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Immune response in cattle inoculated with the recombinant complete polyprotein of foot-and-mouth disease virus from *Bombyx mori* larvae

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The intact open reading frame (ORF) of foot-and-mouth disease virus (FMDV) Asia I/XJ strain was amplified by RT-PCR and inserted into the transfer vector pVL1393 to generate plasmid pVL-ORF. *Bm*-N cells were transfected with pVL-ORF and linearized *Bm*-BacPAK6 DNA, and the recombinant silkworm baculovirus *Bm*-ORF containing the full ORF of FMDV was obtained. The results of indirect immunofluorescence assay (IFA) showed that *Bm*-ORF could be expressed efficiently in *Bm*-N cell. After inoculating the early 5th instar larvae of silkworm, the polyprotein of FMDV could be detected by sandwich ELISA and empty capsid-like particles could be observed under the electron microscope. Expression products from silkworm were used as the antigen to immunize the cattle. The specific antibody was induced in all vaccinated animals. The immunized cattle were challenged with the virulent FMDV Asia I/XJ strain, two of the four cattle were completely protected and clinical symptoms were alleviated and delayed in the others. The results suggest that this strategy might be used to develop the new subunit FMDV vaccine.

foot-and-mouth disease virus, open reading frame, silkworm-baculovirus expression system, immunogenicity, subunit vaccine

Foot-and-mouth disease (FMD) is the most contagious disease of mammals that causes severe economic loss in susceptible cloven-hoofed animals. FMD virus (FMDV) is a member of genus *Aphthovirus* of the family *Picornaviridae*. Seven distinct serotypes (A, O, C, Asia I, SAT I, SAT II and SAT III) have been identified, within which there is extensive antigenic diversity. FMDV has a positive-sense RNA genome approximately 8500 nt in length, which contains 5′ untranslated region, a single open reading frame (ORF), 3′ untranslated region and Poly(A) tail. The ORF encodes a polypeptide that is cleaved into mature polypeptide product by viral proteinases[1].

Vaccination is a major means to control FMD outbreak in developing countries. The current FMD vaccine is a chemically inactivated whole virus antigen. Although the traditional inactivated vaccine has proved effective, it may lead to new outbreak of FMD because of the incomplete inactivation of FMDV or the escape of live virus from vaccine production workshop[2]. Thus, it is urgent to develop a novel FMD vaccine that is safer, more effective and economic than traditional vaccines.

It has been demonstrated that empty capsids were as immunogenic as 140S virons, which implies that empty capsids of FMDV could be used to mimic virions immunologically[3,4]. Therefore it is a feasible choice to develop empty capsids vaccine. Expression products of baculovirus expressing system are generally considered to be well immunogenic and possess the ability to assemble empty capsids[5]. The silkworm baculovirus ex-

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pression system has several advantages such as high yield of recombinant protein, non-pathogenic to any vertebrate host, and production of proteins with post-translational processing similar to that of mammalian cells[6,7].

Different expression systems such as DNA vaccine, adenovirus vector, vaccinia virus vector, and transgenic plant had been used for expression of antigen of A-type (or O-type) FMDV to produce empty capsids[8−13]. But to our knowledge, no research on empty capsids of type Asia I has ever been reported. In the present paper, virus like particles (VLPs) of FMDV Asia I/XJ strain were well produced in silkworm-baculovirus expression system. These particles are highly immunogenic and are able to stimulate humoral immune responses and provide protection against virulent FMDV.

1 Materials and methods

1.1 Viruses and cell lines

FMDV Asia I/XJ strain that propagated in BHK-21 cell line was isolated and preserved in Lanzhou Veterinary Research Institute of the Chinese Academy of Agriculture Sciences. The parental virus *Bm*-BacPAK6[14], *Bm*-N cell line and silkworm variety JY1 used for the experiment were maintained in Biotechnology Research Institute of the Chinese Academy of Agriculture Sciences. The *Bm*-BacPAK6 and recombinant virus were maintained in *Bm*-N cells at 27℃ in TC-100 insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen).

1.2 Construction of baculoviral transfer plasmid pVL-ORF

Genomic RNA was extracted from the viral supernatant with RNeasy (Qiagen) and used immediately for cDNA synthesis. The cDNA was synthesized with AMV reverse transcriptase and Oligo$(dT)_{18}$ primer (Takara) at 42℃ for 1 h following the recommended protocol. PCR was used to amplify the intact ORF from cDNA using a pair of specific primer: Forward primer: 5′-GCGACTAGTACCATGGAATTCACACTTCACAACGGTGAG-3′ (*Spe* I site was introduced); Reverse primer: 5′-ATAGCGGCCGCAGGGATTATGCGT CACCGCACAC- 3′ (*Not* I site was introduced).

The optimized thermal program was one cycle of template denaturation (95℃ for 1 min); 30 cycles of denaturation (94℃ for 40 s), annealing (60℃ for 40 s) and extension (72℃ for 8 min) followed by one cycle of final extension (72℃ for 10 min). The size of PCR product was verified by 10 g/L Agarose gel electrophoresis.

The purified PCR product was digested with *Spe* I and *Not* I and the transfer vector pVL1393 with *Xba* I and *Not* I. All the digested fragments were ligated by T4 DNA ligase to yield construct. The construct was transformed into *E. coli* JM109 in a low salt LB plate with amp selection. The resultant plasmid was named pVL-ORF and identified by using restriction enzyme digestions and PCR. Further confirmation is performed by sequencing with the pVL1393 sequencing primers. In pVL-ORF, the ORF is placed under control of the *polyhedrin* promoter.

1.3 Construction and screening of recombinant baculovirus

The baculoviral transfer plasmid pVL-ORF was co-transfected with linearized *Bm*-BacPAK6 DNA into *Bm*-N cells by liposome-mediated method using transfection reagent lipofectAMINE2000 (Invitrogen). The co-transfection supernatant was subjected to plaque assays to screen the individual viral plaques. PCR amplification was conducted to examine whether the ORF had been incorporated into the baculoviral genome. After another two rounds of purification, the pure virus clone (*Bm*-ORF) was obtained.

1.4 Expression of FMDV polyprotein in *Bm*-N cells

The expression of FMDV polyprotein 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*-ORF. After 48 h post infection (hpi), IFAT was conducted to analyze the expression of FMDV polyprotein. Cells were then rinsed with PBS for 1 or 2 times and fixed in 100% cold acetone (−20℃ for 30 min). Samples were incubated with rabbit serum against FMDV (37℃ for 30 min) in humid box, washed with PBS for 5 times, and then stained with fluorescein-conjugated goat anti-rabbit serum at 37℃ for 30 min. The cover slips were coated with glycerin and observed via an Olympus fluorescence microscope. *Bm*-N cells infected with *Bm*-BacPAK6 were taken as control.

1.5 Expression of polyprotein in silkworm

Early 5th-instar silkworms were infected with the re-

combinant virus at about 10^5 pfu per larva. The dying silkworm's haemolymph was collected on ice and stored at −20℃ for sandwich-ELISA. 96-well plat-bottomed plates (Costar) were coated with the rabbit serum against FMDV overnight at 4℃ and blocked with defatted milk powder for 1 h. Then the plates were washed 5 times. FMDV antigen, haemolymph lysates of infected silkworm with *Bm*-ORF and *Bm*-BacPAK6 were diluted with two-fold serial and incubated at 37℃ for 1 h. Subsequently, the plates were washed thoroughly and guinea pig sera against FMDV were added to each well. The plates were incubated at 37℃ for 60 min, and then rabbit anti-guinea pig IgG peroxidase conjugate (Sigma) at 1∶10000 dilution was added and reacted at 37℃ for 1 h. Substrate (0.05% H_2O_2 plus orthophenylene diamine) was added, reacted for 15 min and stopped by adding 1 mol/L sulphuric acid. Absorbance was determined at 492 nm.

1.6 Observation of viral empty capsids

The harvested silkworm haemolymph was centrifuged and the supernatant was examined to observe viral empty capsids. Samples were observed in an EM at 80000× magnification according to the test protocol[15].

1.7 Vaccination of cattle

Silkworm haemolymph was lysed ultrasonically and cell debris was removed by centrifugation. The diluted supernatant was used to produce vaccine following the conventional preparation method. Liquid-phase blocking ELISA (LPBE) (http://www.oie.int/eng/normes/MMANUAL/A_00024.htm) was performed to determine the antibody titer for screening of candidate cattle according to the standard method of World Organization for Animal Health, Office International desEpizooties (OIE) before vaccination. Candidates with a potency lower than 8 were housed in disease-secure isolation facilities in Lanzhou Veterinary Research Institute. Detection kit was prepared by Lanzhou Veterinary Research Institute. Six cattle were immunized by intramuscular inoculation at the site in the neck. Four cattle were vaccinated with 3 mL/animal of vaccine with *Bm*-ORF's, while two control cattle were vaccinated with the same dose of vaccine with *Bm*-BacPAK6's.

1.8 Detection of specific antibody by LPBE

Cattle sera were collected at 21 d postvaccination (dpv). Antibody against FMDV was detected by LPBE method as above.

1.9 Challenge with virulent homologous FMDV

According to the descriptions by standard protocol of OIE (http://www.oie.int/eng/normes/MMANUAL/A_00024.htm), all animals were challenged by intradermal inoculation at two sites in the tongue with 10000 bovine infectious doses (BID_{50}) of Asia I/XJ at 21 dpv. The restrained animals were carefully examined in the mouth, and feet in 3, 5, 7 and 10 d postchallenge (dpc).

2 Results

2.1 Construction of the recombinant virus *Bm*-ORF

Intact ORF from Asia I/XJ strain were amplified by RT-PCR and inserted into the transfer vector pVL1393 to generate plasmid pVL-ORF. The plasmid was verified by restriction enzyme analysis and PCR. The results indicated that pVL-ORF was correctly constructed. Sequence analysis indicated that the ORF was approximately 6.9 kb containing full P1, P2 and P3 coding regions.

The baculoviral transfer plasmid pVL-ORF was co-transfected with linearized *Bm*-BacPAK6 DNA into *Bm*-N cells. By 4 d post-transfection, the supernatant was collected as the viral stock for screening of recombinant virus. 24 isolated virus plaques from the plaque assays were cultivated in 24 wells plate for about 4—5 d and recombinant virus were further identified via added x-gal to every well. PCR amplification was also conducted to see whether the ORF had been incorporated into the viral genome. After another round purification, the pure virus clone was obtained.

2.2 Expression of polyprotein in *Bm*-N cells

The expression of polyprotein in *Bm*-N cells was analyzed by IFAT. The results demonstrated that *Bm*-N cells infected with *Bm*-ORF could produce specific fluorescence, while only very weak fluorescence background appeared in the control cells (Figure 1). This indicated that polyprotein was expressed validly in *Bm*-N cells.

2.3 Expression of polyprotein in silkworm

The dying silkworm's haemolymph was collected (about 4—5 d post infection, corresponding to rearing mean

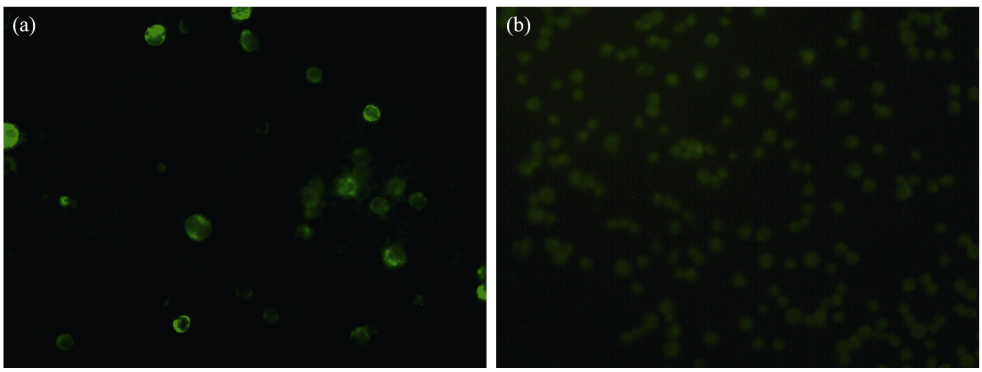

Figure 1 Expression of FMDV polyprotein in *Bm*-N cells by IFAT. *Bm*-N cells were analyzed for expression of FMDV polyprotein by IFAT 48 h post infection: (a) *Bm*-N cells infected with *Bm*-ORF; (b) *Bm*-N cells infected with *Bm*-BacPAK6.

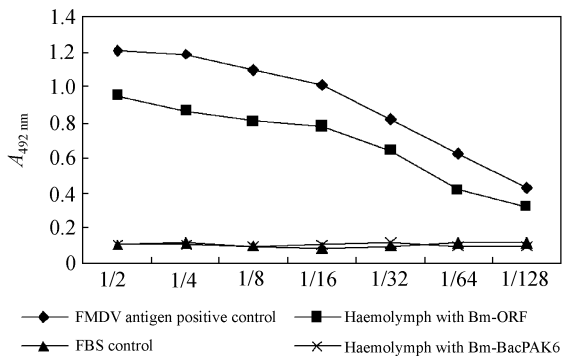

Figure 2 Expression of FMDV polyprotein in silkworm larvae was estimated by the sandwich-ELISA. The haemolymph was diluted with two-fold series.

temperature of about 27－25℃). Sandwich- ELISA was conducted to evaluate the expressed antigen in silkworm. The results indicated that *A* value of the harvested haemolymph from silkworm infected with *Bm*-ORF decreased as dilution rate increased, which was in good agreement with variation of positive control of FMDV antigen. The antigen expressed in haemolymph was about half of that in the positive control (BHK-21 cell vaccine), but was undetectable in the negative control of *Bm*-BacPAK6 infected silkworm's haemolymph (Figure 2).

2.4 Observation of empty capsids

Empty capsid-like particles could be observed in haemolymph with a diameter size of about 30 nm (Figure 3). These structures were absent in the haemolymph infected with *Bm*-BacPak6. The identity of these particles will be confirmed by the immunomicroscopy observation.

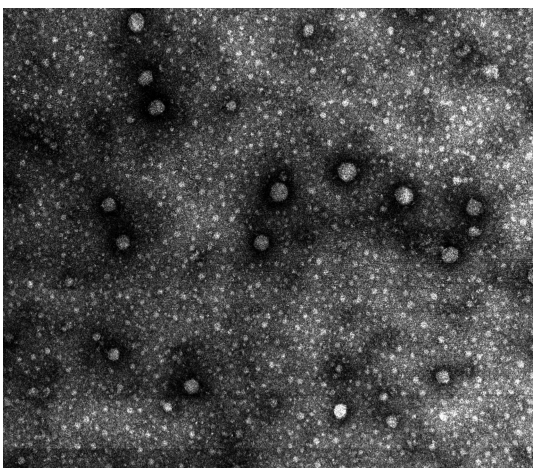

Figure 3 Empty capsid-like particles in the haemolymph infected with Bm-ORF (80000×).

2.5 The anti-FMDV antibody in cattle

The animals were vaccinated by the vaccine prepared from expressed antigens. LPBE-antibody titer was determined at 21dpv following LPBE method. It was found that all four cattle vaccinated with *Bm*-ORF antigen developed a detectable FMDV-antibody response. By 21 dpv, antibody titers reached 128 in cattle No 115. In contrast, valences of antibody in the two control cattle (vaccinated with vaccine prepared from *Bm*-BacPAK6) were not boosted (Table 1).

2.6 Challenge with FMDV Asia I/XJ

All animals were challenged with 10000 BID_{50} of Asia I/XJ at 21 dpv. Mouth and feet were observed for ten days to evaluate the incidence of disease. The results are shown in Table 1.

Table 1 FMDV- antibody response after vaccination with *Bm*-ORF's and clinical signs in cattle after challenge with Asia I/XJ

Animal number	Vaccine[a)]	LPBE-Antibody[b)] (21 dpv)	Lesion scores[c)] (dpc)				Protection[d)]
			3	5	7	10	
115	*Bm*-ORF	128	none	none	none	none	+
140	*Bm*-ORF	90	small vesicle at the site of inoculation	vesicle was limited	vesicle was limited	vesicle was limited	+
126	*Bm*-ORF	32	big vesicle at the site of inoculation	mouth	2+mouth	convalescence	–
141	*Bm*-ORF	45	big vesicle at the site of inoculation	2	2+mouth	convalescence	–
103	*Bm*-BacPAK6	<8	big vesicle at the site of inoculation, 4+mouth	4+mouth	convalescence	convalescence	–
117	*Bm*-BacPAK6	<8	big vesicle at the site of inoculation, 4+mouth	4+mouth	convalescence	convalescence	–

a) Bovines were vaccinated with vaccine prepared from expressed antigens (*Bm*-ORF) or the control (*Bm*-BacPAK6); b) FMDV-specific antibody titer reported as the serum dilution by LPBE method; c) the lesion score is the number of feet on which the cattle exhibited; d) protection was determined that cattle did not take on the clinical signs of FMD during observation period (10 dpc).

3 Discussion

The FMDV serotype Asia I was first isolated in Pakistan in 1954 and is epidemic within Southeast Asia and Indian peninsula, disseminating among Near East, Middle East and Far East[16], but in 2005, the disease broke out in China[17]. Since its simultaneous occurrence in two independent cattle fields, respectively in Tai'an, Shandong Province and Wuxi, Jiangsu Province, this epidemic quickly spread among 10 provinces, causing great economic losses. This outbreak attracted considerable public and scientific concern and made it an urgent need to develop safer and more effective FMD vaccine. Among the candidates that were used to develop subunit vaccines, VLP is a better choice.

A number of intermediate particles have been identified in FMDV-infected cells, including 5S protomers, 12S pentamers, and a particle with an uncleaved VP0 lacking RNA (75S empty capsid), and 140S virion. Among them, the empty capsids are as immunogenic as the 140S virion[3,4]. Though the mechanism of encapsidation and maturation is not clear, it provides us a better strategy to design a novel vaccine. Zhang et al.[18] constructed a recombinant adenovirus containing the polyprotein coding region of O-type FMDV, and the empty capsids were observed under electronmicroscope. The intact polyprotein of AsiaI-type FMDV in Bombyx mori larvae was expressed in current research by using baculovirus vectors and its antigenicity was detected by using IFA and sandwich-ELISA. Moreover empty capsid-like particles could be observed in haemolymph under electronmicroscope.

Thousands of diverse proteins have been expressed successfully in the baculovirus expression system, which has many advantages over others. For example it can contain approximately 10-kb foreign gene without affecting viral duplication and assembling[19]. So, several genes necessary for viral assembly could be simultaneously inserted into vector to produce the VLPs. The VLPs produced in baculovirus expression system was found to be safe and immunogenic. Therefore the system is a good candidate for producing VLPs[5,20−22]. Compared with AcNPV-*Sf* cell expression system, silkworm-baculovirus expression system has its own advantages. The silkworm larvae, which can be used for *in vivo* expression, and the expression yield in silkworm haemolymph could be at least 50 folds higher than that in the same volume infected cells. In addition, silkworm does not contain any pathogen that can crossly infect with vertebrate, and silkworm rearing is cost-effective (0.8 $ per 100 larva) in China. The production-cost is one of the important limited factors restricting new type subunit FMD vaccine in the market. In this study even a gene about 6.9 kb could yield high level of antigen in haemolymph, and the expression products were post-translationally processed and assembled well. This leads to the conclusion that it is feasible to employ silkworm-baculovirus expression system for large scale production of FMD vaccine.

To make vaccination useful as part of a control strategy in an FMD outbreak, a single inoculation would be required to induce protection. In this vaccine trial, we followed the bovine potency test protocol described by the OIE and used by vaccine manufacturers to test the traditional inactivated FMD vaccines with some modifications to test this subunit vaccine potency. Four cattle were vaccinated with the vaccine prepared from *Bm*-ORF's and two controls were vaccinated with vaccine prepared from *Bm*-BacPAK6's. Three weeks after

vaccination, the antibody level of the four vaccinated cattle has some ascension, while the control group remained low. After been challenged with virulent homologous virus, two of the *Bm*-ORF's subunit vaccine immunized cattle were considered to be protected, and the other two delayed the disease and eased the clinical symptom. But the controls developed lesions on all the feet and inside the mouth on the third day post infection. Since the cattle are the most susceptible cloven-hoof animal to FMDV and the challenge dose could be 10000 BID_{50}, the vaccine in cattle can hardly get ideal efficacy. Only adenovirus vectored vaccine known for its best protective effects can keep vaccinated cattle at 5/5[10]. But this vaccine is unacceptable in developing countries because of safety and preservation problems. The antibody titers are obviously different between animals in this experiment, which might be due to the following reasons. (1) The specific antigen produced in silkworm haemolymph per milliliter was still lower than that contained in traditional inactivated vaccine, thus failing to offer enough protection to animals. (2) The uneven distribution of antigen in the preparation process by manual preparation is likely to lead to the difference in immunity dosage to each animal. (3) Although the humoral immunity plays a main role in the course of FMDV anti-infection immunity, the factors of cellular immunity may also play a part in anti-virus immunity. To overcome these shortcomings and enhance the immune response, some work is yet to be done to improve the quantity of the expression products, such as concentrate antigen (or enhance efficiency of expression system), delete the unnecessary regions of ORF that were irrespective of synthesis, process and assemble viral structural proteins into empty viral capsids, and introduce cellular immunity factor for successful preparation of new type FMD.

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