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Asian Journal of Animal and Veterinary Advances



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## High Expression of Type O FMDV Target Protein by Gene Replacement in *Baculovirus* Expression Vector System

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### ABSTRACT

The structural protein VP1 of foot-and-mouth disease virus (FMDV) play an important role in the construction of a high immunogenic subunit vaccine. The objective of this study was to obtain higher expression of type-O FMDV target protein, this will provide further experimental evidence through animal immunization. The VP1 which in *Bombyx mori Baculovirus* recombinant transfer vector pVL-P12A3C of Asia I FMDV was replaced by VP1 of type-O FMDV and the recombinant plasmid pVL-OP12A3C was created. The *Bombyx mori* N (Bm-N) cells was co-transfected with pVL-OP12A3C and linearized DNA of insect virus expression vector. Indirect immune fluorescence tests (IFAT) demonstrated that target proteins were expressed. The recombinant virus was used to infect silkworm and the target protein in haemolymph was characterized by western blotting and double-antibody sandwich Enzyme-linked Immune Sorbent Assay (ELISA) analysis. The results indicated that gene replacement is an effective way to enhance gene expression.

**Key words:** FMDV, P12A3C gene, VP1 gene, replacement genetics, subunit vaccine

### INTRODUCTION

Foot-and-mouth Disease (FMD) is highly contagious disease of cloven-footed mammals which can result in huge economic losses (Alexandersen *et al.*, 2001; Liu *et al.*, 2011). The disease spreads rapidly through livestock populations such as cattle, pigs and sheep. FMD has the potential to cause explosive epidemics with heavy economic implications to the animal industry worldwide. FMD is caused by the Foot-and-mouth Disease Virus (FMDV), which belongs to the *Aphthovirus* genus of the Picornaviridae family. The virus exists as seven immunologically distinct serotypes (i.e., O, A, C, Asia 1 and SAT 1-3) with multiple subtypes within a serotype (Pereira, 1977). There is one copy of single-strand RNA and 60 copies each of VP1-VP4 which are the four structural proteins of FMDV. The VP1 carries important epitopes against FMDV inducing specific immune responses (Bittle *et al.*, 1982; Brown, 1992). Two major B-cell epitopes, VP1 (141-160) and VP1 (200-213) are able to induce neutralizing antibodies (Collen *et al.*, 1991; Van Lierop *et al.*, 1992). The VP1

(141-160) also contains at least one T-cell epitope that is able to induce FMDV-specific T-cells (Zamorano *et al.*, 1995). In view of the above, the VP1 is of much importance in the research of FMDV.

Due to the degree of risk associated with FMDV and its agricultural importance, safe and effective vaccines are necessary. The use of conventional vaccines has raised safety concerns, as inactivated virus vaccines have been related to outbreaks of FMD caused by the release of incompletely inactivated viruses (King *et al.*, 1981), therefore, genetically-engineered vaccines provide a safe and efficient alternative.

Empty capsid vaccine as a genetically-engineered vaccine has successfully been used in clinical trial (Li *et al.*, 2008). In earlier reports, many kinds of empty viral capsids in *Baculovirus* expression system has been developed (Maranga *et al.*, 2002; Noad and Roy, 2003). Furthermore, there are many advantages of the silkworm-*Baculovirus* expression system, such as high level expression of recombinant protein, safe to any vertebrate host and even post-translational modifications are similar to that of mammalian cell (Choi *et al.*, 2000; Lin *et al.*, 2006).

Through series of experiments and years of testing, we found that the expression of recombinant protein of type O is much lesser than FMDV of Asia I in the same silkworm-*Baculovirus* expression system. In this study, FMDV VP1 gene of Asia I was replaced by VP1 gene of type O and results showed that expression of the target gene in new recombinant plasmid can be developed.

## MATERIALS AND METHODS

This study was carried out during the period from December 2010-October 2011.

**Viruses and cell lines:** FMDV strains Asia I/JQ strain, O/May/8/2005 propagated in BHK-21 cell line are isolated and preserved in Lanzhou Veterinary Research Institute of the Chinese Academy of Agriculture Sciences Chengguan District Lanzhou City, Gansu Province. The parent virus Bm-Bac PAK6, Bm-N cell line and silkworm variety Qiufeng×Baiyu used for the experiment were maintained in the Biotechnology Research Institute of the Chinese Academy of Agriculture Sciences Beijing, China. The Bm-BacPAK6 and recombinant virus were maintained in Bm-N cells at 27°C in a TC-100 insect medium (Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with a 10% heat-inactivated fetal bovine serum (Invitrogen Corporation, Carlsbad, CA).

**Construction of recombinant *Baculovirus*:** Genomic RNA was extracted from the viral supernatant with RNeasy QIAGEN China Shanghai Co., Ltd. and used immediately for cDNA synthesis. The cDNA was synthesized with AMV reverse transcriptase and Oligo(dT)18 primer (Takara Bio Inc., Shiga, Japan) at 42°C for 1 h following the recommended protocol. PCR was used to amplify P12A 3C VP1 from its corresponding cDNA using a pair of specific primers:

- **P12A forward primer:** 5'-ATAGGATCCACCATGGGAGCCGGGCAATCCAGCC-3' (BamHI site was introduced)
- **P12A reverse primer:** 5'-CGCGAATTCTGACATGTCCTCCTGCATCTGGTTG-3' (EcoRI site was introduced)
- **3C forward primer:** 5'-GCGGAATTC AAGAAACCTGTCGCTTTGAAAGT-3' (EcoRI site was Introduced)
- **3C reverse primer:** 5'-ATAAGATCTCTACTCGTGGTGTGGTTCGG GAT-3' (BglII site was introduced)

- **OVP1 forward primer:** 5'-ATATCTAGACAACACCACCAACCCAA CAGCTT-3' (XbaI site was introduced)
- **OVP1 reverse primer:** 5'-ATAGAATTCTGGGTCCTTGGACC GTGCTGCCA-3' (EcoRI site was introduced)

The optimized thermal program was one cycle of template denaturation (94°C for 5 min). Amplifications were comprised of P12A: 30 cycles of denaturation at 95°C for 45 sec, primer annealing at 58°C for 2 min and extension at 72°C for 1 min. Finally, three polishing step were performed as follows: (1) at 72°C for 10 min; VP1: 30 cycles of denaturation at 95°C for 1 min, primer annealing at 63°C for 1 min and extension at 72°C for 1 min, (2) at 72°C for 10 min; 3C: 30 cycles of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min and extension at 72°C for 1 min and (3) at 72°C for 10 min.

The purified PCR products were ligated with T vector and then transformed into *Escherichia coli* DH5 $\alpha$  competent cells successfully. T-P12A was digested with BamHI and EcoRI and the transfer vector pVL1393 was digested with the same restriction enzyme and the digested fragments were ligated by T4 DNA ligase to yield a construct. The plasmid was named pVL-P12A by enzyme digestion and PCR. 3C was ligated to pVL-P12A by the same method. The VP1 of pVL-P12A3C was replaced by OVP1 was digested with XbaI and EcoRI and the plasmid was named pVL-OP12A3C after identification.

The baculoviral transfer plasmid pVL-OP12A3C was co-transfected with linearized Bm-BacPAK6 DNA into Bm-N cells by a liposome-mediated method using transfection reagent lipofectAMINE2000 (Invitrogen). The co-transfection supernatant was subjected to plaque assays to screen the individual viral plaques. After another two rounds of purification, the pure virus clone (Bm-OP12A3C) was obtained.

**Expression of FMDV composite protein in Bm-N cells:** The expression of FMDV composite protein in Bm-N cells was analyzed by immunofluorescence test (IFAT). Bm-N cells were cultured on cover slips and inoculated at an MOI of 10 pfu with Bm-OVP1. After 48 h post infection, IFAT was conducted to analyze the expression of FMDV composite protein. Cells were then rinsed with PBS for 1 or 2 times and fixed in 100% cold acetone (-20°C for 30 min). Samples were incubated with rabbit serum against OVP1 of FMDV (37°C for 30 min) in a humid box, washed with PBS 5 times and then stained with fluorescein-conjugated goat anti-rabbit serum at 37°C for 30 min. The cover slips were coated with glycerin and observed via an Olympus fluorescence microscope (Olympus America, Inc., Melville, NY, USA. Bm-N cells infected with Bm-BacPAK6 were used as the control.

**Expression of composite protein in silkworm:** Fifth instar silkworms were infected with the recombinant virus at about 10<sup>5</sup> plaque forming unit per silkworm. The dying silkworm's hemolymph was collected on ice and stored at -20°C. The VP1 fusion protein were analysed by SDS-PAGE and Western-blotting.

The pupae were grown in conventional conditions for 6 to 8 days, injected with the recombinant virus rBm-NPV (Bm-OP12A3C) by 10<sup>5</sup> pfu/pupa and grown at 27°C for 4 to 5 days for protein expression and then the pupae were collected and preserved at -20°C for sandwich-ELISA. 96-well plat-bottomed plates were coated with the rabbit serum against type O FMDV overnight at 4°C and blocked with BSA (Solarbio) powder for 1 h. Then the plates were washed 5 times. FMDV antigen,

haemolymph lysates of infected silkworm with Bm-OP12A3C and Bm-BacPAK6 were diluted with two-fold serial and incubated at 37°C for 1 h. Subsequently, the plates were washed thoroughly and guinea pig sera against type O FMDV were added to each well. The plates were incubated at 37°C for 60 min and then rabbit anti-guinea pig IgG peroxidase conjugate at 1:500 dilution was added and reacted at 37°C for 1 h. Substrate (0.05% H<sub>2</sub>O<sub>2</sub> plus ortho-phenylene diamine) was added, reacted for 15 min and stopped by adding 1 mol L<sup>-1</sup> sulphuric acid. Absorbance was determined at 492 nm. Comparative expression of Bm-P12A3C(O) which has constructed before and Bm-OP12A3C of this study by sandwich-ELISA.

## RESULTS

**PCR amplification of P12A, 3C and VP1 gene:** The P12A, 3C and VP1 sequence were amplified by RT-PCR. Sequence analysis indicated that the specific PCR products of 690, 639 and 2256 bp were amplified (Fig. 1a-c).

**Cloning of P12A3C in recombinant *Baculovirus*:** The amplified products were purified and subsequently cloned into pMD18-T. After verification by sequencing, they were subcloned into the *Baculovirus* transfer vector pVL1393 to generate plasmid pVL-P12A3C. The VP1 of type was replaced and the baculoviral transfer plasmid pVL- OP12A3C was constructed. A specific band of about 3000 bp was digested with BamHI and BglII (NEB) (Fig. 2a); a band of about 600 bp that was confirmed by double restriction enzyme digestion with XbaI and EcoRI(NEB) (Fig. 2b).

**Expression of composite protein in Bm-N cells:** The expression of composite protein in Bm-N cells was analyzed by IFAT. The results demonstrated that Bm-N cells infected with Bm-OP12A3C could produce specific fluorescence, while only very weak fluorescence background appeared in the control cells (Fig. 3). This indicated that composite protein was expressed validly in Bm-N cells.

**Expression of composite protein in silkworm and pupae:** The dying silkworm's hemolymph and pupae were collected (about 4-5 days post infection, corresponding to rearing mean

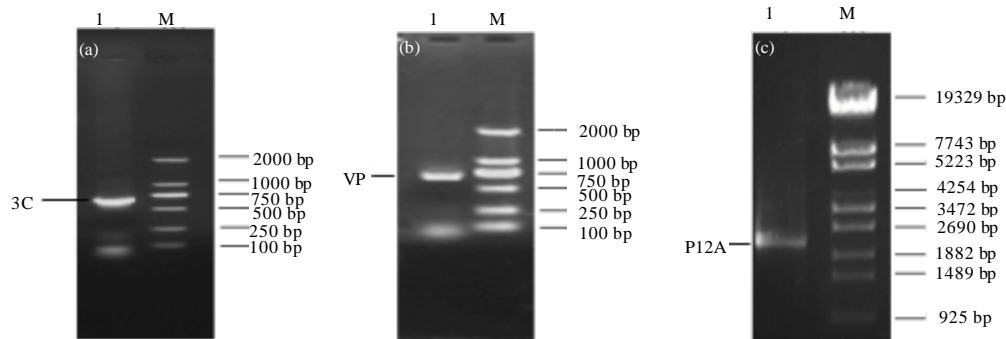


Fig. 1(a-c): (a) The agarose gel electrophoresis of amplified PCR products are shown, (a) Amplified PCR product of 3C; Lane 1: PCR product (3C) amplified from cDNA, Lane M: Standard DNA molecular weight marker (DNA Marker 2000; Takara), (b) Amplified PCR product of 3C; Lane 1: PCR product (VP1) amplified from cDNA, Lane M: Standard DNA molecular weight marker (DNA Marker 2000; Takara) and (c) Amplified PCR product of 3C; Lane 1: PCR product (P12A) amplified from cDNA, Lane M: Standard DNA molecular weight marker ( $\lambda$ -EcoT14 I digest; Takara)

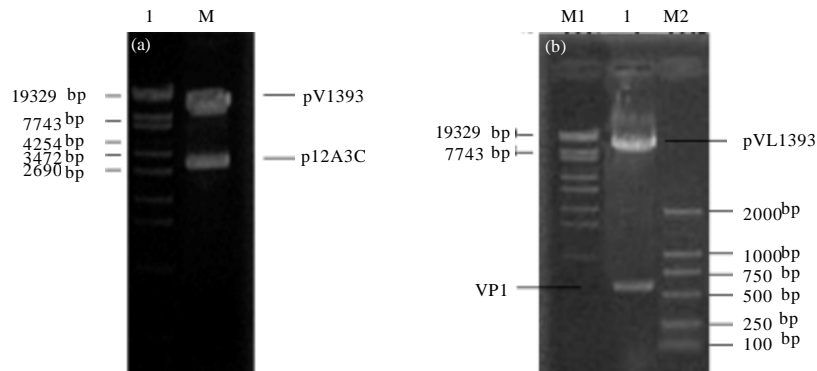


Fig. 2(a-b): (a) The agarose gel electrophoresis of enzyme digestion is illustrated, The recombinant plasmid of pVL-P12A3C identified by enzyme digestion; Lane M: Standard DNA molecular weight marker (-EcoT14 I digest; Takara), Lane 1: Restriction fragment of P12A3C and (b) The recombinant plasmid of pVL-OP12A3C identified by enzyme digestion; Lane M<sub>1</sub>: Standard DNA molecular weight marker (-EcoT14 I digest; Takara), Lane M<sub>2</sub>: Standard DNA molecular weight marker (DNA Marker 2000; Takara), Lane 1: Restriction fragment of OVP1

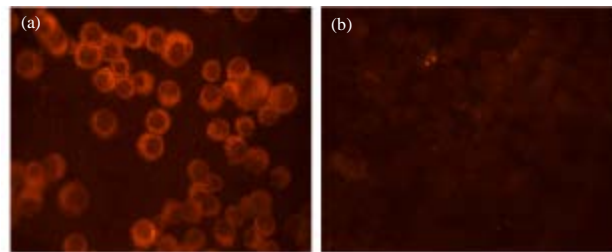


Fig. 3(a-b): The expression of FMDV composite protein in Bm-N cells by IFAT is shown (a) The Bm-N cells co-transfected with pVL-OP12A3C and Bm-BacPAK6 and (b) The Bm-N cells infected with Bm-BacPAK6

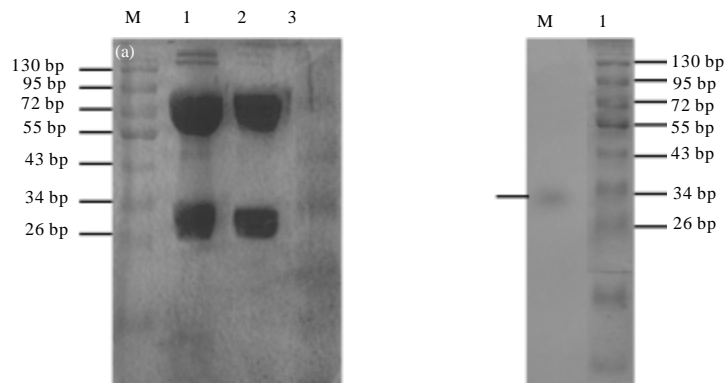


Fig. 4(a-b): (a) SDS-PAGE analysis of expressed FMDV proteins, Lane M: Standard prestained protein marker III (GenStar), Lane 1: 1:5 dilution of hemolymph, Lane 2: 1:50 dilution of hemolymph, Lane 3: Bm-BacPAK6 vector control and (b) Western blot analysis of expressed FMDV protein; Lane M: Standard prestained protein marker III (GenStar), Lane 1: Protein of VP1 in silkworm hemolymph

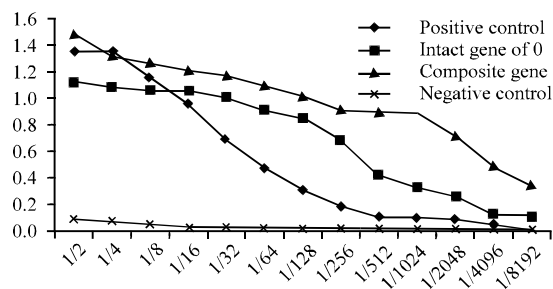


Fig. 5: Comparative expression of FMDV polyprotein in silkworm larvae was estimated by the sandwich-ELISA, The haemolymph was diluted with two-fold series

temperature of 27°C). The hemolymph was diluted by PBS and determined by SDS-PAGE (1:5 dilution of lane 1, 1:50 dilution of lane 2, Bm-BacPAK6 vector control of lane 3) (Fig. 4a). The protein interacted with rabbit anti-VP1(FMDV) antibodies and Goat anti-Rabbit IgG (H and L)-HRP (Bioord) in the Western blot. A band of about 27.4 kDa protein in the expression product of VP1 gene in silkworm (Fig. 4b).

Sandwich-ELISA was conducted to evaluate the expressed antigen in silkworm pupae. The results indicated that a value of recombinant *Baculovirus* after replacement of OVP1 was higher than intact P12A3C gene of type O, which was in good agreement with variation of positive control of the FMDV antigen, but was undetectable in the negative control of *Bm-BacPAK6* infected silkworm's hemolymph (Fig. 5).

## DISCUSSION

This experiment was conducted to find an effective method for increasing expression of specific antigen and VP1 region is the most relevant in the immunogenicity of FMDV (Burroughs *et al.*, 1971). It was shown that mice immunized with protein that synthesized in vitro of FMDV VP1 gene can induce specific immune responses in mice. In addition, the ability of the FMDV structural protein VP1 or capsid polyprotein P1, produced in transgenic plants to invoke virus-neutralizing antibodies, protection has already been demonstrated in a mouse model (Carrillo *et al.*, 2001; Dus Santos *et al.*, 2002; Dus Santos *et al.*, 2005). This experiment thus provides a suitable method to replace VP1 between the different types of FMDV.

But separate VP1 does not attempt to achieve a maximum effect. In adenovirus expression system, full structure of P1-2A and 3C, can be expressed and assembled into empty capsids (Mayr *et al.*, 1999). In the past few years, P12A3C genes of type-O FMDV have been expressed in *Baculovirus* expression system, but the expression did not get the expect level. In this study, the VP1 gene which in P12A3C of Asia I FMDV was replaced by VP1 of type O FMDV, which achieved the higher experssion of target protein.

This study could find a safer and more effective vaccine to immunize animal. Expression antigen of *Baculovirus* expressing systems are generally considered to be well immunogenic and possess the ability to assemble empty capsids (Rueda *et al.*, 2000; Maranga *et al.*, 2002; Noad and Roy, 2003; Mortola and Roy, 2004). Exactly the subunit vaccine have distinct advantages such as safety, efficiency, broad spectrum, economic and operability, all of which are incomparable when it comes to conventional vaccines (Zhao *et al.*, 2003). Moreover, the silkworm *Baculovirus*

expression system has obvious advantages: the recombination of a foreign gene not only can infect *Bombyx mori* cells and express in vitro, but also can express in vivo through injecting silkworm larva or pupa (Ikonomou *et al.*, 2003).

The data obtained in the study provided a new method to develop FMDV subunit vaccine of type-O. Of the four immunized cattle, two were completely protected FAMD challenge and the clinical symptoms of the other two were alleviated (Li *et al.*, 2008; Li *et al.*, 2011). Besides, more experiments related to test the potency of the vaccine are needed.

#### ACKNOWLEDGMENTS

This work was supported by grants from the National 863 Project of China (Grant No. 2011AA10A211-3), International Science & Technology Cooperation Program of China (Grant No. 2011DFA31830) and the National Natural Science Foundation of China (Grant No. 31201943). We appreciate the valuable comments from other members of our laboratories.

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