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Immunogenicity of transmembrane deleted-G protein of bovine ephemeral fever virus (BEFV) expressed in insect cells

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Abstract: Bovine ephemeral fever (BEF) is a common febrile disease in cattle in tropical countries causing loss of milk production and reproductive performance. Recently, severe outbreaks have occurred in many countries in East Asia, the Middle East, and Southeast Asia, especially in the rainy season, while limited vaccines and diagnostic tools are available. Thus, the aim of this study was to produce a secreted, soluble form of BEFV G glycoprotein in insect cells for further applications. BEFV G gene expressing transmembrane-deleted G glycoprotein (GΔTM) was constructed and cloned into the baculovirus vector. The recombinant GΔTM protein was expressed in High5 insect cells and then purified by affinity chromatography. The purified GΔTM reacted specifically with a convalescent bovine serum. Four 8-week-old Wistar rats were injected subcutaneously with the purified GΔTM protein. Postimmunized rat serum samples also strongly reacted with the G protein by western blot and immunoperoxidase mono player assay (IPMA). These results indicated the potential use of the transmembrane-deleted G protein to develop protein based-diagnostic tools for BEFV control programs.

Key words: Bovine ephemeral fever virus, truncated G protein, protein expression, baculovirus expression system, diagnostic tool

1. Introduction

Bovine ephemeral fever virus (BEFV) is classified in the genus *Ephemerovirus*, family *Rhabdoviridae*. It causes an acute febrile disease in cattle and water buffalo transmitted by *Culicoides* biting mosquitoes (1,2). The BEFV genome is 14.9 kb in length and contains a nonsegmented, negative-sense, single-stranded RNA encoding five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and a large multifunctional enzyme (L) (3,4). The G protein is a class I transmembrane glycoprotein located on the virion envelope. It induces protective immunity including virus-specific neutralizing antibodies (5,6). BEFV G protein comprises an N-terminal signal peptide (amino acid 1–12), a C-terminal transmembrane domain, and a cytoplasmic tail (amino acids 522–623). The native BEFV G protein present on the virion is 81 kDa but the recombinant G protein expressed in insect cells appears to be either 76 or 71 kDa (7). Four antigenic sites on the G protein (G1–G4) were well characterized by sequence analysis and protein mapping using specific monoclonal antibodies (7–9). G1–G3 sites are located at amino acid 18–521 on the ectodomain between the signal peptide and transmembrane domain of the G protein (7,9).

Bovine ephemeral fever (BEF) is endemic in tropical and subtropical regions of Africa, Asia, and Australia. Outbreaks occur mostly during the rainy season (10–12). The clinical signs of BEF in cattle include fever, inappetence, lameness, recumbency, and decreased milk production (13). Effective disease prevention and control require both vaccines and diagnostic tools. Various diagnostic procedures have been developed for BEFV such as the loop-mediated isothermal amplification assay (LAMP) (14) and enzyme-linked immunosorbent assay (ELISA) (15–18). ELISA, which is based mainly on the reactivity between protein antigens and theirs cognate antibodies, has been widely established according to high sensitivity and simplicity. G1-epitope, a highly conserved

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and antigenic region located on amino acids 487–503 of BEFV G protein, was previously expressed in *E. coli* (15,19) and yeast (17,20). This epitope has been shown to be a candidate antigen for ELISA development.

Genetic analysis revealed three clusters of BEFVs (21). Cluster I contains BEFVs from East Asia. Most BEFVs in clusters II and III were from Israel and Australia, respectively. The recent whole genome analysis of the highly pathogenic bovine ephemeral fever virus isolated in Turkey classified it in cluster II (22). Thai BEFV is in cluster I (our unpublished data). Antigenic properties of different BEFV strains were dissimilar as some amino acids on neutralizing epitopes were different (11,21). Thus, a protein-based diagnostic assay developed from the local strain would be a more effective tool for disease control (23).

In the present study, we examined the expression and purification of the baculovirus-expressed transmembrane-deleted G protein (GΔTM) of BEFV cluster I in insect cells. The purified GΔTM reacted well with a convalescent bovine serum. Immunogenicity of the protein was examined by rat immunization. The results show that the GΔTM protein has a potential use for BEFV diagnosis.

2. Materials and methods

2.1. Cells

Spodoptera frugiperda (Sf9) cells were maintained in Grace's Insect Medium/Hink's TNM-FH (Caissonlabs) supplemented with 10% fetal calf serum, 2% pluronic acid (Sigma), 12 mM L-glutamine, and 1X antibiotics/antimycotics (Invitrogen). *Trichoplusia ni* (High5) cells were maintained in a serum-free medium, Express Five SFM (Invitrogen), at 26 °C.

2.2. Cloning of the full-length G gene

BEFV RNA was isolated from a serum sample obtained from an infected calf by using a viral nucleic acid extraction kit II (Gene-aid) following the manufacturer's instruction. The isolated total RNA in RNase-free water was measured by Nanodrop (Thermo Scientific). One microgram of the total RNA was reverse transcribed using a random-hexamer primer and Superscript III reverse transcriptase (Invitrogen). cDNA was used as the template to amplify a full-length G gene with specific primers, BEF_G1F and BEF_G1R (Table). The PCR reaction contained 1X HF buffer (Thermo Scientific), 0.5 mM dNTP, and 0.2 µM of each primer and Phusion tag DNA polymerase (Thermo Scientific). The PCR cycle was 98 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 min plus an extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized under UV light. The full-length G gene was purified and cloned into pGEM-T easy vector (Promega), resulting in pBEF_G. The plasmid was submitted for sequencing (Macrogen). the G gene was then amplified from pBEF_G and cloned into pFastBac HT/b (Invitrogen).

2.3. Construction of the transmembrane-deleted G gene (GΔTM)

pFastBac_HTB vector (Invitrogen) was modified by insertion of secretory peptide of Drosophila, BiP, so called pFB_Bip, using a QuikChange Site-Directed Mutagenesis Kit (NEB) following the manufacturer's instruction. Bip31_HisF and Ph_Bip1_R primers used for pFB_Bip construction (Table) were designed according to the BiP sequence in the pMT/BiP/V5-His A, B, and C manual (Invitrogen). His8- tag was also inserted into pFB_Bip by using QuikChange Site-Directed Mutagenesis Kit (NEB) with His_AgeApaI_F and EK_His_ R primers as shown in the Table, resulting in the so-called plasmid pFBip_His8.

N-terminal signal peptide (amino acids 1–12), C-terminal transmembrane domain, and cytoplasmic tail (amino acids 522–623) were removed from the plasmid containing the full length G gene, pBEF_G, to generate the GΔTM gene fragment (8) by PCR with primers AgeI_G13F and XhoI_G522R as shown in the Table. The GΔTM fragment was cloned into pFBip_His8 between *Age*I and *Xho*I restriction enzyme sites. The recombinant plasmid was named pFBip_GΔTM and it contains 5'-Bip (18

Table. Primers used in this study.

Primers	Sequences
BEF_G1F	ATGTTCAAGGTCCTAATAATTAC TTAATGATCAAAGAATCTGTCATC
BEF_G1R	GCCTTTGTTGGCCTCTCGCTCGGGAGATCTACCGGTCATCACCATCACCATCACGATTACGATATCCCAAC
Bip31_HisF	CACGACGGCCAGTAATATGCATAACTTCATCCCGGGGGTTTCGGACCGAGATCCGCGCCCGATGGTGGGAC
Ph_Bip1_R	CATCACCATCACTGAGATATCGGGCCCAAGCTTGTCGAGAAGTACTAGAGGATCATAATCAGC
His_AgeApaI_F	GTGATGGTGATGCTTATCGTCATCGTCGGTACCGCATGCCTCGAGACTGCAGGCTCTAGA
EK_His_ R	TGCAACCGGTGGAATTCATTTTGAAAAAATTTAC
AgeI_G13F XhoI_G522R	CAGTCTCGAGCTTCTTTCCTCCTCTTTGAATTTCTC

amino acids) and GΔTM (512 amino acids) followed by a His8 (tag)-3'.

2.4. Generation of the recombinant baculovirus expressing the GΔTM protein

The Bac-to-Bac (baculovirus) expression system (Invitrogen) was used for the expression of the BipGΔTM. The recombinant plasmid, pFBip_GΔTM, was transformed into *E.coli* DH10Bac (Invitrogen), where the BipGΔTM was transposed into the bacmid. The purified recombinant bacmid was transfected into Sf9 cells (Invitrogen) to generate a recombinant baculovirus (AcMNPV) carrying the BipGΔTM, AcMNPV_BipGΔTM.

2.5. Expression and purification of truncated G protein

AcMNPV_BipGΔTM was inoculated onto High5 cells for protein expression at multiplicity of infection (MOI) of 3 and 5. The secreted GΔTM in cell supernatant was collected at 48 and 72 h postinfection (hpi). The level of protein expression at each condition was compared by western blot analysis. The supernatant was clarified by centrifugation at 10,000 rpm at 4 °C for 15 min. The clarified supernatant was precipitated with 50% (w/v) PEG 6000 (Sigma) to the final concentration of 8% (w/v). The supernatant was then stirred at 4 °C for 2 h and centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant was discarded and the pellet containing GΔTM was re-suspended in 30 mL of the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4).

The concentrated GΔTM protein was purified by affinity chromatography using a HisTrap FF column (GE Healthcare) with the application provided in a protein purification platform, AktaStart machine (GE Healthcare). The column was equilibrated with 15 mL of the binding buffer at a flow rate of 1 mL/min. The re-suspended pellet was filtrated through a 0.45 μm filter (Sartaurious) and diluted with the binding buffer before loading onto the column at a flow rate 0.8 mL/min. The column was washed with 15 mL of the binding buffer at a similar flow rate. The bound proteins were then eluted by a step gradient (13%, 50%, and 100%) with an elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). The eluted fractions containing the GΔTM protein were pooled. Salts in the eluents were decreased by exchanging with 0.01 mM PBS pH 7.4. The purified GΔTM protein was concentrated using Amicon Ultra (Millipore) with 30 kDa cut-off.

The purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel. The presence of GΔTM protein was confirmed by western blot using anti-his epitope tag antibody (Thermo Scientific) as described previously (24).

2.6. Rat immunization

The rat immunization protocol was approved by Kasetsart University – Animal Care Unit Center (ID-ACKU 59-VET-043). Eight-week-old Wistar rats purchased from the National Laboratory Animal Center (NLAC), Thailand, were primed subcutaneously once with 40 μg of the purified GΔTM protein mixed with an equal volume of complete Freund's adjuvant (CFA). At 4 weeks after priming, the rats were boosted subcutaneously with 40 μg of the purified GΔTM protein mixed with an equal volume of incomplete Freund's adjuvant (IFA). The rats were bled before immunization (d0), before boosting (d28), and after boosting for 28 days (d56). Two rats were immunized with PBS-CFA or IFA and served as controls. During blood collection and antigen immunization, the rats were sedated by isoflurane inhalation.

2.7. Western blot analysis

Purified GΔTM protein was separated by SDS-PAGE in a 12% gel. Western blot analysis was performed by using anti-GΔTM rat sera (1:200 in 3% bovine serum albumin; BSA) and anti-mouse IgG-HRP (Millipore) (1:2000 in 3% BSA) as primary and secondary antibodies, respectively. After each step, the membrane was washed with PBST (0.01 mM PBS pH 7.4 and 0.1% tween 20) three times. The protein bands were visualized by adding 3,3'-diaminobenzidine (DAB).

2.8. Immunoperoxidase monolayers assay (IPMA)

IPMA was performed as previously described (25). Briefly, AcMNPV_BipGΔTM was inoculated onto High5 cells in a 96-well plate at an MOI of 3. The plate was incubated at 26 °C in an incubator (Thermo Scientific). The infected cells were fixed with methanol when 50% cell death was observed. The cells were washed once with 1X PBST at room temperature for 5 min before being incubated with 100 μL of 1% H_2O_2 in PBS at room temperature for 30 min. After washing, anti-GΔTM rat sera diluted to an optimal concentration were incubated with the infected High5 cells at 37 °C for 1 h. Anti-mouse IgG-HRP (Millipore) diluted at an optimal concentration was incubated with the bound antibodies at 37 °C for 1 h. The cells were stained with DAB (Sigma) and observed for the presence of a brown color.

3. Results

3.1. Expression of BEFV GΔTM in insect cells

A secreted BEFV G protein containing deleted transmembrane domain and cytoplasmic tail was successfully produced from insect cells with the application of the baculovirus expression system. The optimal condition for GΔTM protein expression was obtained by inoculating High5 cells with 3 MOI of AcMNPV_BipGΔTM and the infected cell supernatant was collected at either 48 or 72 hpi as shown in Figure 1a. The results of SDS-PAGE and western blot analysis using anti-histidine tag and anti-mouse IgG-HRP as primary and secondary antibodies, respectively, showed that the GΔTM proteins

had a molecular weight of approximately 68 kDa. It reached the maximum expression level at 48 hpi. When the amount of the recombinant AcMNPV_BipGΔTM was increased from 3 to 5 MOI, the expression level was not different as shown in Figure 1b.

To study the expression of GΔTM in insect cells, AcMNPV_BipGΔTM was inoculated onto High5 cells at an MOI of 3 in a 96-well plate for 48 h. The presences of GΔTM protein in mock- and AcMNPV_BipGΔTM-infected cells were examined by IPMA using either anti-histidine tag or a convalescent bovine serum as primary antibodies. The results showed that the GΔTM protein appeared mostly at cell periphery and on the plasma membrane. The protein reacted well with both anti-histidine tag and a BEFV-convalescent bovine serum as shown in Figure 2.

3.2. Purification of GΔTM from cell supernatant

The secreted, soluble form of GΔTM proteins was purified from infected cell supernatant by PEG precipitation followed by affinity chromatography. Figure 3 demonstrates fractions of GΔTM proteins eluted from a nickel column by a step gradient method. The majority of GΔTM protein was eluted at 100% elution buffer containing 500 mM imidazole. The eluted fractions containing GΔTM proteins were pooled and concentrated before being analyzed by SDS-PAGE using 12% acrylamide gel. Specificity of the GΔTM protein was confirmed by western blot. The results showed that the protein reacted specifically to both anti 8X-his epitope tag antibody and a convalescent bovine serum as shown in Figure 4.

3.3. Immunogenicity of GΔTM protein

To determine whether GΔTM protein could induce the specific immune response in animals, rats were subcutaneously injected with 40 µg of purified GΔTM protein. Rat serum samples were examined for the presence of BEFV G protein specific antibodies by western blot analysis and IPMA. The results showed that all serum samples from the immunized rats reacted specifically to the purified GΔTM protein. Sera from preimmunized rats and nonimmunized control rats did not react to the protein as shown in Figure 5. The immunized rat sera also bound specifically to the G protein presented in the positive cells as shown in Figure 6.

4. Discussion

BEF is one of the most important infectious diseases of livestock in tropical regions. Cattle infected with BEFV show clinical signs such as sudden onset of fever and lethargy, which become a serious management problem especially in large-scale farming. Vaccines have been developed and vaccination was applied as a successful strategy for disease prevention and control (5,23,26–29). BEFV glycoprotein (G) is an important protein to induce a protective immune response in cattle (28). Among the types of vaccines used for BEFV protection, the G protein expressed from recombinant vaccinia virus and partially purified virion had promising results in experimental and field studies (6,30). In fact, the G protein contains four antigenic sites (G1, G2, G3, and G4) with neutralizing activities (5,9). All four neutralizing epitopes remained

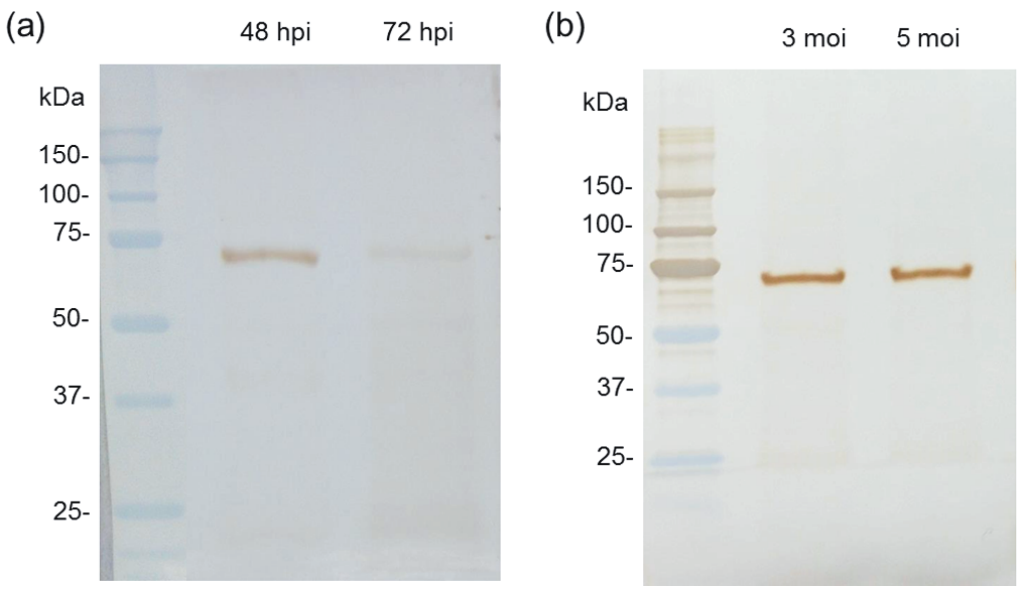

Figure 1. Soluble GΔTM protein collected from the supernatant of AcMNPV_BipGΔTM infected High5 cells. (a) The secreted GΔTM proteins were collected at 48 and 72 h postinfection (hpi). (b) The proteins were obtained from High5 cells infected with AcMNPV_BipGΔTM at MOIs of 3 or 5 for 48 hpi.

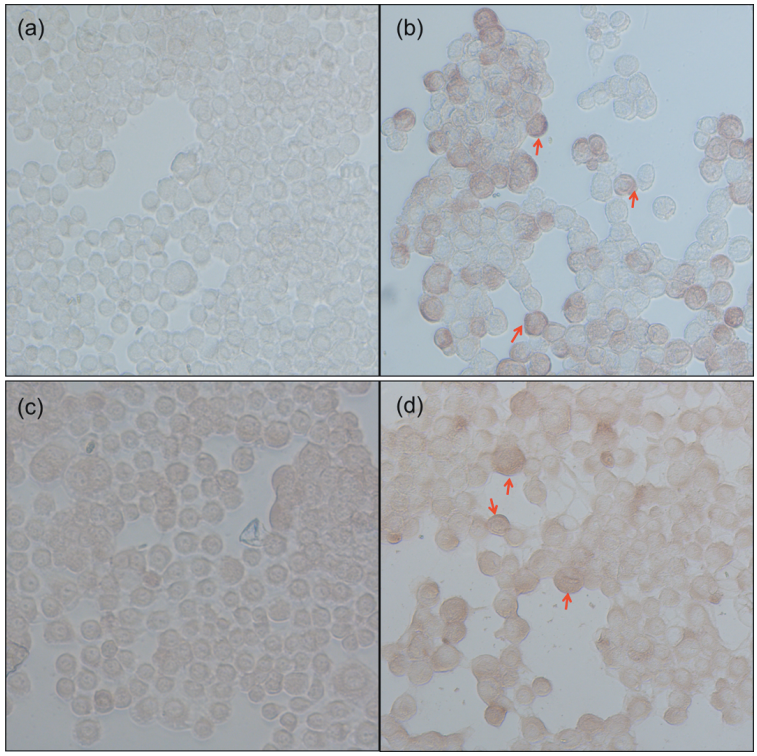

Figure 2. GΔTM proteins expressed in infected insect cells examined by immunoperoxidase monolayer assay (IPMA). High5 cells were mock-infected (a, c) or infected with AcMNPV_BipGΔTM (b, d) at multiplicity of infection (MOI) of 3. The cells were allowed to react with anti-histidine tag (a and b) or a BEFV convalescent bovine serum (c and d) as primary antibodies.

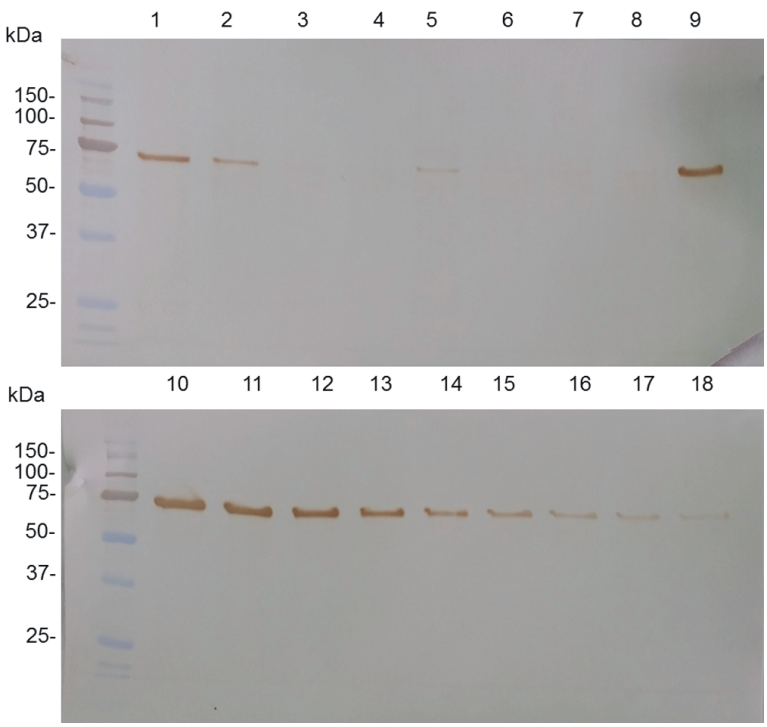

Figure 3. Immuno-blot photographs demonstrating fractions of secreted GΔTM proteins during purification step. Lanes 1 and 2 are total protein; lane 3 is flow-through; lanes 4 and 5 are wash fractions containing 20 mM imidazole; lanes 5 and 6 are wash fractions with 65 mM imidazole; lanes 7 and 8 are wash fractions with 250 mM imidazole; lanes 9–18 are elution fractions with 500 mM imidazole.

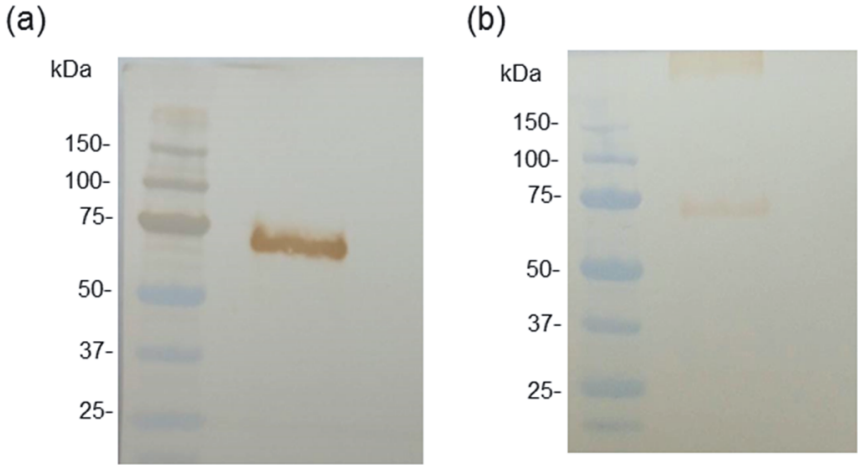

Figure 4. Immuno-blot photographs of purified GΔTM protein. The protein band of approximately 68 kDa reacted specifically to anti-histidine tag antibody (a) or a BEFV convalescent bovine serum (b).

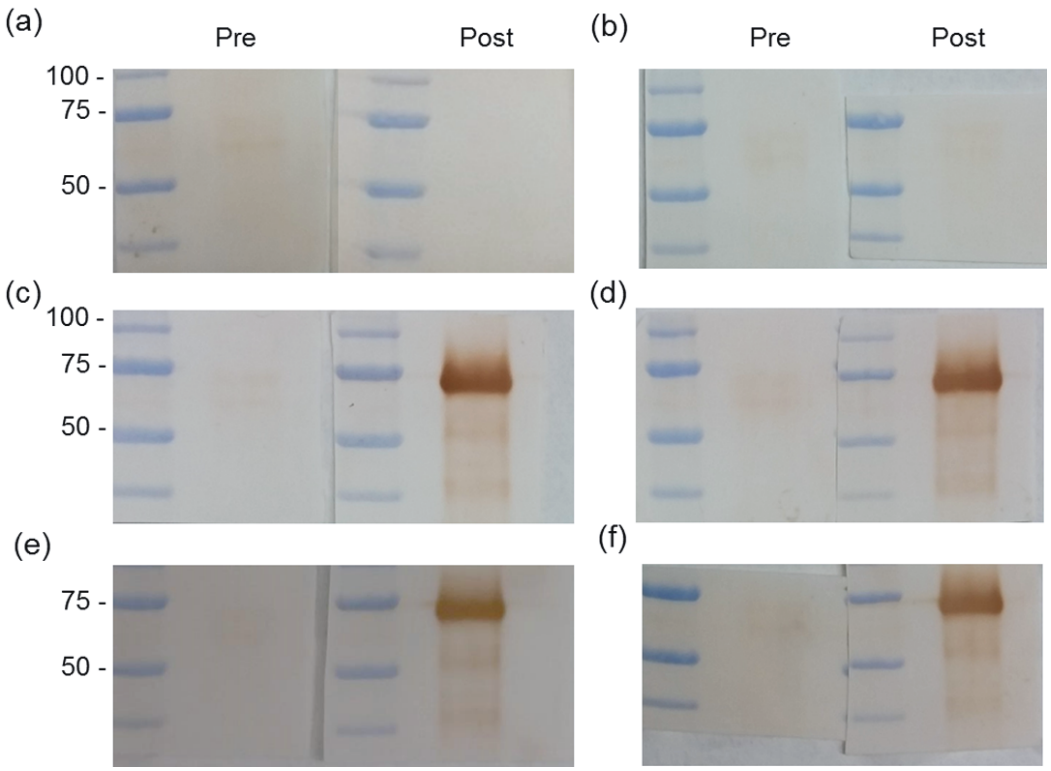

Figure 5. Immuno-blot photographs showing specific reactivity between GΔTM proteins and immunized rat sera. The purified GΔTM proteins were incubated with pre- or mock-immunized sera from two control rats (a and b) or pre- and postimmunized sera from each of four rats (c–f).

on the structure of the soluble form of G protein lacking C-terminal transmembrane and cytoplasmic tail (amino acids 522–623) (7).

In our study, we generated baculovirus-expressing BEFV truncated G protein (GΔTM) as previously described (7) with a modification on the commercial Bac-to-Bac expression vector by adding the Drosophila secretion peptide, namely BiP. The AcMNPV_BipGΔTM-infected cells secreted recombinant GΔTM protein into supernatant and the protein was purified as a native form.

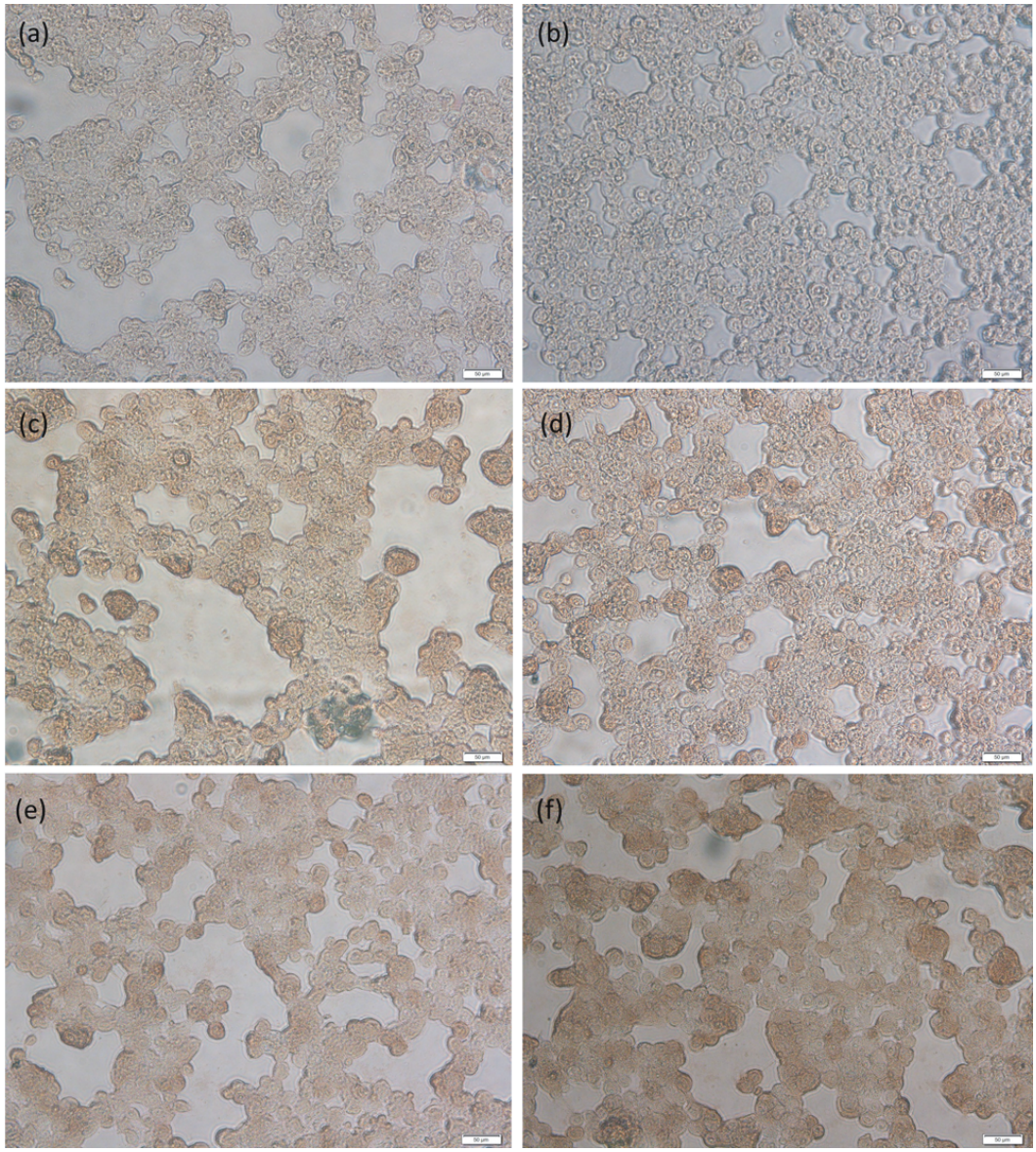

Figure 6. GΔTM proteins expressed in AcMNPV_BipGΔTM infected insect cells examined by IPMA assay. AcMNPV_BipGΔTM was inoculated onto overnight seeded High5 cells in a 96-well plate at an MOI of 3 for 48 h. The infected cells were then fixed and incubated with mock-immunized sera from two control rats (a and b) or postimmunized sera form four rats (c–f). Horse-radish peroxidase-labeled anti-mouse IgG was used as a secondary antibody. The brown color was developed by the reactivity between peroxidase and 3,3'-diaminobenzidine (DAB).

The expressed GΔTM protein has approximately 68 kDa and reacted specifically to either anti-histidine tag or a BEFV convalescent bovine serum. As demonstrated by IPMA, the GΔTM localized in cytoplasm and on cell membrane of the recombinant AcMNPV_BipGΔTM-infected insect cells. Our results also showed that the recombinant GΔTM was immunogenic as it could induce the specific antibody production in rats and the protein could react specifically with a BEFV convalescent serum. In addition, it was shown previously that this form of the G protein could react specifically with a panel of monoclonal antibodies raised against BEFV (7). This indicates the potential use of the soluble, secreted GΔTM protein to develop a protein-based diagnostic assay for detecting BEFV infecting cattle.

Ephemeral fever has been found in cattle in Southeast Asia (4) and may be closely related to East Asian viruses. Taiwanese BEFV isolates were grouped into the same

cluster as Japanese and Chinese isolates and were genetically distant from Australian and Israel isolates (11,31). Antigenic variations among these strains or even between field and vaccine strains occurred as amino acid substitutions were found on the epitopes of G proteins (11,32). Our GΔTM protein derived from the Asian cluster of BEFV containing all four antigenic sites on G protein would be a good candidate antigen for diagnostic assay development.

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