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Production of Monoclonal Antibody against Prokaryotically Expressed G1 Protein of Bovine Ephemeral Fever Virus

Research Article

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ABSTRACT

Epitope-G1 of bovine ephemeral fever virus (BEFV) G glycoprotein has been genetically and antigenically conserved among various isolates of BEFV and only reacts with anti-BEFV neutralising antibodies. Therefore, it is a candidate antigen for development of the enzyme linked immunosorbent assay (ELISA) for serological identification bovine ephemeral fever (BEF)-infected animals. The aim of this study was to produce a monoclonal antibody (MAb) against recombinant G1 antigen expressed into *Escherichia coli*. For this purpose, somatic cell hybrids between SP2/0 myeloma cells and spleen cells derived from Balb/c mice immunized with maltose-binding protein (MBP)-G1 fusion protein were established. After three rounds of cloning, the stability of antibody secretion in the positive clones was confirmed by ELISA and the reactivity of the MAbs against recombinant G1 was verified by Western blot analysis. The specific MAbs produced against recombinant G1 antigen in this study could be used for establishing BEFV diagnostic experiments in the future.

KEY WORDS bovine ephemeral fever virus, hybridoma, monoclonal antibody, recombinant G1 protein.

INTRODUCTION

Bovine ephemeral fever (BEF), usually known as three-day sickness, is a viral disease of cattle and buffaloes. It is endemic in most tropical and subtropical regions of Africa, Australia, the Middle East and Asia (Aziz-Boaron *et al.* 2013). The clinical signs of the disease may be indistinctive or mild to severe including a bi-phasic fever, muscle stiffness, lameness, ocular and nasal secretion, cessation of rumination and constipation (Zheng and Qui, 2012). The mortality is low but infection can cause significant economically losses through reduced milk production in dairy herds, loss of body condition in beef cattle and the immobilization of draught animals. Bovine ephemeral fever is caused by an insect borne virus namely bovine ephemeral fever virus (BEFV), which is classified as a member of the genus *Ephemerovirus* in the family Rhabdoviridae (Aziz-Boaron *et al.* 2013). The virus has a negative single-stranded RNA genome which encodes five structural proteins including N, P, M, L, and G spanning the viral envelope and a nonstructural glycoprotein (GNS) (Uren *et al.* 1994). The G protein is the main protective antigen of the virus and the target of anti-BEFV neutralising antibodies (Walker and Klement, 2015). Five major neutralisation sites namely G1, G2, G3a, G3b, and G4 were located on the surface of G protein (Dhillon *et al.* 2000). Epitope-G1, a linear site (Y^{487}-K^{503}) in the C-terminal end of the ectodomain (Trinidad *et al.* 2014), only reacts with the sera against BEFV but other antigenic sites have cross-reactions with the antibodies against BEFV-related viruses (Yin and Liu, 1997). The

prevention and control of BEF disease can be performed through vaccination and treatment of the affected animals (Wallace and Viljoen, 2005; Aziz-Boaron *et al.* 2013). Drug treatment will be very effective if applied in the early stages of BEF diagnosis. Bovine ephemeral fever is typically diagnosed from history and clinical symptoms during outbreaks in the endemic areas (Nandi and Negi, 1999). Isolation of BEF virus from blood samples is rarely used because the viral culture is difficult in laboratory systems (Kirkland, 2002).

The detection of BEFV can be confirmed by fluorescent antibody technique, immunoperoxidase, and real-time PCR assay. However, most cases of BEF are identified by serological assays such as virus neutralization (VN) test and enzyme-linked immunosorbent assay (ELISA) (Stram *et al.* 2005). During the last decades, advances in hybridoma technology led to develop monoclonal antibodies (MAbs) against infectious disease agents. The application of MAbs in competitive ELISA, also known as blocking ELISA, provides a rapid specific diagnostic method to detect the presence of anti-organism antibody. In addition, MAbs can be used as immunodiagnostic reagents to directly demonstrate the pathogens in an antigen capture ELISA (AC-ELISA) (Siddiqui, 2010; Deb *et al.* 2013). So far, MAbs have been produced against the G, M1, M2, and N proteins of BEFV using immunized mice with the whole virus (Cybinski *et al.* 1990; Eto *et al.* 1991). The main objective of the present study was to produce MAbs, for the first time, against the recombinant G1 antigen of BEFV, produced in an efficient bacterial expression system, to design a local AC-ELISA for detecting infected animals in the future.

MATERIALS AND METHODS

Expression and purification of maltose-binding protein (MBP)-G1 fusion protein

The G1 encoding sequence derived from BEFV was previously cloned into a prokaryotic expression vector, pMalc2x, under the control of the *lac* promoter and expressed into *Escherichia coli* Rosetta Strain (Beygi Nassiri *et al.* 2016). Briefly, the optimal expression conditions for G1 gene were obtained at a reaction temperature of 37 ˚C, incubation time of 18 h, and a final concentration of 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG, CinnaGen, Iran). The pMalc2x vector encodes a maltose binding protein (MBP) at the N-terminus part of the expressed genes by this vector. The MBP in this vector has been engineered for tighter binding to amylose (Kapust and Waugh, 1999). Therefore, purification of the recombinant MBP-G1 fusion protein was done using an amylose resin column (BioLabs, UK).

For this purpose, after expression, the bacterial pellet was resuspended in column buffer (20 m*M* TrisHCl, 200 m*M* NaCl, 1 m*M* EDTA, and 700 µL of β-mercaptoethanol, Merck, Germany) and sonicated to release the bacterial proteins. After centrifuging at 10000 rpm for 20 min, the supernatant was diluted 1:6 with column buffer and passed from the amylose resin column. Then, the MBP-G1 fusion protein was detached through amylose resin using column buffer containing 10 m*M* maltose (Kapust and Waugh, 1999). Finally, the result of purification of the expressed protein was tested using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunization

Three 4 to 6-week-old female Balb/c mice (obtained from the Razi Vaccine and Serum Research Institute, Iran) were injected intraperitoneally with 100 µg of purified MBP-G1 protein in complete Freund's adjuvant (Razi Institute, Karaj, Iran). Immunization was repeated two more times with two-weeks interval in incomplete Freund's adjuvant (Razi Institute, Karaj, Iran) to enhance the immune responses. One week after the third immunization, mice were bled and the resulting sera were examined by an in-house indirect ELISA using the MBP-G1 protein as the antigen. The mouse with the highest optical density in ELISA was chosen and three days before the fusion, a booster injection of MBP-G1 without adjuvant was done. Then, the immunized animal was sacrificed and lymphoid cells (including progenitor antibody-producing cells) were isolated from its spleen (Goding, 1980).

Preparation of myeloma and mouse feeder cells

SP2/0 murine myeloma cell line (Razi Institute, Iran) has optimal growth rate in medium and high fusion efficiency; therefore it was used for production of hybridoma cells. After removal from liquid nitrogen storage, the SP2/0 cells were grown overnight in Roswell Park Memorial Institute (RPMI, Gibco, Scotland) complete medium (containing 1X penicillin-streptomycin and 15% fetal bovine serum) at 37 ˚C in a humidified incubator with an atmosphere of 8% CO_2. To ensure the sensitivity of cells to the hypoxanthine-aminopterin-thymidine (HAT, GIBCO, Grand Island, NY) for the selection process following fusion, the complete culture medium was supplemented with 8-azaguanine at 20 µg/mL during maintenance. One week prior to cell fusion, the SP2/0 cells were grown in medium without 8-azaguanine. A total of 1×10^7 SP2/0 cells in the log phase of growth and with high viability (more than 95%) at the time of collection were used for fusion (Ausubel *et al.* 1992). Mouse peritoneal cells were used as feeder cells to enhance hybridoma formation. Briefly, an adult Balb/c mouse was killed by cervical dislocation and 8 mL of 0.34 M chilled sucrose solution was injected intraperitoneally,

entering directly at the base of the sternum and placing the tip of the needle over the liver. After two or three times mild massage of the abdomen, the solution containing feeder cells was withdrawn and viable cells were counted and diluted with chilled HAT medium to 1×10^5 feeder cells/mL. This cell suspension was distributed in the wells of microtiter plates (100 µL/well) 1 day before cell fusion (Ausubel *et al.* 1992).

Fusion of myeloma and spleen cells

During the fusion process, 1×10^8 cells isolated from immunized mouse spleen were mixed with 1×10^7 myeloma cells and centrifuged at 300 g for 7 min to obtain a tight pellet. Then, one mL of 50% polyethylene glycol (PEG, Sigma, USA) dissolved in the RPMI medium was added slowly (1 mL in 1 min) into the drained pellet while stirring. After pipetting for 1-2 min, 1 mL of RPMI was added in 1 min and then 3 mL of RPMI was added in 3 min and finally, 10 mL of RPMI was gently added to the cell suspension. After incubation at 37 ˚C for 5 min, the cells were centrifuged at 100 g for 7 min. The resulting hybridomas were then resuspended in 35 mL of tissue culture medium containing HAT, and incubated for 30 min at 37 ˚C in an incubator with an atmosphere of 8% CO_2. Then, 100 µL of the fusion mixture was added to each of the 60 inner wells of the 96-well plates containing feeder cells and the peripheral 36 wells were filled with sterile phosphate buffered saline (PBS). Incubation continued for five days at 37 ˚C in a CO_2 incubator. After this time, 100 µL of HAT medium was added to each well and replaced with fresh HAT medium every other day. This step kills any unfused myeloma cells that might outgrow the other weaker hybridoma cells. Unfused B cells have limited powers of division and will die naturally in culture. Therefore, only the B cell-myeloma hybrids will survive. These hybridoma cells will produce antibodies (a property of B cells) and remain immortal (a property of myeloma cells) (Harlow and Lane, 1988).

Detection of hybridoma cells secreting anti-G1 antibody

Two weeks after the fusion process, when hybrid cells reached 10% to 50% confluence, culture supernatants were collected and tested for the presence of the desired antibody using an indirect ELISA. Ninety-six-well immune-plates were first coated with recombinant MBP-G1 diluted 1/50 in carbonate coating buffer (0.5 M $NaHO_3/Na_2CO_3$, pH 9.3) overnight at 4 ˚C (MBP-G1 ELISA). Also, recombinant MBP was coated concurrently and separately with dilution of 1/200 in coating buffer (MBP ELISA). The final concentrations of coating antigens were 0.1 µg/well by calculation. After washing three times with PBS supplemented with 0.05% Tween 20 (PBST), the plates were post coated with 300 µL of blocking buffer consisting of PBST containing 5% skim milk for 4 h at 37 ˚C. The plates were washed three times with PBST and then the hybridoma supernatants were added (50 µL/well) and incubated for 45 min at room temperature, with sera (1:2000 in PBST containing 5% skim milk, Merck, Germany) from non-immunized and immunized mice as negative and positive controls, respectively, and also a supernatant of an unrelated hybridoma as a negative control. The plates were washed three times with PBST, and then the bound antibodies were detected using an anti-mouse IgG (H+L)-HRP (Bio-Rad, USA) solution (1:3000 in PBST containing 5% skim milk) for 30 min at room temperature. After washing four times with PBST, 50 µL of chromogen substrate (tetramethylbenzidine 1%, 0.1 M sodium acetate (pH 6), and H_2O_2 3%) was added to each well and incubated for 10 min at room temperature in the dark. The reaction was stopped by adding 50 µL of chloridric acid (0.1 M) and finally, the absorbance was measured at 450 nm by an ELISA spectrophotometer (Metertech, Taiwan). Positive hybrids in MBP-G1 ELISA, which did not react with recombinant MBP in MBP ELISA, were considered as positive.

Isolation and cloning of hybridoma cells

The selected positive cells were sub-cultured *in vitro*, using limiting dilution method to ensure that each culture consists of only one single clone secreting anti-G1 antibody. Cloning by this method is based on the Poisson distribution. Briefly, a day before cloning, 24 and 96-well plates were incubated with mouse feeder cells in RPMI medium. Then, all of the cells from each well containing anti-G1 antibody were transferred into a separate well of the prepared 24-well plate and cultured overnight at 37 ˚C in a humidified CO_2 incubator. After examining cell viability, the positive hybrids were cloned by limiting dilution (8 cells/mL) in hypoxanthine thymidine (HT, Sigma, USA) medium and distributing 100 µL of the dilution in the prepared 96-well plates. Two to three single positive clones from each plate were selected using indirect ELISA and sub-cloned at least three times until a stable and single hybridoma cell line was established and all sub-cloned supernatants were positive for anti-G1 antibody production. The desired cells were then propagated for liquid N_2 storage (Ausubel *et al.* 1992).

Reactivity of MAbs to recombinant G1 antigen by Western blotting

The reactivity of MAbs to recombinant G1 protein was evaluated using Western blot analysis. For this purpose, the recombinant G1 and MBP antigens were electrophoresed on 12% SDS-polyacrylamide gel and the protein bands were then transferred to a nitrocellulose membrane (Sigma,

USA) by electro blotting at 60 V for 3 h. The blotted membrane was blocked with PBST containing 5% skim milk overnight at 4 ˚C and then rinsed three times with PBST.

The nitrocellulose membrane was cut into strips and treated with the supernatant of each positive hybridoma for 2 h at room temperature, separately. Likewise, the supernatant of the unrelated hybridoma as the negative control and also sera (1:2000) from non-immunized and immunized mice were used as negative and positive controls, respectively. After washing as previously mentioned, the strips were dipped in an anti-mouse IgG (H+L)-HRP (Bio-Rad, USA) solution (1:3000 in PBST containing 5% skim milk) for 1 h at room temperature. After rinsing three times with PBST, the strips were submerged in a substrate solution (PBS containing H_2O_2, 4-chloro-l-naphthol, and methanol, Merck, Germany) to stain antigens bound to MAbs.

RESULTS AND DISCUSSION

Expression and purification of recombinant MBP-G1 fusion protein

The expression of MBP-G1 fusion protein was checked by SDS-PAGE analysis. The results showed that the gene encoding epitope-G1 was effectively expressed using the prokaryotic expression vector, pMalc2x, under the control of the strong *lac* promoter into *E. coli* Rosetta strain (Figure 1). The pMalc2x vector adds a MBP tag at the N-terminus part of the expressed protein that facilitates its purification. Therefore, MBP-G1 fusion protein was successfully purified by a column of amylose resin based on maltose affinity chromatography. The band of the purified protein was evidenced at ~58 kDa that was consistent with the expected molecular weight of the fusion protein including ~16 KDa for G1 protein and ~42 KDa for MBP (Figure 2).

Hybridoma production and isolation

The resulting sera of immunized mice with the recombinant MBP-G1 protein were examined by an in-house indirect ELISA on day 45 and the absorbance was measured at 450 nm with the ELISA reader (Metertech, Taiwan). The highest optical density (OD) value of immunized serum (1:2000 dilution) was 1.89 compared to 0.06 which was the OD value of non-immunized serum (negative control). The immunized mouse, with the highest titer of anti-G1 antibody, was used for the fusion of spleen cells with SP2/0 myeloma cells using PEG. Culture supernatants of hybrids in HAT selection medium were screened by MBP-G1 ELISA and MBP ELISA. Results indicated that three hybridomas secreting antibodies were positive in MBP-G1 ELISA and did not react in MBP ELISA. After three times cloning, ELISA OD values of these hybridoma cells increased with no reaction towards MBP. Therefore, three hybridoma clones were established and isolated with the highest OD values (average 2.1) for MAbs reactive to recombinant G1 protein of BEFV.

Reactivity of MAbs to recombinant G1 antigen

After three rounds of cloning, the positive hybridoma clones were selected and the reactivity of the MAbs with recombinant G1 antigen was confirmed by Western blot analysis. In the Western blotting, anti-G1 MAbs specially reacted with a protein with approximate molecular weight 58 kDa, which was consistent with the molecular weight of MBP-G1 fusion protein. However, there was no reaction with the recombinant MBP that indicated specificity of the MAbs to expressed G1 protein of BEFV (Figure 3).

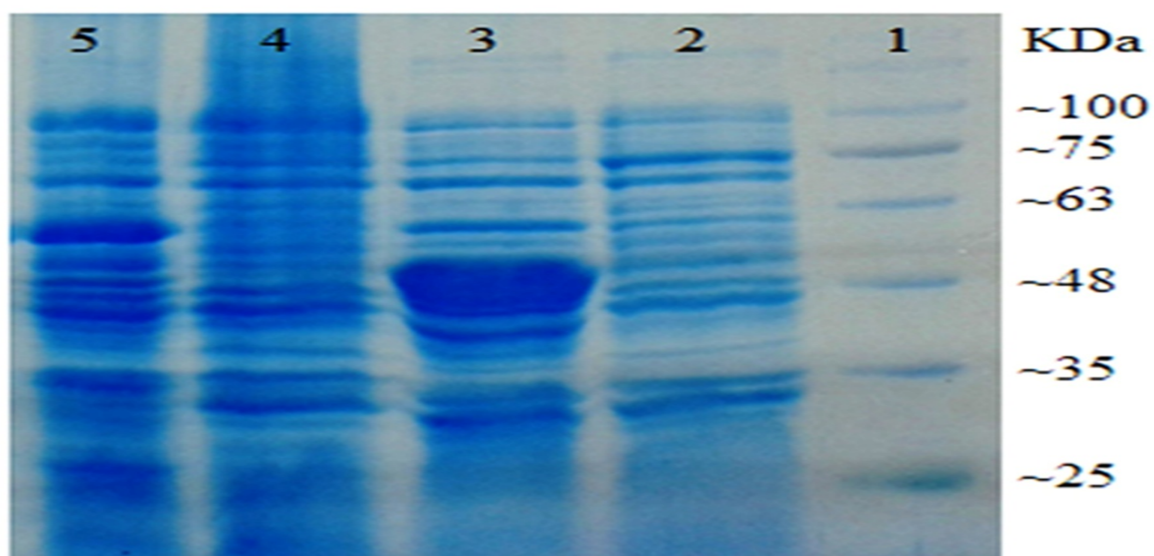

Figure 1 Induction of protein expression in *E. coli* containing pMalc2x-G1 construct
Lane 1: CinnaGen molecular weight marker; Lanes 2 and 3: *E. coli* containing pMalc2x plasmid before and after induction by IPTG, respectively and Lanes 4 and 5: Transformed colony with pMalc2x-G1 before and after induction by IPTG, respectively

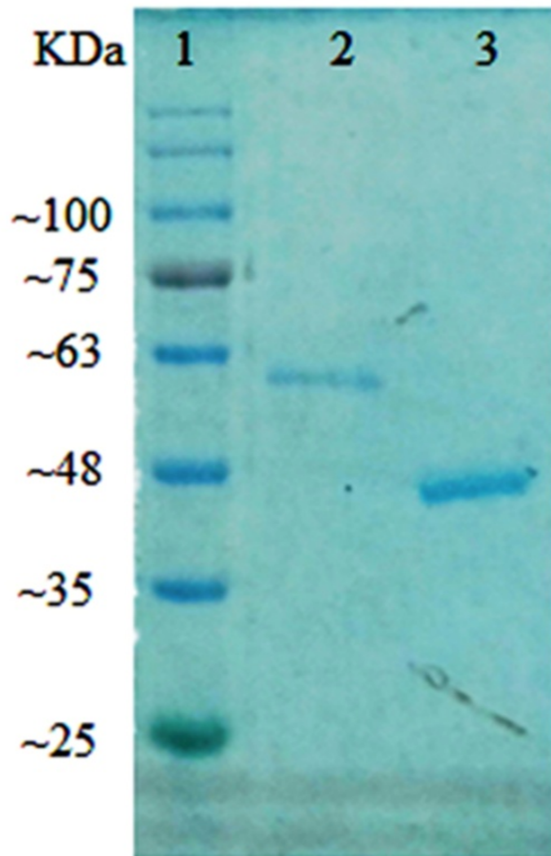

Figure 2 SDS-PAGE analysis of purified MBP-G1 fusion protein by the amylose resin column
Lane 1: CinnaGen molecular weight marker; Lane 2: purified MBP-G1 fusion protein and Lane 3: purified MBP from the control colony containing only pMalc2x plasmid

Bovine ephemeral fever virus (BEFV) can spread rapidly and lead to significant economic losses in the cattle industry (Walker and Klement, 2015). Identification and treatment of the affected animals can reduce the losses of bovine ephemeral fever (BEF). Rational treatment will be very successful if applied in the early stages of the disease. Therefore, diagnosis of the virus and BEF-infected cases in the early of an outbreak can be especially important. There are various clinical and laboratory assays for diagnosis of BEF disease. In regions where BEFV is endemic, a diagnosis usually is made based on clinical history and symptoms of disease (St George *et al.* 1984).

Virus culture from the suspicious blood is difficult, time consuming, and costly in laboratory systems, therefore it is not usually employed. Polymerase chain reaction (PCR) assay has been successfully used for the rapid detection of BEFV nucleic acid in blood samples in some Australian laboratories (Kirkland, 2002). A new reverse-transcription, loop-mediated isothermal amplification (RT-LAMP) assay displayed higher sensitivity for the detection of BEFV compared with traditional RT-PCR and virus isolation procedures (Zheng *et al.* 2011).

Stram *et al.* (2005) developed a real-time RT-PCR assay as a quick, accurate, and sensitive test for detecting infected animals and identifying vectors of BEFV. However, serological tests are the most commonly used methods to identify BEFV (Kirkland, 2002). Serological diagnosis can be performed through virus neutralization test but paired serum samples in the early stages of the disease and 3 weeks later are needed.

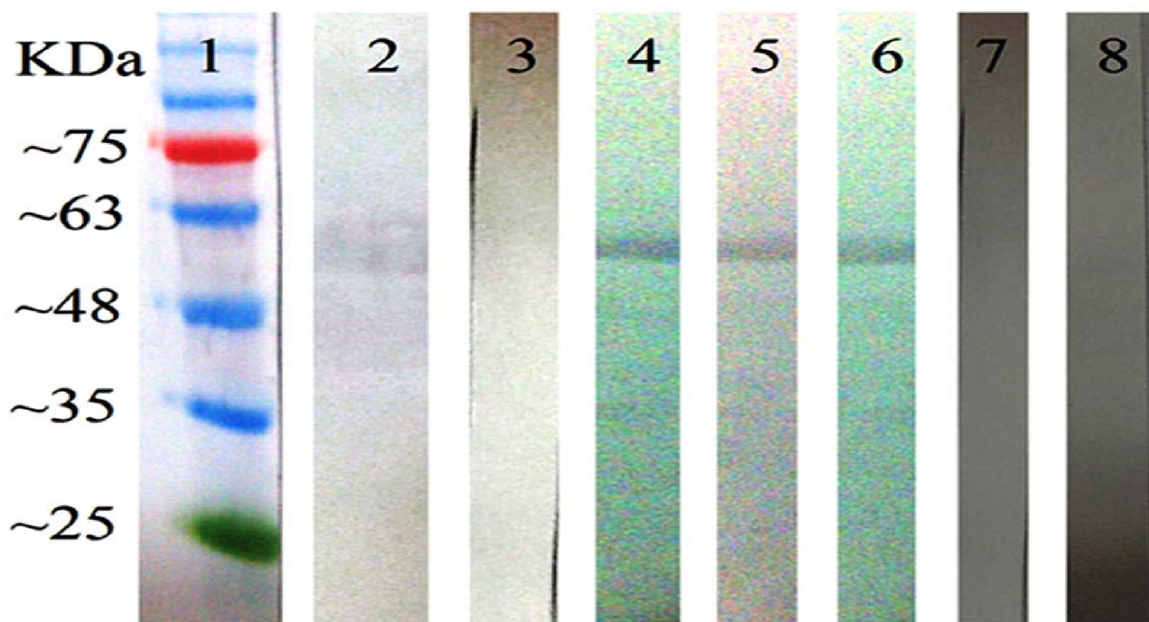

Figure 3 Reactivity of supernatants of subclones secreting anti-G1 MAbs with recombinant G1 and MBP antigens in Western blot analysis
Lane 1: CinnaGen molecular weight marker; Lanes 2 and 3: immunized and non-immunized mice sera as positive and negative controls, respectively; Lanes 4 to 6: supernatants of subclones containing anti-G1 MAbs; Lane 7: supernatant of an unrelated hybridoma as negative control and Lane 8: the blotted membrane with MBP antigen exposed to supernatant of a subclone containing anti-G1 MAb

In addition, it can be complicated due to prior infection with other ephemeroviruses and accordingly false positive results occurred (St George *et al.* 1984). Serological detection of BEFV can also be performed using various types of ELISA.

In the previous studies, indirect ELISAs based on expressed G1 protein into *E. coli* and *Pichia pastoris* were successfully developed to detect antibodies against BEFV (Zheng *et al.* 2009; Zheng *et al.* 2010). The ELISA is a specific, low-cost and rapid serological diagnosis assay that can be appropriate for screening of BEF-infected animals on a large scale (Zheng *et al.* 2010). During the last decades, the monoclonal antibodies (MAbs) have been proposed as an invaluable tool for rapid diagnosis of different livestock diseases.

The monoclonal antibodies can be used directly as immunodiagnostic reagents to detect the pathogens or indirectly for serological diagnosis of produced antibodies against them (Deb *et al.* 2013). Cybinski *et al.* (1990) produced MAbs against the G, M2 and N proteins of BEFV using two types of antigens, BEFV-infected suckling mouse brain and BEFV nucleocapsids, in mice. Eto *et al.* (1991) established two types of MAbs against the M1 protein of BEFV using immunization of mice with crude BEFV, but they could not neutralize the viral infectivity. Zakrzewski *et al.* (1992) developed a blocking ELISA for detection of antibodies produced against BEFV using a MAb, DB5, established against G1 epitope.

The G protein of BEFV is placed mainly on the virion surface and is therefore a target for antibodies (Walker and Klement, 2015). G1 epitope of G glycoprotein is specific to BEFV and only reacts with the anti-BEFV neutralising antibodies (Zakrzewski *et al.* 1992; Yin and Liu, 1997). In our previous study, it was found that G1 protein expressed by a recombinant construct induced specific neutralising antibodies against BEFV in mice (Pasandideh *et al.* 2018). The nucleotide and amino acid sequences of the G1 antigenic site have been highly conserved among all isolates, except for an amino acid substitution at position 499 for a few strains (Kato *et al.* 2009; Zheng and Qiu, 2012). However, amino acid variations detected in the main neutralisation sites (G1, G2, and G3) of the G protein did not influence the neutralisation properties of epitopes targeted by the MAbs (Trinidad *et al.* 2014). The fact that G1 epitope has been genetically and antigenically conserved among various isolates of BEFV allowed the use of G1 as a useful marker for identification of infected animals.

CONCLUSION

In conclusion, in this study for the first time, expressed G1 antigen into a prokaryotic system was employed to produce MAbs by the hybridoma technology and subsequently three MAbs reactive with this antigen were established. The specific MAbs produced against recombinant G1 antigen could be used for establishing BEFV diagnostic experiments particularly AC-ELISA in the future.

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