An Applicable Method for Mapping Epitopes on Viral Protein*

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Abstract By the means of overlapping peptides expressed in *Escherichia coli* in combination with Western blotting, two linear epitopes were identified on non-structural protein 3ABC of foot-and-mouth disease virus (FMDV), which covering amino acid residues 106 ~ 155 and 156 ~ 190 on 3ABC. The epitopes gave positive reaction with sera from pigs or guinea pigs infected with different serotypes of FMDV, but not with sera from vaccinated or naive animals. The method based on expressed peptide for mapping epitope on viral protein was reliable and applicable.

Key words epitope mapping, foot-and-mouth disease virus, non-structural protein

Viral epitope is useful for developing new vaccines and site-directed serological diagnosis. Method used now for mapping epitopes of virus protein is mainly based on synthetic peptide[1].

Foot-and-mouth disease virus (FMDV) is a causative agent of highly infectious and economically important disease in cloven-hoofed animals. Identifying animals that have been infected with foot-and-mouth disease virus is important because it is well established that infected cattle and sheep frequently become carriers of the virus and consequently may become the source of new outbreak of the disease. In non-vaccination countries, the infected animals can easily be stamped out by detecting structural protein antibodies in them. But situation is compromised by the difficulty in distinguishing infected animals from those that have been vaccinated against the disease since both groups contain structural antibodies in their sera. It is known that currently available inactivated FMDV vaccines mainly consist of capsid proteins, thus being less likely to induce antibody response to non-structural protein (NSP) *in vivo* after vaccination. The detection of antibody to NSP is therefore, the preferred diagnostic method to distinguish virus- infected carrier from vaccinated animals. The polyprotein 3ABC was immunogenic and abundantly expressed during infection and proved by several groups to be the most reliable marker of viral replication[2~7]. The polyprotein 3ABC consists of 3A, 3B and 3C. Some groups had observed that anti-3A antibodies were present in some of the sera from vaccinated swine and cattle[1,5]. This may be due to two reasons: (1) there might be 3A protein left in vaccine; (2) antigenic sites, which result in non-specific reactivity, might exist on 3A. So it is necessary to screen more specific infection-related epitopes on 3ABC. By the means of overlapping peptides expressed in *E. coli* in combination with Western blotting, two immunodominant epitopes specific for FMDV infection were localized on 3ABC, which covered amino acid residues 106 ~ 155 and 156 ~ 190.

1 Materials and methods

1.1 Serum samples

The infected sera were collected from 5 pigs between 20 to 40 days after experimental infection with FMDV of serotype O. The convalescent guinea pig sera of FMDV serotype A and AsiaI were from Lanzhou Veterinary Research Institute.

Sera of vaccinated animals were collected from pigs in FMD free herds in Shanghai, which had been inoculated with a standard dose of commercial vaccine of serotype O for twice. The FMDV commercial vaccine were manufactured in Lanzhou Biomedicine Factory, Lanzhou.

1.2 Construction of recombinant expression plasmids carrying overlapping DNA fragments of 3ABC

The 3ABC gene of a FMDV strain of serotype O had been cloned into pMD18-T vector (Takara) and the plasmid pMD18T-3ABC served as a template for the following PCRs. The full-length 3ABC gene was dissected into 12 overlapping DNA fragments by PCR kit(Takara,). Figure 1 showed the construction map of 3ABC fragments (represented with F1 ~ F12). Each fragment was amplified with a forward primer containing a *Bam*H Ⅰ restriction site and a reverse primer with a stop codon and a *Hind*Ⅲ restriction site. After digested with *Bam*H Ⅰ and *Hind*Ⅲ (Takara), the PCR products were cloned into expression vector, pET-32a(+) (Novagen).

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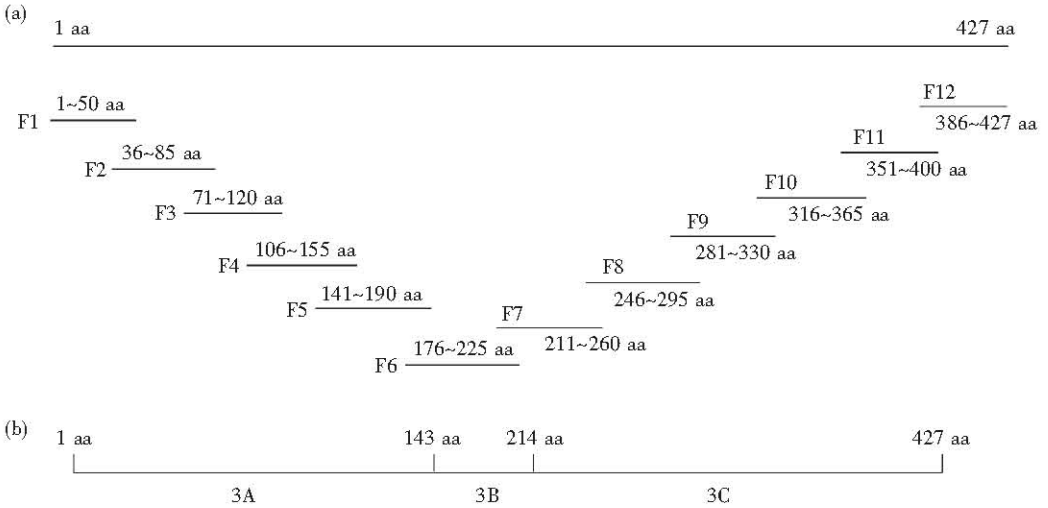

Fig. 1　The construction of FMDV 3ABC fragments (a) and structure of 3ABC protein (b)

(a) The 3ABC protein was truncated into 12 overlapping fragments (F1 ~ F12) and each fragment was expressed as Trx-fusion protein in *E. coli* BL_{21}(DE3). The numbers represented the position of amino acids. (b) 3A: 1 ~143aa; 3B: 144 ~214aa; 3C: 215 ~427aa.

1.3　Expression and purification of TrxA-fusion proteins in *E. coli*

Recombinant expression plasmids carrying overlapping DNA fragments of 3ABC gene were transformed into *E. coli* BL21 (DE3) respectively. Bacteria were cultured in luria-Bertani (LB) medium with ampicillin (100 mg/L). An overnight bacterial culture was diluted to 1∶ 100 in LB medium and grown at 37℃ until A_{600} reached 0.5. Induction from the T7 promoter was achieved by the addition of 1 mmol/L isopropyl -B-D- thiogalactoside (IPTG), and an additional 4 h of induction was preformed. The induced bacterial cells were harvested by centrifugation at 10 000 *g* for 5 min. The pellets were washed with phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.9), 0.5 mol/L NaCl, 5 mmol/L imidazole. Following sonication, total bacterial lysates were centrifuged at 20 000 *g* for 20 min at 4℃. Target proteins had been determined to be expressed in soluble forms before purification, so the supernatants were collected and purified by affinity chromatography on a Ni-IDA-Sephorose 6B column.

1.4　Epitope mapping

The purified TrxA-fusion proteins and TrxA were separated by electrophoresis on 12% SDS-polyacrylamide gel (SDS-PAGE) using a discontinuous Tris-glycine buffer system. These proteins, resovled on a 12% SDA-PAGE, were electrophoretically blotted onto nitrocellulose filters and examined for serological reactivities to convalescent sera of type O, AsiaI or A. Western blotting procedures were performed as previously described[8]. The convalescent sera were used as the primary antibody and diluted to 1∶200. Horseradish peroxidase (HRP)-conjugated rabbit anti-guinea pig or pig antibodies (Sigma) were diluted 5000-fold or 6000-fold respectively prior to use as secondary antibodies; the HRP substrate, DAB (3,3′-diaminobenzidine tetrahydrochloride) (Fluka) and H_2O_2, were used for color development. The control sera were obtained from pigs vaccinated twice with commercial vaccine of serotype O or a healthy guinea pig.

1.5　ELISA protocal for testifying the epitopes

The TrxA-fusion proteins showing positive reactivity with FMDV convalescent sera were diluted to 5.0 mg/L respectively in coating buffer (0.015 mol/L carbonate/bicarbonate pH 9.6) and bound to 96 well microtitre plates with 50 μl by overnight incubation at 4℃. Purified TrxA were used as control. Plates were rinsed with phosphate buffered saline containing 0.05% Tween 20 (PBST). The coated wells were blocked by 150 μl blocking buffer (PBST containing 3% non-fat dried milk) at 37℃ for 2 h. Washed three times with PBST after blocking, a 1∶200 dilution of each test sample in blocking buffer was reacted in duplicate. 50 μl of the diluted samples were added to each well and incubated for 2 h at 37℃. 15 vaccinated and 15 convalescent swine sera samples were used for immunoassay. The wells were then washed three times with PBST. Bound pig IgG was detected by incubation with rabbit anti-swine antiserum conjugated with peroxidase (Sigma) at an optimal dilution of 1∶6 000. A solution of 0.4 g/L of O-Phenylenediamine dihydrochloride (OPD) containing 0.05% H_2O_2 was used as the chromogen/substrate mixture and color

development. Reaction was stopped after 15 minute by addition of 1.25 mol/L H_2SO_4. The absorbance of each well was read at 492 nm (A_{492}). Statistical analyses were performed by using SAS software 6.12.

2 Results

2.1 Expression and purification of TrxA-fusion proteins in *E. coli*

Overlapping fragments of full-length 3ABC protein were expressed in *E. coli* (Figure 2). The recombinant proteins were purified using Ni^{2+}-IDA-Sepharose 6B affinity columns (Figure 3).

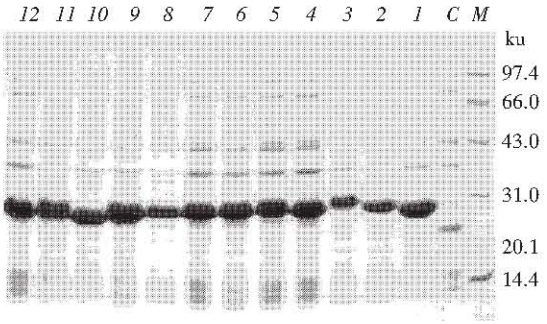

Fig. 2 SDS-PAGE of TrxA fusion proteins expressed in *E. coli*

M: Low molecular mass protein marker; *C*: Control; *1 ~ 12*: Expression product of Trx- F1 to F12.

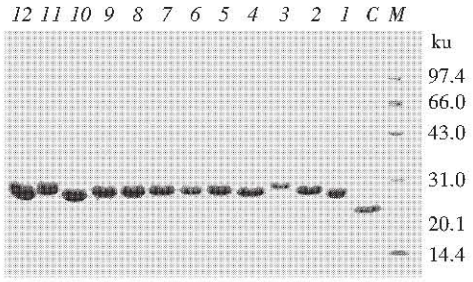

Fig. 3 SDS-PAGE of the purified fusion proteins

M: Protein marker; *C*: Purified TrxA. *1 ~ 12*: Proteins from TrxA- F1 to F12.

2.2 Epitopes identified by Western blotting

When purified Trx-fusion proteins reacted with convalescent swine sera of serotype O, TrxA-F4, F5 gave strong positivity (Figure 4a), whereas the control sera from vaccinated swine did not react with all the fusion proteins. F4, F5 also reacted with sera of serotype AsiaI and A from infected guinea pig (Figure 4b, c) and none of the overlapping fragments react with the naive guinea pig serum. So, F4 (105 ~ 155aa) and F5 (140 ~ 190 aa) could be the candidate linear epitopes on 3ABC protein.

Since F4 and F5 were adjacent and the overlapping peptide may be the common eptitope of them, another fusion protein was constructed with 15 amino acids deletion at N terminal of F5 and it was called TrxA -F5′. The new fusion proteins also showed positive reactivity when reacted with the convalescent sera by Western blotting (Figure 5), but negative with vaccinated swine sera or normal guinea pig sera.

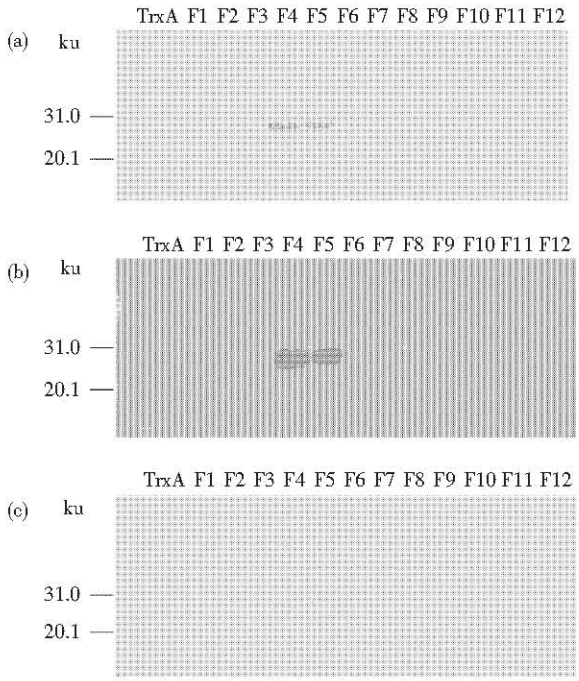

Fig. 4 Identification of antigenic sites on 3ABC by Western blotting

When purified TrxA-fusion proteins reacted with convalescent sera of serotype O, A and Asial, F4 and F5 were positive. (a), (b) and (c) represented serotype O, Asial and A respevtively. F1 to F12 represented the purified fusion proteins from TrxA-F1 to F12. TrxA represented purified thioredoxin.

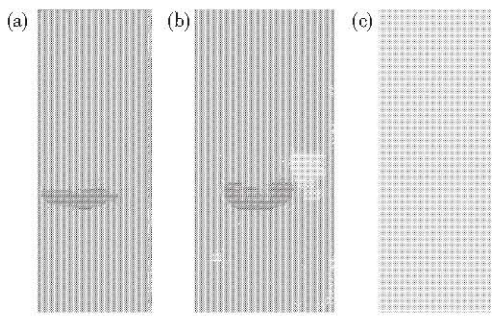

Fig. 5 Identification of antigenicity of TrxA-F5′ by Western blotting

(a), (b) and (c) represented reactivity of TrxA-F5′ with serotype O, Asial and A respevtively.

2.3 Immunoassay for testifying the infection - specific epitopes

The two epitopes found by Western blotting were also testified by ELISA. As shown in Table 1, there

Table 1 A_{492} **of ELISA for TrxA, TrxA-F4 and F5′**

	TrxA		TrxA-F4		TrxA-F5′	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Vaccinated sera	0.240	0.047	0.117	0.047	0.208	0.081
Convalescent sera	0.272	0.092	0.645	0.192	0.713	0.183

s: standard deviation.

were significant differences between the positive and negative populations when TrxA-F4, F5′ were used as coating antigen for ELISA ($P < 0.05$), whereas there existed no difference between vaccinated and convalescent sera when the plate was coated with TrxA ($P > 0.05$). So, F4, F5′ were definitely the infection specific epitopes on 3ABC and their amino acid sequences were listed in Figure 6.

2.4 Amino acid Sequence of two infection-connected fragments(F4, F5′)

Amino acid sequence of two infection-connected fragments were showed in Figure 6.

(a) ASGASAVGFRERSPTEQKTCDDVNTEPVVPGREQPRAEGPYAGPLERQKP

(b) LKVRARLPQQEGPYAGPMERQKPLKVKAKAPVVKE

Fig. 6 Amino acid sequences of epitopes

(a) F4; (b) F5′.

3 Discussion

By overlapping peptides expressed in *E. coli* combined with Western-blotting, two infection-related immunodominant epitopes were identified on non-structural protein 3ABC of FMDV. The epitopes were further testified to be infection-specific by indirect ELISA. There were clear differences between the convalescent and vaccinated sera when TrxA-F4, F5′ were used as coating antigen for ELISA, so the antigenic determinants could be used for setting up site-directed serology to differentiate FMDV infected animals from vaccinated ones. The assay directly targeted at infection-related epitope has some advantages since the epitope sequence was specific for FMDV infection, whereas the region beyond the epitope on 3ABC that can result in non-specific reaction is removed.

There are often two kinds of plasmids for high-level expression of peptide sequences in prokaryotic expression system. One is pET-32 series (Novagen company), the other is pGEX series (Pharmacia company). Fused with the foreign proteins, such as thioredoxin (TrxA) or glutathione S- transferase (GST), short peptide (<50aa) could be expressed in *E. coli*. In our study, the plasmid pET-32a(+) was used for producing fusion proteins. A 109 aa TrxA tag was fused with the N-terminals of overlapping peptides. Since the epitopes were identified under denaturing condition, they were considered as linear rather than conformatonal epitopes, just like epitopes found by synthetic peptide.

The locations of the two immunodominant epitopes identified in our study were similar to those found by Shen et al. [9] by means of synthetic peptide based on FMDV A12 strain. This means the method based on expressed peptide for mapping epitopes is reliable and applicable, without complex equipment such as peptide synthesizer.

Sequence alignments showed that the antigenic regions on 3ABC is conserved among different FMDV strains. Sera of different serotype from different animals can recognize the antigenic regions. Therefore, it is a potential target to monoclonal antibody and RNA interference.

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一种实用的筛选病毒抗原表位方法的建立*

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摘要 采用基因分段克隆、表达结合蛋白质印迹，筛选到了口蹄疫病毒非结构蛋白3ABC上高结合力、保守的感染相关线性表位，分别位于3ABC蛋白上第106～155和156～190位氨基酸. 这两个表位可与感染不同血清型口蹄疫病毒动物康复血清反应，但不与来自健康免疫动物和未接触病毒动物的血清发生反应. 实验表明，用基因工程表达的多肽筛选抗原表位的方法是可行的.

关键词 表位，口蹄疫病毒，非结构蛋白
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