and conformational antigenic sites and may be a useful vaccine antigen. Vaccinated rabbits and cattle with recombinant vaccinia viruses expressing the glycoprotein G developed high level of antibodies which neutralised BEFV in either mammalian or insect cells (Uren *et al.*, 1994; Hertig *et al.*, 1996). In addition, neutralising G protein monoclonal antibodies injected intraperitoneally conferred passive protection against intracerebral infection of suckling mice (Cybinski *et al.*, 1990). Hence, it appears that the G protein delivered in a properly folded form and with an appropriate adjuvant is adequate to induce protective immunity (Walker & Klement, 2015). Amino acids variations have been detected in the major neutralisation sites (G1, G2, and G3) of the G protein but they did not affect on the neutralisation phenotypes of epitopes targeted by the MAbs (Trinidad *et al.*, 2014). Consequently, the G1 epitope of BEFV can be as a convenient candidate to construct a DNA vaccine for immunisation of animals against the infection.

The molecular weight of expressed protein in transfected cells was approximately 26.0 kDa which was much bigger than the predicted size (~16.1 kDa). The G1 gene sequence includes three potential glycosylated sites (Jin *et al.*, 2000), so the recombinant G1 is a glycoprotein that is

certainly glycosylated in host cells to obtain the natural configuration. This finding is consistent with recombinant glycosylated G1 protein expressed in *Pichia pastoris* GS115 (Zhang *et al.*, 2007b). Several studies have confirmed that glycosylation can significantly influence the structure, function, stability, antigenicity, and immunogenicity of various viral glycoproteins. In addition, it has been demonstrated that in many infections, immune responses are mostly generated against the glycosylated portions of protein (Rudd *et al.*, 2001; Goffard *et al.*, 2005; Dowling *et al.*, 2007; Marth & Grewal, 2008; Vigerust, 2011). The expressed G1 glycoprotein by pcDNA3.1-G1 construct is structurally similar to native protein, thus it may induced considerably the immune responses in the form of DNA vaccine.

There are inconsistent reports on the role of neutralising antibodies in protection against BEF. It is probable that cell mediated immunity is also involved in protection, especially for the longer term sequelae that occur in some animals (Walker & Klement, 2015). Della-Porta & Snowdon (1979) proposed that cell mediated responses may also be required to protection against the disease. DNA vaccination can induce both humoral and cellular immune responses against infection agents (Khan, 2013). DNA vaccines have been successfully used to immunise a number of various animal species against many infectious agents (Fynan *et al.*, 1993; Robinson *et al.*, 1993; Corr *et al.*, 1996; Sakaguchi *et al.*, 1996). Considering above results and successful expression of G1 glycoprotein, our constructed recombinant pcDNA3.1-G1 vector in present study can be used as a DNA vaccine to evaluate its potential for immunogenicity and protection against BEFV in future studies.

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