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An improved Bac-to-Bac/BmNPV technology expressing envelope E2 glycoprotein of classical swine fever virus (CSFV) in the silkworm, *Bombyx mori*

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ABSTRACT. The envelope E2 glycoprotein of classical swine fever virus (CSFV) is an immunodominant protein which neutralizes the virus. Previous trials to eradicate CSFV have been expensive, inefficient and time-consuming process without complete success. In contrast to DNA transfection into cultured cells, the efficacy of gene transduction in *in vivo* organ is very low because of the presence of cortical laminar structures and multilayered cells followed by quality and quantity of DNA. Therefore, to eradicate CSFs, developing an effective and inexpensive vaccine targeting E2 glycoprotein is important. Here, we reported using different quantity of DNA with lipofectamine-2000 reagents that could markedly enhance the effectiveness of gene transfer in particular experiment while we are looking for long term development of animal vaccine and an

alternative strategy for large scale production of CSFV E2 glycoprotein using baculovirus (bac-to-bac) system in silkworm, *Bombyx mori,* L. Our results show successful expression of E2 glycoprotein in BmN cell lines and silkworm larvae. The direct injection of recombinant rBacmid/BmNPV/ E2 DNA with lipofectamine-2000 reagent infecting the silkworm larvae are varied in different groups and clear symptoms of infection were found and polyhedrons were counted by hemocytometer in individual and different batch. Confocal and electronic microscopy further revealed the expressed polyhedral, followed by SDS-PAGE and western blot further supporting our data. Our study provides an alternative strategy to produce large scale protein against CSFV. Current work to purify the E2 protein for elucidating its structure and development of vaccine is underway.

Key words: E2 glycoprotein; Classical swine fever virus (CSFV); *Bombyx mori* L; Baculovirus; Insect cell line

INTRODUCTION

Classical swine fever virus (CSFV) is an extremely infectious disease of swine (Xu and Liu 2008). The virion of CSF envelope composes of three glycoproteins namely E1, E2, and E3. (Hulst and Moormann 1996), among which the E2 (an immunodominant glycoprotein) plays an important role in neutralizing the virus (Nig et al., 1995) (Yu et al., 2001). CSFV is classified under the genus of Pestivirus (Family Flaviviridae) (Becher et al., 2003). CSFV is a RNA virus (positive-stranded), with a genomic size ranging 12.3 kb, lacking poly (A) tail in the 3'and 5'UTRs regions. Interestingly, the RNA genome carries a single open reading frame (ORF) (Poole et al., 1995; Rijnbrand et al., 1997; Moennig et al., 2003). To eradicate CSFV, several studies have applied without much success, in an expensive and inefficient way. For instance, to control disease eruptions, an efficient serological method of screening was employed (Hulst and Moormann, 1996). In fact, the E2 envelop protein has a perplexing role within a target cell. Meanwhile, efforts to develop new CSFV vaccine based on E2 glycoprotein are on in pharmaceutical industries. Unfortunately, the hydrophobic and low abundance nature of E2 glycoprotein further provides obstacles to fully elucidate its protein functions. However, emerging tool, for example, SDT (Serological diagnostic test for E2 double water-in-oil sensitive) is safe and efficient for developing CSFV's marker vaccines (Van Rijn et al., 1999). Meanwhile, alternative methods to eradicate CSFV like SV's (subunit vaccines) could be available in the market at reasonable rate. In fact, the vaccine produced in China against CSFV using rabbit is available already in the market; and interestingly, in term of pork production, China is the world largest market (National Bureau of statistics of China, 2014). Some vaccines like the Chinese lapinized vaccine (C-strain), the Japanese GPE strain and the French Thiverval strain, have been developed and used in several countries (Beer et al., 2007). However, due to high complexity involve, CSF is not properly regulated in China. Still, some pocket areas of China is not free from swine fever. Therefore, a holistic approach comprising identification

of susceptible pigs and immunization would benefit both veterinary science and economical condition (Luo et al., 2014).

BVES (Baculovirus expression system) system is widely used for expressing recombinant DNA and prokaryotic proteins and provides modification of translationally expressed proteins (under favorable condition) to produce ample glycoproteins (Sanderson et al., 1999). BVES systems have been widely used in silkworm, *Bombyx mori* L including BmN cell line for heterologous protein expression. Interestingly, silkworm animal model is an alternative tool for human pathological bacterial infection (Kaito et al., 2002). Delivery of direct baculovirus DNA in the silkworm system is considered safer tools compared to traditional methods (Wu et al., 2004). Besides, silkworm is easy to rear throughout the year using mulberry leaves and artificial diets; and silkworm is promising for producing large-scale recombinant protein at low-cost. Meanwhile, recent findings have shown powerful immunogenicity among baculovirus expressing E2 glycoprotein (Dong and Chen, 2007), thus, further proving for quick and economical production of vaccines in veterinary industry (Wu et al., 2010). Later, Lee et al. (2012) achieved production of recombinant E2 glycoprotein using BVES system in silkworm larvae.

Large-scale and mass low-cost industrial recombinant proteins production is promising using silkworm (Minkner and Park, 2018). However, gene transduction is vastly used to execute gene expression of foreign genes from a recombinant plasmid. Several methods such as microinjection, viral vector delivery etc. have been devised to transduce and express exogenous genes in silkworm. In contrast to DNA transfection into cells line, the efficacy of gene transduction into silkworm organ is less because of the presence of cortical laminar structures and multilayered cells followed by DNA purity. Therefore, with the objective to develop suitable long term animal vaccine, recombinant baculovirus was transfected *in vitro* and *in vivo* using different doses of lipofectin-2000.

What is differing from Lee's et al. (2012) is that our current study achieved high efficiency of E2 glycoprotein expression at various doses of Lipofectamine, and we were able to measure infection ratio of the polyhedra. Our results demonstrated highly successful expression of E2 glycoprotein in both BmN cell line and silkworm, *B. mori* larvae. Findings from this research could help generate CSFV vaccine at reasonably low-cost and efficacy, besides bio-medical drug discovery.

MATERIAL AND METHODS

Animals system

The *B. mori* L Qingsong×Haoyue hybrid strain was used in this study. Silkworms were cultured at 25℃ with a relative humidity of 65±5% by using fresh mulberry leaves at photoperiod 12 h L: 12 h D (light: dark). When larvae reached 5^{th} instar stage, several subgroups were divided and injections were resumed with recombinant DNA virus after 5 min on ice. All injected larvae were fed post-injection.

Cell lines

BmN cell line was cultured using GIBCO media (TC-100) (GIBCO, Grand Island, NY) by adding fetal bovine serum (FBS) 10% (v/v) at 27℃.

Cloning and Synthesis of E2 gene from CSFV

GeneBank No. DQ907718 and BLAST search from http://www.ncbi.nlm.nih.gov (Figure 1a) were used to design primers of the E2 gene. Total RNA synthesis and cloning of the E2 gene was done from CSFVs provided by Dr. Guang qing Liu, Zhejiang Academy of Agriculture Science, China. *In silico* analysis showed the start and end of transmembrane domain at 342 and 364 with low complexity (Figure 1b). Swiss-model analysis showed 74% sequence identity and alignment with the CSFV protein (Figure1c).

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1       cggctagcctgcaaggaagattacaggtacgcaatatcgtcaaccgatgagataggcta
20       R  L  A  C  K  E  D  Y  R  Y  A  I  S  S  T  D  E  I  G  L
61      cttggggccggaggtctcaccaccacctggaaggaatacaaccacgatttgcaactgaat
40       L  G  A  G  G  L  T  T  T  W  K  E  Y  N  H  D  L  Q  L  N
121     gacgggaccgtcaaggccagttgcgtggcaggttcctttaaagtcacagcacttaatgtg
60       D  G  T  V  K  A  S  C  V  A  G  S  F  K  V  T  A  L  N  V
181     gtcagtaggaggtatttggcgtcattgcataagaaggctttacccacttccgtgacattc
80       V  S  R  R  Y  L  A  S  L  H  K  K  A  L  P  T  S  V  T  F
241     gagctcctgttcgacgggaccaacccatcaactgaggaaatgggagatgacttcaggtcc
100      E  L  L  F  D  G  T  N  P  S  T  E  E  M  G  D  D  F  R  S
301     gggctgtgcccgtttgatacgagtcctgttgttaagggaaagtacaatacgaccttgttg
120      G  L  C  P  F  D  T  S  P  V  V  K  G  K  Y  N  T  T  L  L
361     aacggtagtgctttctatcttgtctgcccaatagggtggacgggtgtcatagagtgcaca
140      N  G  S  A  F  Y  L  V  C  P  I  G  W  T  G  V  I  E  C  T
421     gcagtgagcccaacaactctgaggacagaagtggtaaagaccttcaggagagacaagccc
160      A  V  S  P  T  T  L  R  T  E  V  V  K  T  F  R  R  D  K  P
481     tttccgcacagaatggattgtgtgaccaccatagtggaaaatgaagatttattctattgt
180      F  P  H  R  M  D  C  V  T  T  I  V  E  N  E  D  L  F  Y  C
541     aagttgggggcaactggacatgtgtgaaaggcgagccagtggtctacacaggggggta
200      K  L  G  G  N  W  T  C  V  K  G  E  P  V  V  Y  T  G  G  V
601     gtaaaacaatgtagatggtgtggcttcgacttcgatgggcctgacggactcccgcattac
220      V  K  Q  C  R  W  C  G  F  D  F  D  G  P  D  G  L  P  H  Y
661     cccataggtaagtgcattttggcaaatgagacaggttacagaatagtagattcaacggac
240      P  I  G  K  C  I  L  A  N  E  T  G  Y  R  I  V  D  S  T  D
721     tgtaacagagatggcgttgtaatcagcacagaggggagtcatgagtgcttgatcggtaac
260      C  N  R  D  G  V  V  I  S  T  E  G  S  H  E  C  L  I  G  N
781     acgactgtcaaggtgcatgcatcagatgaaagactgggccctatgccatgcagacctaaa
280      T  T  V  K  V  H  A  S  D  E  R  L  G  P  M  P  C  R  P  K
841     gagattgtctctagtgctggtcctgtaatgaaaacctcctgtacattcaactacacaaaa
300      E  I  V  S  S  A  G  P  V  M  K  T  S  C  T  F  N  Y  T  K
901     actttgaagaacaggtactatgagcccagggacagctacttccagcaatatatgcttaag
320      T  L  K  N  R  Y  Y  E  P  R  D  S  Y  F  Q  Q  Y  M  L  K
961     ggtgagtatcagtactggtttgacctggatgcgactgaccgccactcagattacttcgca
340      G  E  Y  Q  Y  W  F  D  L  D  A  T  D  R  H  S  D  Y  F  A
1021    gaatttgttgtcttggtggtggtagcactgttaggaggaagatatgtcctgtggctgata
360      E  F  V  V  L  V  V  V  A  L  L  G  G  R  Y  V  L  W  L  I
1081    gtgacctacgtagttctaacagaacaactcgccgctggtctacca
380      V  T  Y  V  V  L  T  E  Q  L  A  A  G  L  P
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Figure 1a. Deduced Classical swine fever virus E2 gene nucleotide and amino acid sequences.

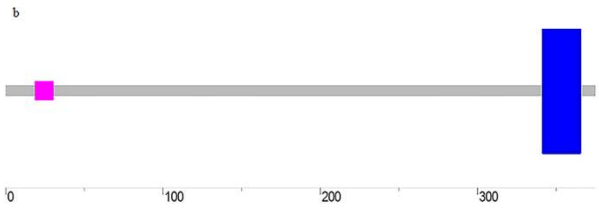

Figure 1b. Predicted domains of classical swine fever virus

The PCR reaction was carried out with incubation for 2 min at 94℃, followed by 32 cycles for 1 min at 94℃, 1 min at 60℃, and 50 s at 72℃, and 10 min at 72℃. The amplified DNA fragments were used as templates in the next PCR reaction with specific primer for 2 min at 94°C, followed by 32 cycles for 1 min at 94°C, 1 min at 60°C, and 90 s at 72°C, and finally, 1 cycle at 72°C for 10 min. Later, PCR product was sub-cloned into the

vector of pMD-18T (TAKARA), followed by correct sequence analysis. Finally, using DNAstar software, the E2 sequence was analyzed and further, the E2 glycoprotein model was generated using SWISSMODEL Repository Database (Figure 1c (Adapted)) (http://swissmodel.expasy. org/repository/).

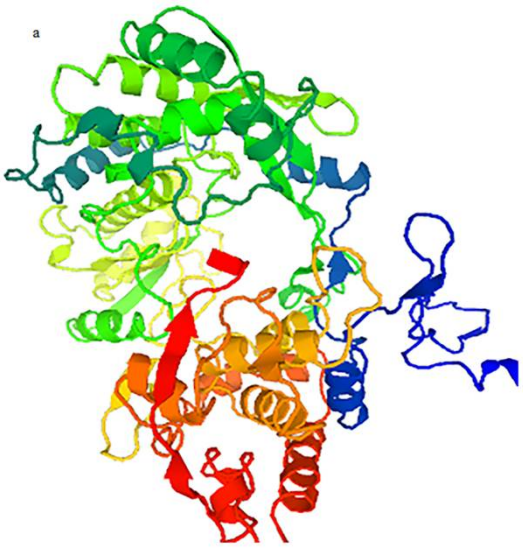

Figure 1c. Three dimensional protein model from classical swine fever virus (CSFV)

Construction of recombinant donor plasmid pFast Bac HTB/E2

We constructed the recombinant donor plasmid pFast Bac HTB/E2 as shown in Figure 2. Using forward and reverse primers of the E2 gene at restriction sites *NCOI* and *HindIII*, the gene was PCR amplified from pMD18-T/E2. Later, it was sub-cloned to pFastBac^{TM}HTB, resulting pFastBac^{TM}HTB/E2. Following sequencing result and open reading frame (ORF) confirmation, it was stored at 20℃ for further use.

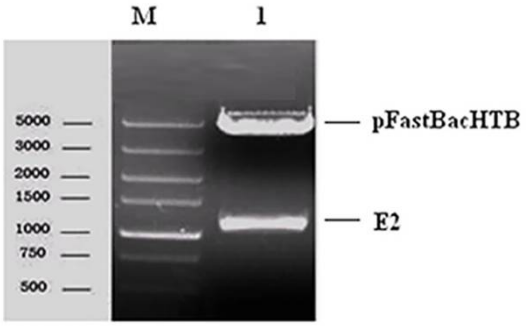

Figure 2. Indication of the recombinant pFastBac HTB/E2 with *NCO1* and *Hind lll* through digestion. M indicates DNA marker (TaKaRa) and Lane 1 shows digestion product in which E2 gene was separated from the vector.

Construction of recombinant virus rBacmid/BmNPV/E2

The construction of recombinant virus rBacmid/BmNPV/E2 was generated according to Figure 3a, 3b. The recombinant donor plasmid pFastBac^{TM}HTB/E2 was transformed into the DH10Bac^{TM} *E. coli* strain competent cells. The *E. coli* DH10Bac baculovirus were cultured in LB medium (Luria-Bertani) containing antibiotics. Later, the selected white colonies were PCR amplified using pairs of M13 forward and reverse primers.

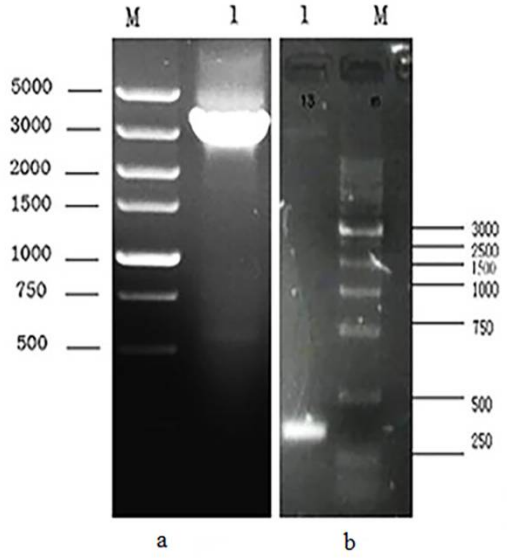

Figure 3. The E2 gene fragment synthesized by PCR a. M indicates DNA marker (TaKaRa). Lane1 is PCR product of white colony which contains E2 genes + bacmid, the analysis of recombinant bacmid using M13 primers. b. Lane 1, PCR of a blue colony. M indicates DNA Marker (TaKaRa).

Recombinant virus generation in BmN cell line

The recombinant virus was generated at our laboratory using BmN cell line cultured in T-25 flask containing 10% FBS solution at 27°C. Lepofectamine-2000 (Roche) was used for transfection of BmN cells. The recombinant viral solution was collected at 96 h post-transfection into the eppendorf tube and stored at 4°C, protecting from direct light. Upon transfection to BmN cells, the first generation virus P1 was generated followed by recombinant virus second P2 stock in six-well plates.

Expression of E2 gene in *in-vivo* condition

The recombinant E2 virus generated in BmN cells post-96 h infection was used to inject on 5th instars first day larvae at about 10 µL volume prior to feeding. Simultaneously, the recombinant bacmid baculovirus DNA with lepofectamine-2000 was microinjected on

5th instars first day larvae. Finally, post-96 h infection, hemolymph from 10 (ten) worms was collected for analysis of SDS-PAGE and western blot.

Assessment of polyhedra in hemolymph

An individual silkworm hemolymph was observed for presence of polyhedra under hemocytometer at 48 h and 96 h after infection and the morphology of the polyhedra was investigated by an electronic microscope. We calculated infection ratio by following the company's standard protocol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

The sample (hemolymph) was boiled and denatured for 3 min, and SDS was performed to a 12% SDS slab gel. Finally, proteins in the gel were visualized with the Coomassie Blue stain. Western blot was performed to nitrocellulose membrane for 1.5 h at 110 mA. Following membrane washing and blocking, an anti-mouse goat IgG polyclonal antibody was added to confirm the protein band.

RESULTS

Construction and generation of E2 bacmid baculoviruses

The target E2 gene was inserted into the MCS of the donor plasmid pFastBac™ HTB (Figure 2) under P10 promoter within *NCOl* and *Hind III* sites by T4 DNA ligation and transformed to *E.coli* DH10Bac/BmNPV. During transposition in bacteria, the target gene (E2) was transferred to a baculovirus shuttle vector (Bacmid) within a mini-*att* Tn7 target site and we generated recombinant bacmid baculovirus containing the gene.

Transfection of rBacmid/BmNPV/E2 DNA (purified recombinant) in BmN cells was performed with lipofectamine-2000 according to the protocol. Cells were monitored on daily basis for possible signs of infection. BmN cells morphology (infected and non-infected) are shown in Figure 4a and 4b. Generally, infected cells show cessation of growth and suspension, besides enlarge cell diameter and nuclei compared to uninfected cells post-96 h transfection.

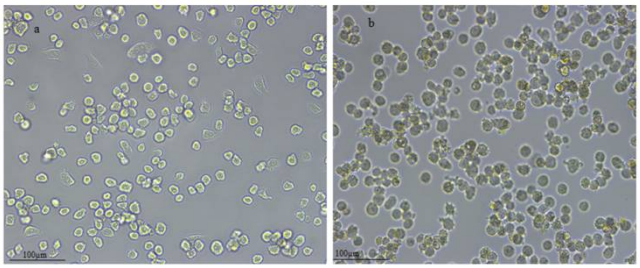

Figure 4. Baculovirus infected BmN cell phenotypes. a. Normal BmN Cells as a control. b. BmN cells infected with recombinant rBacmid/BmNPV/E2 plasmid DNA 96 h post-infection, which become recombinant rBacmid/BmNPV/E2 baculovirus.

Expression of E2 gene in silkworm larvae by Lipofectamine 2000

Post-96 h injected silkworm larvae show signs of NPV infection in treatment group resulting blackening of body color, sluggish growth, bulged body, and whitish hemolymph (Figure 5a and 5b) compared to control group (Figure 5a). The collected hemolymph is shown in Figure 6a and 6b. Moreover, we show single silkworm polyhedron density in the hemolymph after treatment with different concentration of lipofectamine-2000 at different times. The main objective was to observe the efficiency of this treatment by microinjection. Table 1 showed that when the lipofectamine-2000 concentration decreases at different time scale, less polyhedral was observed and few silkworms were infected (Figure 6c). Now, we can conclude that lipofectamine had high ability to introduce viral DNA into the animal system. All treatment groups later resulted acute infection, tore body and died. Hemolymph from the treatment larvae was collected post-96 h for further SDS-PAGE and western blotting analysis.

Figure 5. 5th instar silkworm larvae after infection with NPV. a. Normal silkworm and b. Injected E2 recombinant virus, on the 1st day of the 5th instar larvae pre-feeding stage (at 4×105 particles/worm in 10 μL volumes). The infected larvae show translucent body, intersegmental and swollen body. Post-infection (after 72 to 96 h) was compared to the normal control.

Figure 6. Picture of observation polyherda from bacmid infected larvae after 96 h post-infection a. Whitish hemolymph of infected 5th instar silkworm larvae after 96 h post infection. b. Infected silkworm larvae hemolymph was collected in the eppendorf tube. c. Picture of Polyhedra in larvae hemolymph was observed under electronic microscope after 96 h post infection. The recombinant bacmid baculoviruses were injected into the 5th instar silkworm larvae.

Table 1. Observation of *Bm*NPV infected polyhedron in hemolymph of silkworm larvae at different intervals under electronic microscope.

Samples	Lepofectamine-2000 (µL)	DNA (µg)	Larva no	Polyhedron observed (no.) (48 h-post-injection)	Infect ratio (%)	Polyhedron observed (no.) (96 h-post-injection)	Infect ratio (%)
1	10	1	50	25	50	40	80
2	5	1	43	17	39.53	38	88.37
3	5	2	35	14	40	26	74.28
4	5	5	55	13	23.63	24	43.63
5	2	4	80	12	15	22	27.5
6	2	3	48	10	20.83	20	41.66
7	1	5	90	8	8.8	17	18.88

SDS and Western Blotting

Result of western blot show band size of about 39.6-kDa (Figure 7a; Lane 1, E2 containing silkworm hemolymph; lane 2, GFP-His Tag (Positive control) and similar band size was also observed in SDS-PAGE from silkworm hemolymph post-96 h infection showing a band size of about 39.6-kDa (Figure 7b; Lane 1, Control hemolymph; Lane 2, E2 expressed hemolymph; Lane 3, protein marker).

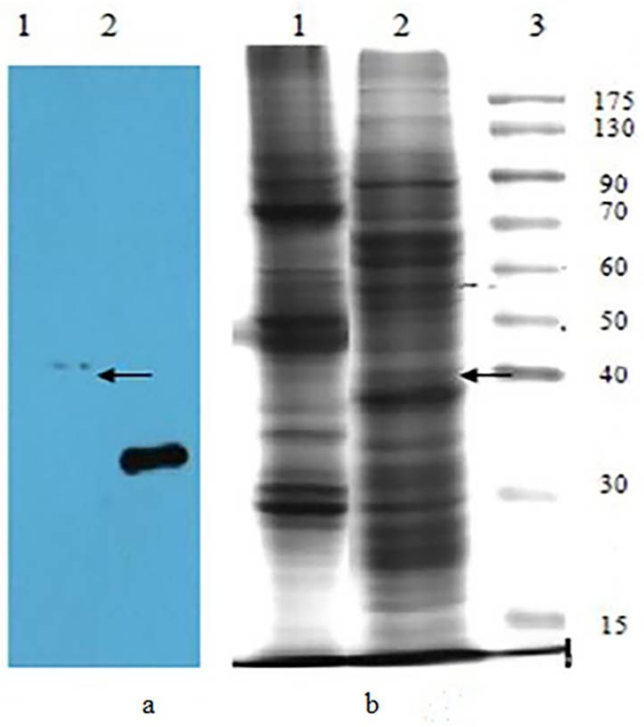

Figure 7. The result of Western–blot showing E2 expressed protein from silk worm hemolymph. a. Western–blot analysis of E2 glycoprotein. Lane 1, hemolymph containing the E2 protein; Lane 2, Positive control (GFP-His). b. SDS-Page. Lane 1, Normal hemolymph; Lane 2, *E2* gene expressed hemolymph; Lane 3, Protein marker.

DISCUSSION

In pig industry, vaccination of CSF (Classical swine fever) is one utmost important step. However, so far, the marker vaccines subunits which are commercially available based on baculovirus expressing E2 envelope glycoprotein against CSFV have only been developed. Unfortunately, such vaccines show slow immune reaction besides least protective than the attenuated CSF vaccines of conventional type (De Smit et al., 2001; Henderson, 2005). Therefore, market demand for such novel vaccine development having high efficiency, cost-effective is important. Interestingly, recently developmental based genetic engineering for novel marker vaccine strategy has been made (Na et al., 2008). Meanwhile, strategies for vaccines development are rapidly changing each day. Underscoring this, we aim here to develop vaccine against classical swine virus by using cost-effective baculovirus system in BmN cell line and silkworm larvae. Our results show successful delivery of the E2 gene in BmN cells and silkworm larvae. We hope this method could play vital role in preventing swine virus in China, in the future. In fact, in biotechnology, recombinant DNA technology for developing viral vaccine is a new era (Bull et al., 2017) in science.

In fact, for protein production understanding cellular metabolism is essential (Morokuma et al., 2017) and for large scale protein production, baculovirus system could be an alternative source. Also, for large scale foreign protein production, the baculovirus expression system is a cheap, quick and easy method (Chico E., 2000). For expression of protein in eukaryotic system, it is one of the most efficient and popular tools (Miao et al., 2006). Therefore, here, we demonstrated ways to create an effective method for producing vaccines against CSFV in pig industry by cloning the E2 gene. The successful delivery of E2 baculovirus in BmN cells and silkworm larvae, confirmed by SDS-PAGE and western blot open up ways for large scale vaccine production.

In summary, we established a method for producing the E2 glycoprotein in an efficient and economy way by using an improved bacmid system in BmN cells and silkworm larvae. Also, we show the potentiality of implementing this method in any established laboratory. Research from our findings could facilitate not only future research on swine virus vaccine production but also for potential testing and application in pig industries. Further study to deduce complete structure of E2 and vaccine in this line is underway.

AUTHOR CONTRIBUTIONS

All authors worked collaboratively on all aspects of this manuscript and agree to publish this manuscript in this journal.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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