Expression and Immunogenicity Assessment of The Immunocompetent FB Tandem Fragment of Foot-and-mouth Disease Virus*

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Abstract The immunocompetent tandem fragment FB of the foot-and-mouth disease virus (FMDV) genome was inserted into the expression vehicle pBAD/TOP, to yield an identified recombinant plasmid pBAD-FB, which was used to transform the host bacteria TOP10 and, after induction with different concentrations of the inductant arabinose for varied duration, samples of the expression product were subjected to SDS-PAGE and Western blot analysis. Results revealed that using a final concentration of 0.002% arabinose for induction, expression peaked at 4 h, yielding a product approximately 26 ku in size. Software scanning demonstrated that the FB fusion protein expressed accounted for 28.9% of total bacterial protein, could react specifically with FMDV antibody, and occurred both in the form of inclusion bodies and soluble protein. The soluble fraction of the fusion protein was purified with 50% Ni-NTA resin affinity chromatography, and the fusion protein inclusion bodies extracted. After washing both fractions were separately used to prepare oil-emulsion vaccines, with which guinea pigs were immunized subcutaneously. Neutralization test in suckling mice was employed to assess the neutralization index of the guinea pig serum, and foot-and-mouth disease virus was used to challenge the guinea pigs. The result proved that when the purified soluble product and inclusion bodies were used to immunize guinea pigs, they could induce a high titer of neutralizing antibodies, and provide 100% immunoprotection against virus challenge.

Key words foot-and-mouth disease virus, immunocompetence, tandem fragment, expression, immunogenicity

Foot-and-mouth disease (FMD) is an acute febrile highly contagious livestock infection, caused by foot-and-mouth disease virus (FMDV). For long, frequent outbreaks of FMD epizootics have occurred worldwide, eliciting huge economic loss. FMDV belongs to the genus Apthovirus and the family Picornaviridae, the virion consisting of a positive single-stranded RNA and nucleocapsid, with no envelope. The nucleocapsid is made up of 4 structural proteins VP1, VP2, VP3 and VP4, each comprised of 60 molecules, whereas the genomic RNA contains 8 500 nucleotides. Earlier studies have shown that the VP1 gene is located at nucleotides 2 977 through 3 615, encoding 213 amino acid residues, and that VP1 is the major component eliciting neutralizing antibodies. X-ray crystal diffraction studies have revealed that the three dimensional structure of the VP1 exists as protuberances exposed on the surface of the virion, thus assuming protuberance a critical role in immunity. VP1 carries critical antigen epitopes capable of inciting immunoreaction, of which the amino acid residues 140 through 160 and 200 through 213 are two major B cell epitopes that can elicit FMDV neutralizing antibodies, whereas amino acids residues 141 through 160 have at least one T cell epitope, which can evoke a FMDV-specific T cell reaction[1~4]. By using synthetic polypeptide corresponding to the amino acid residues 135 through 160 of VP1 from type 01 FMDV to immunize cattle, it has been demonstrated that the B cell antigen epitope inducing the generation of neutralizing antibodies lies at the residues 135 through 144, and the T cell epitopes at the residues 135 through 144, and 150 through 160[4].

In this study, a tandem fragment consisting of nucleotides coding for the VP1 amino acid residues 132 through 159 and 200 through 213 was synthesized in the order 132 through 159-200 through 213-132 through 159, with nucleotide linkers encoding 2 amino acids and 11 amino acids respectively inserted between the components. The tandem set was named FB, cloned in the pBAD/TOPO ThioFusion expression system, and the immune response mounted by guinea pigs against the expression product assessed.

1 Materials and Methods

1.1 Expression system and immunocompetent tandem fragment

The pBAD/TOP ThioFusion Kit was bought from Invitrogen Company. The immunocompetent tandem fragment FB was designed and synthesized in this laboratory, consisting of nucleotide sequences encoding the amino acid residues 132 through 159 and 200 through 213 of FMDV VP1 protein. In order to augment immunogenicity the two fragments were arranged in tandem order coding for amino acid residues 132 through 159-213 through 213-132

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through 159, with a linker fragment encoding 2 amino acid residues and another encoding 11 residues inserted between the tandem components.

1.2 Virus strain, serum and experimental animals

Type O FMDV is preserved in this laboratory.

Swine type O FMDV positive serum was kindly provided by Guangdong Provincial Veterinary Disease Prevention and Quarantine Station.

Guinea pig FMDV antiserum was prepared in this laboratory.

Guinea pigs weighing about 500 g and Kunming strain 3 to 5-day-old suckling mice were purchased from the Guangdong Military Medicine Research Institute.

1.3 Enzymes and other reagents

Ex Taq DNA polymerase (5 U/μl), dNTPs (2.5 mmol/L each), DNA Marker DL 2000 were TaKaRa products; EZNA miniprep kit and EZNA Gel Extraction Kit were Omega products; IgG L-arabinose, and anti-guinea pig IgG (secondary antibody) were procured from Sigma Co.; Precision Protein Standards, Prestained, broad Range was a Bio-Rad product; Anti-swine IgG (secondary antibody) and nitrocellulose filter were bought from Gene Co.

1.4 Construction of expression vector

Based on the sequence of the tandem fragment a pair of primers was designed, namely FB1: GGTAGCAGTAAGTACGGTGACAC and FB2: CAACGTGCGCTCTGCCTTCTGAG, which were used to obtain the FB fragment contained in the plasmid MB by amplification. After recovery and tailing the FB fragment was ligated to the expression vector pBAD/TOP, and the ligated product utilized to transform the host bacteria TOP10. The transformant was subjected to PCR identification and nucleotide sequencing, and a recombinant expression vehicle pBAD-FB with correctly oriented open reading frame acquired.

1.5 Induced expression of FB fragment

Bacteria containing pBAD-FB were grown in LB medium with ampicillin added until an A_{600} value of about 0.5 was reached, when L-arabinose in concentrations of 0.2%, 0.02%, 002%, and 0.000 2% was added to induce expression; bacterial suspension was withdrawn after 4 h induction period for SDS-PAGE identification of expression product.

1.6 Western blotting of fusion protein

Western blot analysis of the fusion protein was done according the method of Wang *et al.*[5] using FMDV positive serum.

1.7 Solubility analysis of fusion protein

Bacteria that have expressed after induction were collected and subjected to 400 W ultrasonic disintegration on ice bath for 5 s at a time for 40 times with intervals of 8 s, followed by 12 000 r/min centrifugation at 4℃ for 20 min, after which the supernatant was used for SDS-PAGE analysis.

1.8 Purification of the fusion protein soluble fraction and extraction of inclusion bodies

The supernatant resulting from ultrasonic disintegration and centrifugation was purified by 50%-NTA resin affinity chromatography, the eluant being collected. The precipitate was subjected to several cycles of ultrasonic washing, after which it was re-suspended in PBS.

1.9 Animal immunization and challenge

To purified soluble fusion protein and inclusion bodies were separately added Freund's adjuvant to prepare vaccines, which were used to immunize guinea pigs twice by subcutaneous inoculations two weeks apart. Two weeks after the second inoculation, blood was collected by cardiac puncture and serum separated. Neutralization test using suckling mice was done to assess the neutralization index of the serum, and FMDV challenge was used to determine the antiviral resistance developed by the vaccinated guinea pigs.

2 Results and analysis

2.1 Cloning and identification of FB fragment in pBAD/TOP

After recovery and tailing, the PCR amplified FB fragment was ligated to pBAD/TOP yielding the recombinant plasmid pBAD-FB. The ligated product was used to transform competent TOP10 bacteria, isolated colonies picked for further cultivation; the bacterial suspension was then utilized as template in PCR identification and when an approximately 261 bp sized fragment (Figure 1) resulted, the bacteria was determined a positive clone. Due to the fact that the

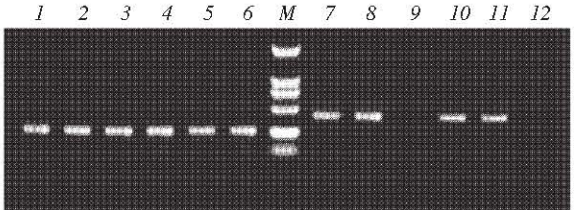

Fig. 1 PCR amplification of recombinant plasmids

M: DNA marker DL 2000; *1 ~ 6*: First PCR amplification of 6 recombinant plasmids; *7 ~ 12*: Second PCR amplification of 6 recombinant plasmids.

PCR product and pBAD/TOP were ligated by T-A tailing, two orientations of the inserted foreign gene would occur. In order to determine whether or not the insert was in the correct orientation, a primer, PA: GATTTAATCTGTATCAGG, located downstream of the foreign insertion site was synthesized, and used in conjunction with the foreign gene upstream primer to

perform a second PCR amplification. Of the six positive clones with FB inserts, four colonies gave the expected fraction (Figure 1) on the second PCR amplification, attesting that their insertion was in the correct orientation. The positive bacterial clone was named TOP-pBAD-FB.

2.2 Expression of recombinant plasmid pBAD-FB in TOP10 host

Optimization of expression conditions was achieved by letting different concentrations of inductant act for different duration; the SDS-PAGE result displayed in Figure 2 demonstrated that L-arabinose is a good inductant for the expression of FB fusion protein, and that the expressed product was about 26 ku, conforming to expectation. A final concentration of 0.000 02% arabinose could induce the expression of FB fusion protein but the level was not high, whereas 0.002% arabinose gave a relatively high level of expression that tend to stabilize with further increase in inductant. Scanning analysis of the electropherogram with the BioID ++ program of the VL gel imaging system revealed that at peak expression level the target protein FB accounted for 28.9%, of the total bacterial protein, the amount of fusion protein increasing gradually and tended to plateau off after 4 h.

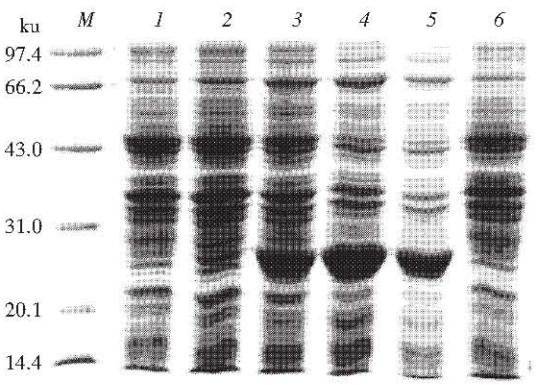

Fig. 2 SDS-PAGE analysis of the FB expression product induced by different concentration of arabinose

M: Protein molecular mass (standard); *1* ~ *5*: Disintegrated expression products of *E. coli* containing pBAD-FB after induction by arabinose of low to high concentration; *6*: Expression products of *E. coli* containing pBAD (control).

2.3 Purification of expression product

The supernatant acquired by ultrasonic disintegration of the TOP-pBAD-FB bacteria and subsequent centrifugation was purified by 50% Ni-NTA resin affinity chromatography; SDS-PAGE analysis of serial aliquots of the elutant disclosed only one protein band, whose size conformed with that of the FB fusion protein (Figure 3), proving that 50% Ni-NTA purification could achieve high purity of FB fusion protein.

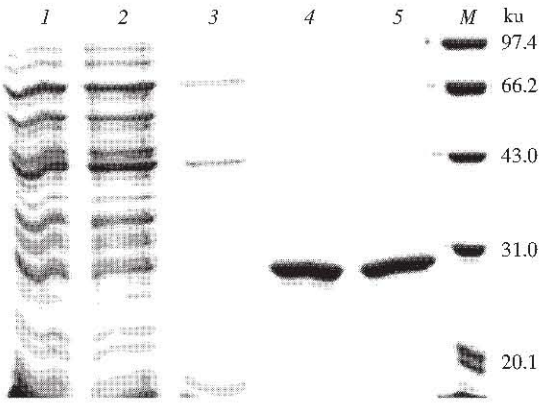

Fig. 3 SDS-PAGE analysis of FB purified products

M: Protein molecular mass (standard); *1*: Lysis buffer eluant; *2* ~ *3*: Successive wash buffer eluant; *4* ~ *5*: Target protein eluant.

2.4 Western blotting of expression product

Western blot analysis of the fusion protein FB using swine anti-FMDV hyper-immune serum and guinea pig anti-FMDV hyper-immune serum prepared in our laboratory revealed, on two attempts, a specific band that conformed to the size of the fusion protein (Figure 4), which demonstrated that the fusion protein could react with specific antibody, and indicating that it possessed good immunogenicity.

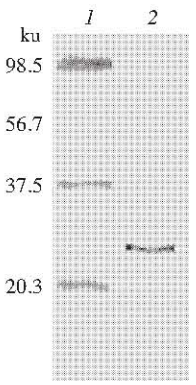

Fig. 4 Western blot analysis of expressed products

1: Prestained protein standards; *2*: Expressed products.

2.5 Dose-related response of guinea pig to immunogen

Table 1 displays the serum neutralization index of guinea pig immune serum. Two weeks after guinea pigs were immunized with 20 μg of FB fusion protein inclusion bodies and purified soluble protein, neutralizing antibodies could be detected, and after 2 immunizations the indices of the serum neutralizing antibodies were 1.96 and 3.04 respectively.

Challenge with type O FMDV was done to assess the antiviral resistance of the immunized guinea pig. The result showed that all the guinea pigs immunized

with the inclusion bodies and purified soluble protein of FB fusion protein did not develop disease, whereas all the non-immunized guinea pig controls became sick (Table 2), attesting that the immunized guinea pigs possessed very strong resistance to the challenge virus.

Table 1 Neutralizing antibody titers in immunized guinea pigs

Groups	Number of animals	Weeks post inoculation	
		2	4
Inclusion body	4	1.27	1.96
Purified soluble protein	4	2.0	3.04
Control	4	0	0

Table 2 Protection of immunized guinea pigs against virus challenge

Groups	Number protected /Number of animals
Inclusion body	4/4
Purified soluble protein	4/4
Control	0/4

3 Discussion

Research on the antigenic structure of FMDV has provided the theoretical basis for developing peptide vaccines, and most earlier work in designing peptide vaccines was directed at B cell epitopes on the nucleocapsid of the virus. The G-H loop is the most important B cell epitope, and utilizing a peptide stretch of the G-H loop for immunization could elicit neutralizing antibody in guinea pigs, and also could confer protection on swine. Based on the gene sequence of O1K VP1, Bitlle chemically synthesized amino acids 140 through 160 and conjugated them to a carrier protein, which he found could induce protective neutralizing antibodies in guinea pigs[6], and subsequently many scholars obtained identical results by using synthesized peptides in trials on guinea pigs and other small animals. It was discovered later that the immunogenicity of the synthetic peptides was weak due to its small size, making them prone to degradation and also difficult to assume a stereometric configuration; this necessitated repeated inoculation before resistance to virulent virus challenge could be attained, and so many studies have come to focus on the augmentation of immunogenicity. Currently it is acknowledged that fusion protein of small molecular antigens and large molecular vehicle can effectively induce immune response in animals. After making a tandem sequence of the VP1 major antigen epitopes, Yang *et al.* in China, ligated it to β-galactosidase gene to construct a recombinant plasmid which was expressed in *E. coli*, and they found that vaccine prepared from the fusion protein could provide resistance in immunized animal against virus challenge. Small molecular peptide antigen conjugated with large molecular vehicle can avoid degradation of the small peptide and increase stability, and also the augmentation of molecular mass favors more effective induction of immune response in the animal. It was found also that the amino acid 200 through 213 sequence had potential significance in bolstering immune response of the amino acids 140 through 160 sequence. Furthermore, peptide containing the two sequences evoked a stronger immune response than either of the sequences alone when the monoclonal neutralizing antibody level is compared with that mounted against the complete virus.

In this work synthetic tandem fragment FB expressed as a fusion product with thioredoxin plus the addition of Freund's adjuvant to make an oil emulsion vaccine, induced high level of neutralizing antibody in vaccinated guinea pigs, and furnished 100% protection against virus challenge. It is a limitation because another synthetic tandem fragment and VP1 were processed in immunization test at the same time, the number of test was large so that the number of guinea pig was limited. It would be considered in the next immunization test of pigs. On the other side, T cell proliferative response was not processed in this work because the guinea pigs were not enough to be killed to get the spleen cell.

In 1997 You *et al.* utilized the fusion protein of VP1 major immunocompetent peptide and pXZ500 to compare the immunization efficacy of the purified soluble protein and inclusion bodies forms in swine and guinea pigs. The result demonstrated that the efficacy was good and almost the same in both species, indicating that inclusion bodies could entirely replace purified soluble protein as immunogen. Subsequently they prepared vaccine from the inclusion body form and achieved satisfactory results in field trials . In this study, vaccine prepared from fusion protein inclusion bodies could also induce protective neutralizing antibodies in immunized guinea pigs, demonstrating that FB fusion protein inclusion bodies can replace purified soluble protein as immunogen. Clinical usage of inclusion bodies as immunogen can dispense with complicated and tedious post-finishing procedures, lowering production costs, and thus of great significance in field application.

References

1 Collen T, Dimarchi R, Doel T R. T cell epitope in VP1 of foot-and- mouth disease virus is immuno- dominant for vaccinated cattle. J Immunol, 1990, **146** (5): 749 ~ 755

2 van Lieop MJC, van Maanen K, Melonen R H, *et al.* Proliferative

lymphocyte response to foot- and- mouth disease virus and three FMDV peptides after vaccination or immunization with these peptides in cattle. Immuonlogy，1992，**75**（3）：406～413

3 Wong H T，Cheng S C S，Chan E W C，*et al*. Plasmids encoding foot- and- mouth disease virus Vp1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. Virology，2000，**278**（1）：27～35

4 Zamorano P，Wigdorovitz A，Peter- Filguerira M，*et al*. A 10-amino- acid linear sequence of VP1 of foot- and-mouth disease virus containing B and T cell epitopes inducesprotection in mice. Virology，1995，**212**（2）：614～621

5 汪家政，范 明. 蛋白质技术手册. 北京：科学出版社，2000. 166～183
Wang J Z，Fan M. Protein Technical Manual. Beijing：Sciences Press，2000. 166～183

6 Bittle J L，Houghten R A，Alexander H，*et al*. Protection against foot- and- mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequences. Nature，1982，**298**（5935）：30～35

7 李光金，严维耀，徐泉兴，等. 以猪 IgG 重链恒定区为抗原载体的抗口蹄疫病毒的 DNA 疫苗的研制. 生物工程学报，2001，**17**（3）：322～324
Li G J，Yan W Y，Xu Q X，*et al*. Bioengineering，2001，**17**（3）：322～324

8 尤永进，徐泉兴，赵洪兴，等. “O”型口蹄疫病毒生物合成多肽苗保护性抗体的免疫应答. 上海农业学报，1997，**13**（4）：1～3
You Y J，Xu Q X，Zhao H X，*et al*. Shanghai J Agri Sci，1997，**13**（4）：1～3

口蹄疫病毒免疫活性串联片段 FB 的表达及免疫原性测定*

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摘要 将口蹄疫病毒（FMDV）免疫串联片段 FB 克隆至原核表达载体 pBAD/TOPO 中，经鉴定后得到重组质粒 pBAD-FB，将此重组质粒转化到受体菌 TOP10 中，用诱导剂阿拉伯醛糖分别以不同的浓度进行诱导，并在不同诱导时间进行采样，经处理后做 SDS-聚丙烯酰胺凝胶电泳（SDS-PAGE）、蛋白质印迹分析. 结果发现以终浓度为 0.002% 的阿拉伯醛糖进行诱导，4 h 后表达可达到高峰，其大小约为 26 ku，软件扫描结果显示，FB 融合蛋白的表达量占细菌总蛋白的 28.9%，能与抗 FMDV 抗体发生特异性反应，融合蛋白以包涵体和可溶形式存在. 将融合蛋白的可溶性组分用 50% Ni-NTA 树脂过柱纯化并抽提融合蛋白的包涵体，经过洗涤后分别制成油乳剂疫苗，经皮下注射免疫豚鼠，用乳鼠中和试验测定豚鼠血清中和指数，并用口蹄疫病毒对豚鼠进行攻毒. 结果表明，用此融合蛋白的纯化产物和包涵体免疫豚鼠能诱导产生高滴度的中和抗体，对病毒的攻击提供 100% 的免疫保护.

关键词 口蹄疫病毒，免疫活性串联片段，表达，免疫原性
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