Veterinary Microbiology 233 (2019) 113–117

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

The immunogenicity of the secretory GΔTM protein of bovine ephemeral fever virus stably expressed by mammalian cells

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ARTICLE INFO

Keywords:
Bovine ephemeral fever virus
G protein
Mammalian stable cell
Vaccine
Diagnosis

ABSTRACT

Bovine ephemeral fever virus (BEFV) causes an acute febrile disease in cattle and water buffalo. The disease has an impact on dairy and beef production in tropical and subtropical countries. Vaccination is used for disease prevention and control. In this study, we developed a recombinant lentivirus to produce mammalian stable cells expressing histidine-tagged BEFV G protein with a deleted transmembrane domain (GΔTM) as a secretory protein. In addition, guinea pigs were immunised with the purified GΔTM protein and booster immunised at a 3-week interval. The mammalian stable cells were able to continuously produce GΔTM protein for a minimum of 25 passages. All of the mammalian stable cells expressing GΔTM protein could react specifically with a BEFV convalescent bovine serum. Serum samples from the immunised guinea pigs could react strongly and specifically with the purified GΔTM protein. Moreover, post-immunised guinea pig sera contained antibodies that could neutralise BEFV. These results indicate that the G protein without a transmembrane domain can be used as a subunit vaccine for the prevention and control of BEFV. The availability of the mammalian stable cells, which constitutively express GΔTM protein, could facilitate the potential use of the secretory protein for BEFV diagnosis and vaccine development.

1. Introduction

Bovine ephemeral fever virus (BEFV) is an arthropod-borne virus belonging to the genus *Ephemerovirus*, in the family Rhabdoviridae. BEFV contains five common structural proteins, including a nucleoprotein (N), polymerase-associated protein (P), matrix protein (M), enveloped glycoprotein (G) and RNA-dependent RNA polymerase (L) (Walker et al., 2011). The G protein is a 81 kDa class I transmembrane glycoprotein and consists of four antigenic sites: G1, G2, G3, and G4 (Cybinski et al., 1990, 1992). It can induce neutralising antibodies and protect animals from BEFV infection (Cybinski et al., 1990; Hertig et al., 1995).

BEFV causes bovine ephemeral fever (BEF) in cattle and water buffalo in the tropical and subtropical areas of Africa, Asia, and Australia. Vaccination is one of the strategies for the prevention and control of BEF. Both live attenuated and inactivated BEFV vaccines have been used in some countries for many years (Aziz-Boaron et al., 2014; Vanselow et al., 1995). The attenuated virus vaccine can induce neutralising antibodies in vaccinated cattle (Vanselow et al., 1995). However, the attenuated viruses can revert to virulent strains because of the high mutation rate of RNA viruses (Lee et al., 2012; Pastoret, 2010). The inactivated vaccine is safe and it is protective against the homologous BEFV strain. Nevertheless, the failure of the inactivated BEFV vaccine to provide full cross-protection in the field has been documented due to variation in the antigenic properties among BEFV strains (Hsieh et al., 2006; Zheng and Qiu, 2012).

BEFVs are genetically classified into three clusters (Zheng and Qiu, 2012). Cluster I contains BEFVs from East Asia, while most BEFVs in clusters II and III are from Israel and Australia, respectively. Thai BEFV is in cluster I and consists of two clades (Chaisirirat et al., 2018). Antigenic properties of different BEFV strains may vary as some of the amino acids on the neutralising epitopes are different (Kato et al., 2009;

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https://doi.org/10.1016/j.vetmic.2019.04.032
Received 21 January 2019; Received in revised form 22 April 2019; Accepted 27 April 2019

Trinidad et al., 2014). Thus, a vaccine developed using a local strain may be more effective for the control of BEF (Aziz-Boaron et al., 2014). A previous study showed that G protein purified directly from the virions and used as a subunit vaccine could protect cattle after a challenge with BEFV (Uren et al., 1994). The subunit vaccine is not infectious as it contains only purified antigenic components; thus, it is safe for use during the production process. In this study, we generated a mammalian cell line that stably expressed the codon optimised G protein without a transmembrane domain (GΔTM) and examined its immunogenicity in an animal model by immunisation with the purified GΔTM protein in guinea pigs.

2. Materials and methods

2.1. Generation of a stable cell line expressing GΔTM protein

The entire BEFV G sequence was optimised and synthesised based on mammalian cell codon usage (GenScript, USA). The codon-optimised BEFV G sequence was used as a template for amplifying the transmembrane deleted G fragment (GΔTM), using the following primers: forward primer containing *Eco*RI site (5′-ATTAGAATTCGCCACC ATGGTCCGGGTCCTGATTATTAC-3′) and reverse primer containing *BamH*I site and 8X histidine tag sequence (5′-ATATGGATCCTCAGTGA TGGTGATGGTGATGGTGATGCACCTTCTTGCCGCCTCTC-3′). The PCR product was purified and cloned into a pLVX-Puro vector (Clontech) at the *Eco*RI/*BamH*I sites to produce a pLVX_GΔTM transfer vector. Then, LentiX-293 T cells (kindly provided by Prof. Dr.Wanpen Chaicumpa from Mahidol University, Thailand) were co-transfected by the pLVX_GΔTM, along with other plasmids: pMDLg/pRRE (addgene, USA); pRSV-Rev (addgene, USA); and pMD2.G (addgene, USA); this was done using polyethylenimine (PEI; Sigma) to generate recombinant lentiviral particles. At 48 h after transfection, the culture medium supernatant was harvested and clarified by centrifugation at 2000 rpm for 10 min. The lentiviral titre was determined using a Lenti-X qRT-PCR titration kit (Clontech).

A stable cell line expressing the GΔTM protein was generated by the transduction of 293 T cells (kindly provided by Dr. Richard J. Webby from St. Jude's Children's Hospital, Memphis, Tennessee, USA) with the recombinant lentivirus at a multiplicity of infection (m.o.i.) of 5. Transduction was facilitated by 10 μg/ml polybrene (Sigma). At 48 h after transduction, the culture medium supernatant was removed and the cells were cultured with a culture medium containing 3 μg/ml puromycin (Sigma) to select the antibiotic resistant and stable cells. The polyclonal stable cells that survived the puromycin selection were pooled and subsequently isolated by a limiting dilution to obtain monoclonal stable cell lines. After selecting a single colony, each of the monoclonal colonies was expanded to a larger culture scale.

2.2. Production and purification of GΔTM protein

To produce the secreted GΔTM, the 293 T_GΔTM stably expressing cells were seeded in four T75 cm^2 cell culture flasks and incubated for 72 h. Then, 60 ml of the culture supernatant was collected and clarified by centrifugation at 10,000 rpm for 15 min using a JA-14 fixed angle rotor (Beckman Coulter). The clarified supernatant was applied to a HisTrap™ excel column (GE Healthcare), according to the manufacturer's protocol. The purified protein in each fraction was analysed using 10% SDS-PAGE and Western blot analyses (Srisombundit et al., 2013) with DAB substrate solution (Dako) for colour development. The eluted protein fractions containing GΔTM were pooled and concentrated using an Amicon® ultra centrifugal filter with a pore size of 30 kDa (Millipore). The protein concentration was determined by a Pierce 660 nm protein assay (Thermo Scientific).

2.3. Immunisation of guinea pigs

Three 8-week-old female guinea pigs were immunised intramuscularly in their rear legs with 100 μg of GΔTM protein mixed with an equal volume of Montanide ISA 206 adjuvant (Seppic, France). The other two guinea pigs were injected with PBS mixed with the adjuvant and served as a control group. All animals received a booster immunisation at a 3-week interval. Serum samples were collected on days 0, 14, 21 and 35 after the initial immunisation. The reactivity between the guinea pig serum samples and the GΔTM protein was examined by Western blot analysis (Srisombundit et al., 2013). A blue colour signal formed by the reaction between the horseradish peroxidase enzyme and TMB substrate solution (Sera Care) was visualised. The animal inoculation protocol was approved by the Animal Care and Use for Scientific Research Committee at Kasetsart University (ACKU61-VET-084).

2.4. Serum neutralisation test

The guinea pig serum samples were 2-fold serially diluted in each well of 96-well plates containing 50 μl of minimum essential medium (MEM) per well. The diluted sera were incubated with 50 μl of MEM containing 1000 $TCID_{50}$ of BEFV at 37 °C, with 5% CO_2 for 1 h. Subsequently, 1×10^5 Vero cells were seeded into each well and further incubated at 37 °C for 48 h. Then, an immunoperoxidase monolayer assay (IPMA) was performed to detect the infected cells, as described previously (Kanpipit et al., 2018), using anti-BEFV bovine serum and horseradish peroxidase-conjugated anti-bovine IgG as the primary and secondary antibodies, respectively. The serum neutralising titre (SNT) was determined by the reciprocal number of the highest dilution of serum that could inhibit virus infectivity.

2.5. Statistical analysis

Data were log-transformed and analysed using commercial software (SAS University edition, SAS Institute Inc., Cary, NC). A repeated measure analysis of variance was used. The analysis for each time point was performed using the generalised linear model method. A P-value < 0.05 was considered significant.

3. Results and discussion

The recombinant lentivirus was successfully generated by the transfection of pLVX_GΔTM and other helper plasmids into LentiX-293 T cells. The recombinant GΔTM-lentivirus had a titre of 1×10^9 copy numbers, as determined by quantitative real-time RT-PCR. In order to produce stable cells expressing GΔTM, 293 T cells were transduced with the lentivirus followed by puromycin selection. Four days after the selection with 3 μg/ml puromycin, previously determined by antibiotic titration (data not shown), only 20% of viable cells with normal morphology remained in each well; these were the transduced cells with an antibiotic-resistant property, whereas all non-transduced cells died. On day 6 after the puromycin selection, viable colonies of antibiotic-resistant cells in the culture well became confluent, which were pooled for a larger culture scale. Then, a single cell cloning was achieved by the limiting dilution technique. The numbers of monoclonal cells were successfully isolated from the polyclonal resistant cell pool. On day 8 after cell isolation, a colony derived from a single cell could be observed in some culture wells of the 96-well plate, while the wells with more than a single clone were discarded. The single cell clone in each well proliferated with a different growth rate. Each colony of the monoclonal cells was the transduced clone that was ready for further sub-culture. At this point, we obtained a total of 17 transduced clones.

To examine whether the expressed GΔTM protein was secreted from each clone, the protein from 10 μl of the culture supernatant was

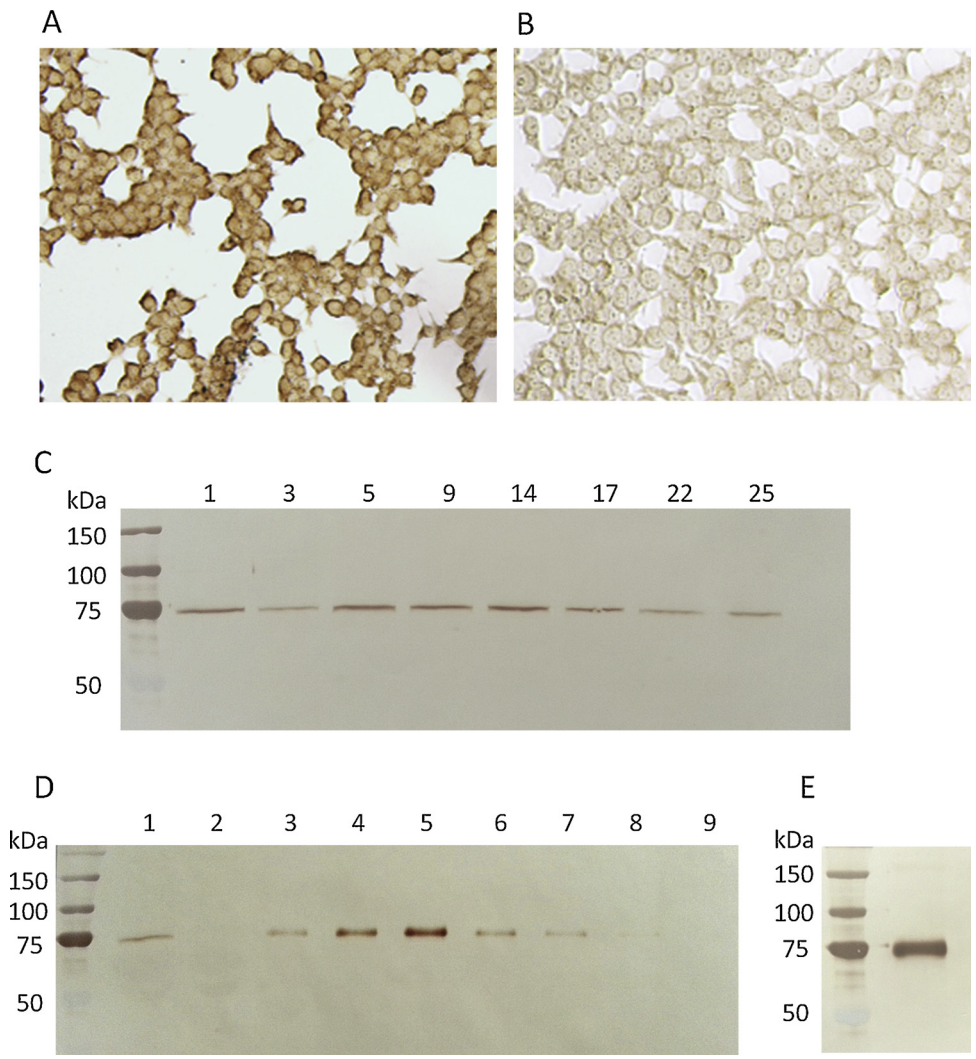

Fig. 1. The expression of GΔTM protein was examined using immunoperoxidase monolayer assay (IPMA). A) The GΔTM protein in 293 T_GΔTM cells reacted specifically with a BEF convalescent bovine serum, B) whereas no such specific reactivity was detected in the parental 293 T cells. C) The immunoblot shows the secreted GΔTM protein expressed from 293 T_GΔTM cells up to 25 culture passages. The 75-kDa band of GΔTM protein reacted specifically with anti-histidine tag monoclonal antibody. The numbers above each well indicate the passage numbers of the 293 T cells stably expressing BEFV GΔTM (293 T_GΔTM). D) The immunoblot shows fractions of purified GΔTM protein obtained from the purification process. Anti-histidine tag monoclonal antibody was used as a primary antibody. Lane 1: clarified culture medium supernatant. Lane 2: flow through fraction of the clarified culture medium after passing through the column. Lanes 3–9: the eluted protein fractions. E) The pool of eluted GΔTM protein fractions after concentration reacted specifically with the anti-histidine tag monoclonal antibody.

analysed directly using SDS-PAGE and a Western blot. The GΔTM protein of approximately 75 kDa was detected from all 17 clones of the stably expressed cells by a monoclonal antibody specific to the histidine tag with different intensity (data not shown). In addition, by following the cell expansion procedure, the stable cells were seeded into each well of a 96-well plate and incubated for 24 h to allow for protein expression. The reactivity between GΔTM in the 293 T GΔTM cells and a convalescent bovine serum was determined by IPMA (Fig. 1A, B). The result showed that the GΔTM stably expressing cells strongly reacted with the anti-BEFV convalescent bovine serum, indicating its immunogenic properties. The GΔTM protein expressed in the 293 T_GΔTM cells was present in both the cytoplasm and cell membrane. It could be a cell-associated protein, which accumulated in the ER, lagged in transport and was slowly secreted from the cells (Crise et al., 1989). Moreover, GΔTM protein was expressed at a minimum of 25 passages (Fig. 1C), confirming the constancy of GΔTM protein expression by the 293 T_GΔTM cells.

The expressed GΔTM protein comprised both cell-associated and secreted forms. We found that the cell-associated GΔTM protein was approximately 30% of the total cellular proteins, whereas the secretory GΔTM protein was around 25% of all proteins in the cell culture medium, as examined by a Western blot. Although the expression of the secretory GΔTM was less than the cell-associated protein, it was more convenient to purify the secreted protein from the culture medium supernatant. Once the expression of GΔTM secreted protein was confirmed, the culture medium supernatant over the 293 T_GΔTM cells was pooled, clarified and purified by affinity chromatography based on the binding between histidine-tagged protein and nickel ions without the extensive protein dissolving process. The result showed that the secretory GΔTM protein could bind well to the nickel ions and that no protein of interest was detected in the flow through fraction after passing through the column. The GΔTM protein was eluted by an elution buffer containing 250 mM imidazole (Fig. 1D). The eluted GΔTM protein fractions were then pooled and concentrated. Fig. 1E shows a single band of the concentrated pooled-protein fractions of the GΔTM protein that was detected by the anti-histidine tag monoclonal antibody.

The GΔTM protein expressed by the mammalian cells in this study has a molecular weight of approximately 75 kDa, which is higher than that of the truncated G protein previously expressed in insect cells (67 kDa; Johal et al., 2008; Kanpipit et al., 2018). Commonly, the protein glycosylation process in insect cells is simpler than that in mammalian cells, as it is unable to produce complex oligosaccharides (Shi and Jarvis, 2007). From 60 ml of culture medium supernatant for purification, the final concentration of the purified GΔTM protein was approximately 400 μg/ml. The yield of the purified GΔTM protein obtained from this study was 6.67 μg per 1 ml of the culture supernatant. By comparison with our previous work, the quantity of the purified GΔTM protein obtained from the mammalian stable cells was higher than that obtained from the transient expression in insect cells (Kanpipit et al., 2018). The mammalian glycosylation process may assist with the proper folding of the G protein, which in turn increases the

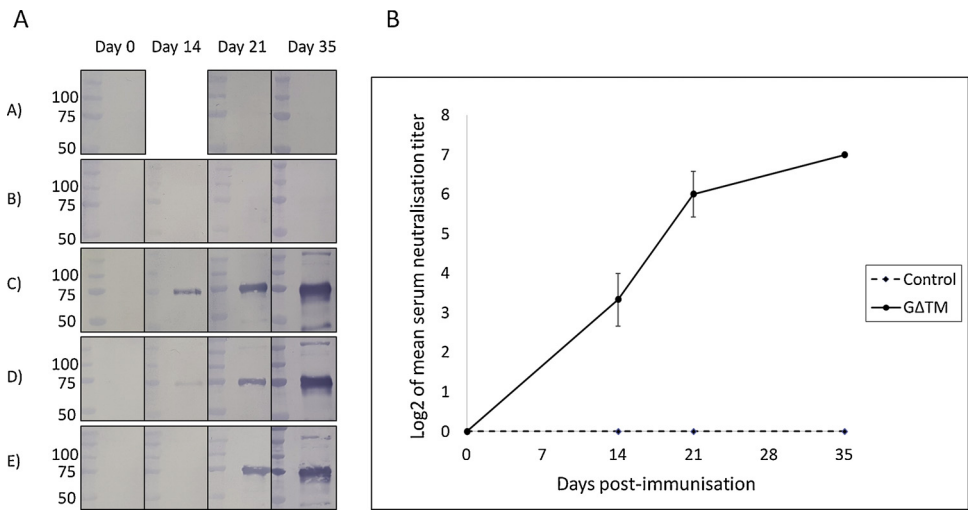

Fig. 2. A) An immunoblot showing the reactivity between purified GΔTM protein and serum samples from mock- (A–B) and GΔTM-immunised (C–E) guinea pigs. Note that the serum sample on day 14 of (A) was not available. B) A serological profile plotted between $\log_2$ of the mean serum neutralising titres (SNT) and the number of days post-immunisation. The dashed line represents the SNT profile of mock-immunised sera and the solid line represents the SNT profile of GΔTM immunised sera. The bar represents the standard error of the mean.

solubility of the GΔTM protein. This could lead to a higher yield of the secretory protein, compared to that expressed by the transient expression (Dalton and Barton, 2014).

In order to examine the immunogenicity of GΔTM, three guinea pigs were immunised with the mixture of GΔTM protein and Montanide ISA 206 adjuvant. The BEFV specific antibody response was examined and compared to that of the control group. Before immunisation, the BEFV GΔTM specific antibody was not observed in any animals. On day 14 after the initial immunisation, serum samples from the immunised guinea pigs could react to the purified GΔTM protein, as examined by a Western blot (Fig. 2A). Furthermore, the positive signals were stronger on days 21 and 35 after immunisation. The increased signal intensity indicates that GΔTM specific antibody titres were gradually higher after the initial and booster immunisations. Serum samples from mock-vaccinated guinea pigs did not react to the purified GΔTM protein throughout the experiment.

A serum neutralisation assay was also performed to determine the neutralising activity in the serum samples after immunisation. All guinea pigs immunised with the GΔTM protein developed specific neutralising antibodies that could inactivate BEFV. The neutralising antibody response in the immunised guinea pigs appeared as early as 14 days after the initial immunisation. However, the mean SNT of animals in the vaccinated group was significantly higher than that of the unvaccinated group from day 21 onward ($P < 0.05$). The level of SNT also increased after the booster immunisation (day 35; Fig. 2B), corresponding to the result from the Western blot analysis (Fig. 2A). In contrast, guinea pigs in the control group did not show the neutralising antibody response at any time point. This result confirmed that the GΔTM protein without a transmembrane domain was able to induce neutralising antibodies similar to the previously reported whole G protein (Uren et al., 1994). Furthermore, a previous study showed that truncated G protein lacking a transmembrane anchor and cytoplasmic tail was able to react with neutralising monoclonal antibodies specific to all antigenic sites (Johal et al., 2008), indicating that GΔTM protein without a transmembrane domain maintains its antigenic property similar to the native G protein.

The generation of a stable cell line is a widely used technique for the production of recombinant proteins. Stable protein expression by mammalian cells is an attractive system for large-scale protein production in terms of quality and homogeneity (Dalton and Barton, 2014). A stable mammalian cell line can produce a reliable and consistent yield of recombinant protein. In addition, it can be stored in a cryogenic condition, recovered and cultured for protein production (Dalton and Barton, 2014). The development of subunit vaccines from recombinant proteins expressed by stable mammalian cells has been reported and these candidate vaccines can induce a protective immune response (Fontana et al., 2014; Hua et al., 2014). In this study, the GΔTM protein was also generated from the stable mammalian cell expression system. The yield of the GΔTM protein produced by the 293 T stable cell line was satisfactory and gained an advantage over the transient expression (Kanpipit et al., 2018). The 293 T stable cells continuously expressing GΔTM protein have growth characteristics similar to the parental 293 T cells, resulting in being easy to culture. Furthermore, this system neither involves the infectious virus nor inactivation agents present in live attenuated or inactivated vaccine preparations. For these reasons, the 293 T stably expressed cell line may be an attractive platform for large-scale GΔTM protein production, and used as a subunit vaccine.

In summary, this is the study on the production of mammalian cells stably expressing BEFV GΔTM as a secretory protein. In a soluble form, the expressed GΔTM protein can react specifically to the convalescent BEFV bovine serum. Notably, the higher level of protein expression and the more convenient protein purification procedure enable it to be a candidate for further upscale development. Moreover, the purified protein was able to induce a protective immune response in the guinea pig model. These results suggest the possible applications of the secretory GΔTM protein produced by the mammalian stable cells for the development of vaccines and diagnostic assays.

Conflicts of interest

None.

Acknowledgements

This study was supported by the Agricultural Research Development Agency (ARDA) with grant number CRP6105021230, the Center for Advanced Studies in Agriculture and Food, the KU Institute for Advanced Studies, Kasetsart University, with grant number PD59(2) CASAF PD015 and the Charoen Pokpan Foundation Thailand. Payuda Hansoongnern received a scholarship from the Thailand Research Fund (TRF) under the Royal Golden Jubilee (RGJ) Ph.D. Program. We are grateful to Seppic (France) for supporting the Montanide ISA 206 adjuvant, as well as Prof. Dr. Wanpen Chaicumpa from Mahidol University, Thailand, and Dr. Richard J. Webby from St. Jude's Children's Hospital, Tennessee, USA, for providing the mammalian cell lines.

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