VIROLOGICA SINICA, February 2010, 25 (1):18-26
DOI 10.1007/s12250-010-3041-4

B Cell Epitopes within VP1 of Type O Foot-and-mouth Disease Virus for Detection of Viral Antibodies*

Shan-dian GAO, Jun-zheng DU, Hui-yun CHANG**, Guo-zheng CONG, Jun-jun SHAO, Tong LIN, Shuai SONG and Qing-ge XIE

(National Foot-and-mouth Disease Reference Laboratory, Key Laboratory of Animal Virology of Ministry of Agriculture, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China)

Abstract: In this study, the coding region of type O FMDV capsid protein VP1 and a series of codon optimized DNA sequences coding for VP1 amino acid residues 141-160 (epitope1), tandem repeat 200-213 (epitope2 (+2)) and the combination of two epitopes (epitope1-2) was genetically cloned into the prokaryotic expression vector pP_{RO}ExHTb and pGEX4T-1, respectively. VP1 and the fused epitopes GST-E1, GST-E2 (+2) and GST-E1-2 were successfully solubly expressed in the cytoplasm of *Escherichia coli* and Western blot analysis demonstrated they retained antigenicity. Indirect VP1-ELISA and epitope ELISAs were subsequently developed to screen a panel of 80 field pig sera using LPB-ELISA as a standard test. For VP1-ELISA and all the epitope ELISAs, there were clear distinctions between the FMDV-positive and the FMDV-negative samples. Cross-reactions with pig sera positive to the viruses of swine vesicular disease virus that produce clinically indistinguishable syndromes in pigs or guinea pig antisera to FMDV strains of type A, C and Asia1 did not occur. The relative sensitivity and specificity for the GST-E1 ELISA, GST-E2 (+2), GST-E1-2 ELISA and VP1-ELISA in comparison with LPB-ELISA were 93.3% and 85.0%, 95.0% and 90%, 100% and 81.8%, 96.6% and 80.9% respectively. This study shows the potential use of the aforementioned epitopes as alternatives to the complex antigens used in current detection for antibody to FMDV structural proteins.

Key words: Foot-and-mouth disease virus (FMDV); Serology; Epitope ELISA

Foot-and-mouth disease (FMD) is a highly contagious and economically significant disease of cattle, pigs, sheep, and wild cloven-hoofed species, which ranks first in the list of infectious diseases of animals published by the Office International des Epizooties (OIE; World Organization for Animal Health)[2, 11]. The causative agent of this disease, Foot-and- mouth disease virus (FMDV), is classified in the genus *Aphthovirus* within the family *Picornaviridae* and exists as seven immunologically distinct serotypes O, A, C, SAT1, SAT2, SAT3 and Asia1 which are further divided into antigenic subtype and molecular

Received: 2009-03-23, Accepted: 2009-10-22

* Foundation item: National key Technology R&D Program (2006BAD06A14).

** Corresponding author.
Phone: +86-931-8342052, E-mail: changhuiyun@126.com

topotype subdivisions [7]. Vaccination against FMD is now a key strategy in the control of the disease in addition to slaughter and movement restrictions in the event of an outbreak. Thus, serological tests are particularly useful for confirming previous or ongoing infection in non-vaccinated animals and for monitoring the immune status as well as discriminating vaccinated and infected animals on a herd basis. Currently, enzyme-linked immunosorbent assay, mostly with purified preparations of FMDV viral structural proteins (SPs) or with recombinant viral nonstructural proteins (NSPs) to detect antibodies to the SPs or the NSPs, have been adopted by a large number of laboratories worldwide for routine serum screening, and some of these tests have been already used routinely at a large scale and are now on the market [1, 3, 19].

Overall antigenic diversity as measured by ELISA and virus neutralization tests appears to be reflected genetically in the VP1 gene, which is one of four structural proteins encoded within the P1 region of the genome. VP1 is immunogenically important as it contains the cell receptor site (RGD) on the GH loop (residues140-160) as well as at the C-terminus (residues 200-213) [22]. The precise location of B cell epitopes of the capsid protein VP1 has been established, flanking amino acid residues 135 - 160 and 200 - 213 [18, 21].These areas have been extensively used as synthetic peptides or as fusion proteins in the formulation of experimental immunogens for the induction of neutralizing antibodies and protection of natural and experimental hosts[5, 8, 23, 27, 28]. Combinations of these epitopes may be ideally suited as replacements of the complex protein antigens used in diagnostics. In order to determine whether the epitopes within the VP1 capsid protein could contain sufficient immunogenic information to replace the conventional antigens in FMDV diagnostics, the epitopes were expressed separately and in combination as fusion proteins in *Escherichia coli* and evaluated for their utility to detect antibody to FMDV structural proteins.

MATERIALS AND METHODS

Antisera and sera samples

Guinea pig antisera to FMDV strains of type A, C and Asia1 was provided by the National FMD Reference Laboratory; Individual pig sera positive and negative to FMDV of type O or swine vesicular disease virus (SVDV) was stored in our laboratory. 80 field pig serum samples were collected from pig farms and used in epitope-ELISA and LPB-ELISA.

Construction of expression plasmids

The studied epitopes covered amino acid residues 141-160 and 200-213 of VP1. These residues had the highest conservation in the VP1 region of type O FMDV. Three panel of primers for the epitope coding fragments with pre-nicked restriction sites and primers for VP1 coding region were synthesized by the Sangon Company (Shanghai). Pre-nicked DNA double-strands coding for epitope1 or epitope2 were generated by annealing primerE1F and primer E1B or primer E2F and primerE2B respectively (Table 1). Primer E2F2 and primer E2B2 were specially designed to form double-strands with pre-nicked *Sal*I and X*ho* I restriction sites and a link sequence (*GGTGGTGGTGGTTCC*) (Table1), which could be inserted after the coding sequence of epitope1 or epitope2 to form a complex epitope or a tandem repeat of epitope2. The link sequence coded a flexible peptide that consisted of four glycines and one serine to help to display the epitope.

Table 1. Primers for generating epitopes

Epitopes	Primer Nucleotide sequences
Epitope1	PrimerE1F *Bam*H I
	5'-GATCCGTGAGCAACGTGAGGGGTGACCTTCAAGTGTTGGCTCAGAAGGCAGAAAGAGCTCTGCCCGG-3'
	primerE1B *Sal*I
	3'-GCACTCGTTGCACTCCCCACTGGAAGTTCACAACCGAGTCTTCCGTCTTTCTCGAGACGGGCCAGCT-5'
AA	V S N V R G D L Q V L A Q K A E R A L P
Epitope2	PrimerE2F *Bam*HI
	5'-GATCCAGACACAAGCAGAAGATCGTGGCACCCGCAAAACAGCTTCTGGG-3'
	primerE2B *Sal*I
	3'-GTCTGTGTTCGTCTTCTAGCACCGTGGGCGTTTTGTCGAAGACCCAGCT-5'
AA	R H K Q K I V A P A K Q L L
Epitope2 with a linker	PrimerE2F2 *Sal*I
	5'-TCGACTC*GGTGGTGGTGGTTCC*AGACACAAGCAGAAGATCGTGGCACCCGCAAAACAGCTTCTGC-3'
	primerE2B *Xho*I
	3'-GAG*CCACCACCACCAAGG*TCTGTGTTCGTCTTCTAGCACCGTGGGCGTTTTGTCGAAGACGAGCT-5'
AA	G G G G S R H K Q K I V A P A K Q L L
VP1 (+)	5'-CCGGGATCCACCACCTCCACAGGTGAGTC-3' *Bam*HI
VP1 (-)	5'-CCGCTCGAGCAAAAGCTGTTTCACAGGCG-3' *Xho*I

"AA" stand for related amino acid sequence

The cDNA of FMDV strain Tibet/CHA/99 (GenBank Accession Number AJ539138) was used as the template for amplication of the VP1 coding fragment. This 639 bp coding fragment was then cloned into prokaryotic expression vector pP_{RO}ExHTb to obtain recombinant expression plasmid pP_{RO}-OVP1. For recombinant expression plasmids of the epitopes, an annealing reaction was conducted in the 50 μL mixture containing 20pmol primerE1F, 20 pmol primerE1B (or primerE2F and primerE2B), 25 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl heated at 96 ℃ for 10 min, followed by slow cooling from 65℃ to room temperature over a time period of 1-2 h. The annealed mixture was used for ligation with pGEX4T-1 plasmid digested by *Bam*H I and *Sal*I, and transformed in *E.coli* DH5α competent cells. The positive recombinant plasmids carrying a single coding sequence of epitope1 or epitope2 were named as pGEX-E1 or pGEX-E2 (+1) respectively. Then pGEX-E1 and pGEX-E2 (+1) were digested by *Sal* I and *Xho* I, and ligated with annealed mixture of primer E2F2 and primer E2B2 to get recombinant plasmids carrying coding sequence of epitope1 linked with epitope2 and that of the epitope2 tandem repeat, which were designated respectively as pGEX-E1-2 and pGEX-E2 (+2). All the positive recombinant plasmids were finally sequenced by the dideoxy chain determination method (Shanghai Sangon Company). The correct pGEX-E1, pGEX-E2 (+2), pGEX-E1-2 and pP_{RO}-OVP1 plasmids were used for recombinant epitope expression.

Expression of recombinant epitopes in *E.coli* and purification

Competent cell BL21 (DE3) was transformed respectively with the recombinant plasmid pGEX-E1, pGEX-E2 (+2), pGEX-E1-2 and pP_{RO}-OVP1, and incubated in LB medium (containing 60 μg/mL ampicillin). After incubation, 2.5mL fresh culture was transferred into 250mL fresh LB medium and incubated at 37 ℃ to reach 0.5 at OD_{600}, then induced by the addition of isopropy-β-D-thiogalactoside (IPTG) at a final concentration of 0.8 mmol/L. The cells were then harvested after 5 h by low-speed centrifugation and resuspended in 25 mL of sonication buffer. The fused epitopes containing Glutathione S-transferase (GST) tags and the recombinant VP1 containing His tag were respectively purified using

GST•Bind™ Kits (Novagen) and the ProBond™ Purification System (Invitrogen), as recommended by the suppliers, then checked by SDS-PAGE and the OD_{280} readings on UV spectrophotometer (UV3000, Eppendof).

Western blot analysis of recombinant epitopes and VP1

Two set of purified recombinant epitopes and GST control were run on SDS-PAGE gels and transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane for 3 h at a current of 160 mA. The membranes were then incubated in 5% Non Fat Dry Milk (NFDM) in phosphate buffered saline with 0.05% tween-20 (PBST) for 1 h at room temperature followed by incubation at room temperature for 1 h in FMDV positive sera and negative sera prediluted to 1:200 with PBST, respectively. The membranes were washed three times with TBST for 10 min, and incubated with horseradish peroxidase-labeled rabbit anti-pig IgG antibodies (Sigma) diluted in PBST (1:5 000) at room temperature for 1 h. The membranes were visualized with a substrate solution of DAB (Sigma) and $CoCl_2$ after washing three times for 10 min with PBST. Western blot analysis of VP1 was performed in the same procedure.

Epitope ELISAs

Positive and negative sera were used for developing VP1-ELISA and epitope ELISAs. Indirect VP1-ELISA was developed as described elsewhere [26]. For Epitope ELISAs, all recombinant epitopes were prediluted to concentration of 4μg/mL in coating buffer (0.05mol/L carbornate/ bicarbornate buffer, pH 9.6) and separately added to wells of a microtiter plates (100 μL/well). The plates were sealed with adhesive plate sealers and incubated at 4℃ overnight. Residual binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). Starting from a 1/10 dilution, FMDV positive and negative sera were separately diluted in a twofold dilution series across the plate; the final dilution of serum samples tested was 1/1 280. Each dilution was added (100 μL/well) to the coated/blocked ELISA plates and incubated at RT for 1 h. After washing three times with PBST, horseradish peroxidase (HRP)-conjugated rabbit anti-pig IgG (Sigma) diluted 1:10 000 (v/v) in PBST were added. The plates were incubated at RT for another 1 h and washed five times with PBST. Color development was achieved by adding 100 μL/ well of ready-to-use tetramethylbenzidine (TMB) chromogen-substrate (Sigma), and incubated for 15 min at RT. The reaction was stopped by adding 100 μL of 0.5mol/L H_2SO_4 per well and the absorbance value (A) at 450 nm was measured using an ELISA reader. The optimal sera dilution was determined according to the seroreactivities of the purified GST-epitopes with FMDV positive and negative sera. Subsequently, the optimal sera dilution was selected for determining the concentration of recombinant epitopes required for coating the ELISA wells. After the optimal dilution of test sera and purified epitopes were determined, an ELISA test was conducted with FMDV-positive and FMDV-negative serum samples (20 sera each) to determine the mean absorbance for each epitope. We took the Mean +/- 2 SD as the cutoff value, so that samples with a value above Mean + 2 SD were defined as positive. Samples with a value less than Mean +/- 2 SD were defined to be negative. Samples with a value within Mean +/- 2 SD were unclassified.

Evaluation of the VP1-ELISA and GST-epitope ELISAs

A set of sera containing the negative and positive pig antisera to SVDV and guinea pig antisera to FMDV of type A, C and Aisa1 was used to evaluate the cross-reactivity of the GST-epitope ELISAs. The procedure was the same as described above except HRP-conjugated goat anti-guinea pig IgG used as the secondary antibody in guinea pig antisera ELISA. The relative specificity and sensitivity of VP1-ELISA and epitope ELISAs for GST-E1, GST-E2 (+2) and GST-E1-2 were estimated with 80 field pig serum samples. LPB-ELISA was taken as a standard test, because the sensitivity and the specificity of this assay were demonstrated respectively to be close to 100% and 95% [1]. All ELISAs were performed in duplicate or triplicate and the assays were repeated to ensure reproducible results.

RESULTS

Construction of expression plasmids

The 639 bp coding regions of FMDV serotype O was amplified by RT-PCR (Fig.1) and then cloned into prokaryotic expression vector pP_{RO}ExHTb. Pre-nicked DNA double-strands coding for epitope1, epitope2 and Epitope2 with a linker were generated by annealing the forward and backward primers (Fig.2). The former two DNA fragments were then cloned into prokaryotic expression vector pGEX4T-1, then the DNA fragment coding for epitope2 with a linker was introduced. The sequencing results demonstrated that all the positive clones of pGEX-E1, pGEX-E2 (+2), pGEX-E1-2 and pP_{RO}-OVP1 had correct reading frames.

Expression of recombinant epitopes in *E.coli* and purification

GST-E1, GST-E2(+2), GST-E1-2 and VP1 were successfully expressed in *E.coli* BL21 (DE3) at high levels. As Fig. 3, Fig. 4 and Fig. 5 show, the fused epitopes and VP1 were partly soluble and purified to a high purity.

Western blot analysis of recombinant epitopes

To evaluate the reactivity of the induced protein, a

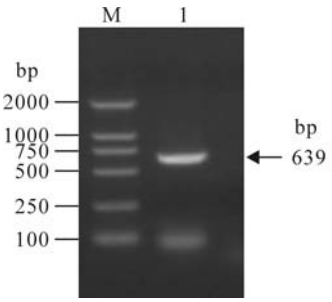

Fig.1. RT-PCR of the VP1 coding region. M, Marker; 1, PCR product.

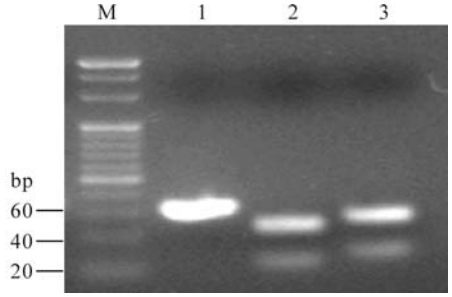

Fig. 2. DNA fragments coding for epitopes. M, DNA Ladder Marker; 1, 2 and 3 are DNA fragments coding for epitope1, epitope2, epitope2 with a linker, respectively.

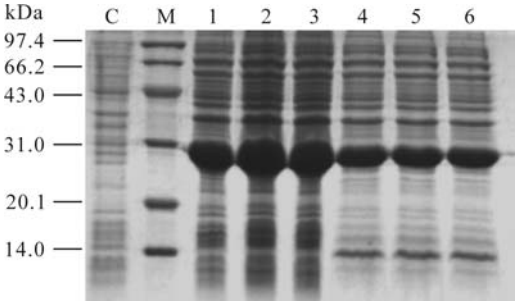

Fig. 3. SDS-PAGE analysis of the expression of GST-epitopes. M, Protein marker; C, control; 1, 2 and 3 are pellets from induced pGEX-E1, pGEX-E2 (+2), pGEX-E1-2 transformants respectively; 4, 5 and 6 are supernatant from induced pGEX-E1, pGEX-E2 (+2), pGEX-E1-2 transformants after sonication respectively.

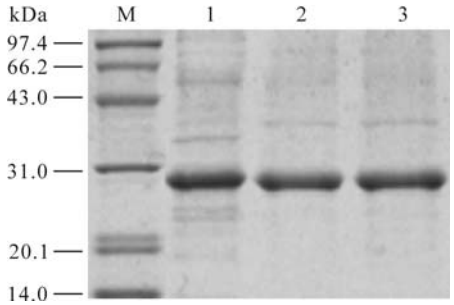

Fig. 4. SDS-PAGE analysis of purified GST-epitopes. M, Protein marker; 1, GST-E1; 2, GST-E2 (+2); 3, GST-E1-2.

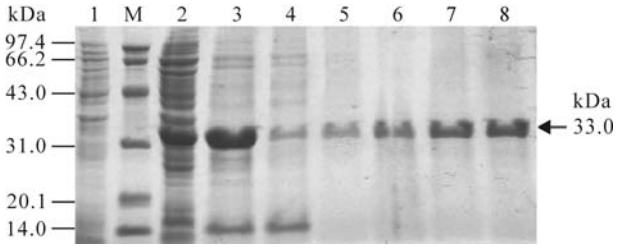

Fig. 5. SDS-PAGE analysis of the expression and purification of VP1. M, Protein marker; 1, Negative control; 2, 5 h pellets from pP_{RO}-VP1transformant; 3, Inclusion bodies; 4, Supernatant after sonication; 5-8, Purificated product.

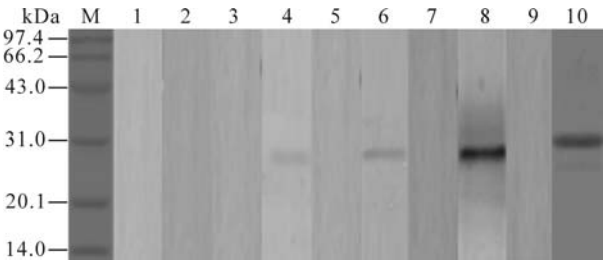

Fig. 6. Western blot analysis of the reactivities of VP1 and GST-epitopes with sera. M, Protein marker; 1 and 2, GST control with negative and positive sera; 3, 5 and 7 are GST-E1, GST-E2 (+2), GST-E1-2 with negative serum, respectively; 4, 6 and 8 are GST-E1, GST-E2 (+2), GST-E1-2 with positive serum, respectively; 9 and 10 are VP1 with negative and positive sera, respectively.

Western blot analysis of purified VP1 and the three GST-epitopes was conducted, with GST tag as controls. Purified VP1 and the three recombinant epitopes could only react with type O FMDV positive serum without cross-reactivity with negative serum, whereas GST could react with neither FMDV positive serum nor negative serum (Fig.6).

Epitope ELISA

Titration assays were performed to determine optimal dilutions of test sera and purified epitopes. The titration plots indicated that the reactivity of FMDV antisera against the recombinant epitopes was optimized at 1:40 sera dilution and there was a significant difference between the positive and negative populations (Fig. 7). The seroreactivity of purified GST-E1 and GST-E2 (+2) was maximum at 4 μg/mL, whereas maximum values for GST-E1-2 and VP1 were 2 μg/mL (Fig.8). The cut-off value was set as 0.224, 0.231, 0.236 and 0.253 for GST-E1, GST-E2 (+2), GST-E1-2 and VP1-ELISA respectively, calculated from mean +2 SDs for the negative control group as described above (data not shown).

Evaluation of the GST-epitope ELISAs

GST-E1, GST-E2 (+2) and GST-E1-2 could recognize FMDV-specific antibodies in clinical samples without cross-reactivity with pig antisera to SVDV or guinea

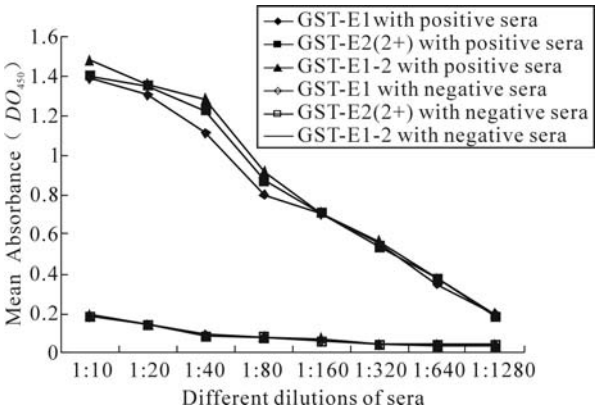

Fig. 7. Seroreactivities of purified GST-epitopes with FMDV positive and negative sera. The mean absorbance (450nm) is the average of 10 ELISA testes with FMDV positive and negative sera. All the concentrations of GST-E1, GST-E2 (+2) and GST-E1-2 used for coating the ELISA plate were 4μg/mL.

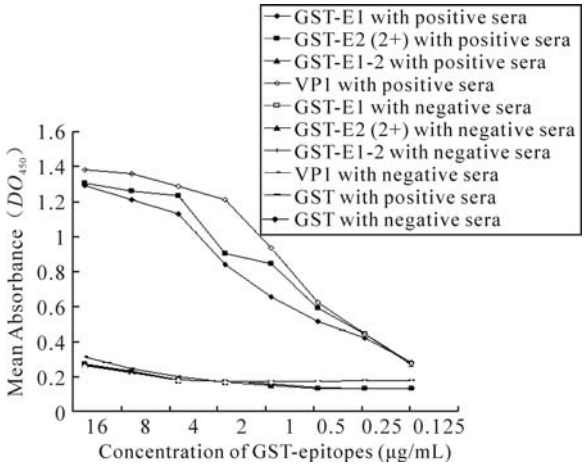

Fig. 8. Seroreactivities of various concentrations of the purified GST-epitopes and VP1 with FMDV positive and negative sera. The mean absorbance (OD_{450}) is the average of 10 ELISA testes with FMDV positive and negative sera in 1:40 dilutions.

pig antisera to FMDV of type A, C, and Aisa1 (Table 2). The relative specificity and sensitivity of the epitope ELISAs when determined using 80 field pig serum samples have shown relatively good agreement with standard LPB-ELISA. The relative specificity and sensitivity for the GST-E1 ELISA, GST-E2(+2), GST-E1-2 ELISA and VP1-ELISA in comparison with LPB-ELISA were 93.3% and 85.0%, 95.0% and 90%, 100% and 81.8%, 96.6% and 80.9%, respectively (Table 3).

DISCUSSION

The internationally accepted tests for foot-and-mouth disease serology are those recognized by OIE as "Prescribed" or "Alternative" tests. Currently the "Gold Standard" is the virus neutralization test [10]. A marked improvement in the laboratory diagnosis of FMD has resulted since ELISA methods were introduced in the mid-1980s[9, 20]. The liquid-phase blocking ELISA (LPB-ELISA), another prescribed test, has been adopted by a large number of laboratories worldwide for routine screening because it is relatively quick, more reproducible and correlates well with the virus neutralization test [12, 13]. Because of the variable stability of inactivated antigens and its relatively low specificity, some improvements were introduced by testing sera in a solid-phase (SP) blocking ELISA [24] or competition ELISA (SPcELISA) [17]. However, in the SP antibody detection ELISAs, the

Table 2. Cross-reactivity of VP1 and GST-epitope ELISAs (*A* value)

Epitope ELISAs	SVDV	FMDV type A	FMDV type C	FMDV type Asia1
GST-E1	0.179	0.193	0.185	0.184
GST-E2(+2)	0.187	0.212	0.187	0.192
GST-E1-2	0.190	0.200	0.187	0.192
VP1	0.176	0.178	0.108	0.232

Table 3. Agreement between LPB-ELISA and epitope-ELISA

LBP-ELISA	GST-E1			GST-E2 (+2)			GST-E1-2			VP1-ELISA		
	P	N	Total	P	N	Total	P	N	Total	P	N	Total
Positive	56	4	60	57	3	60	58	0	58	57	2	59
Negative	3	17	20	2	18	20	4	18	22	4	17	21
Total	59	21	80	59	21	80	62	18	80	61	19	80

"P" stands for positive; "N" stands for negative. In GST-E1 ELISA, relative sensitivity=93.3% (56/60); relative specificity=85.0% (17/20). In GST-E2 (+2) ELISA, relative sensitivity=95.0% (57/60); relative specificity=90% (18/20). In GST-E1-2 ELISA, relative sensitivity=100% (58/58); relative specificity=81.8% (18/22). In VP1-ELISA, relative sensitivity=96.6% (57/59); relative specificity=80.9% (17/21).

main antigen in the FMD vaccine, a 146 S ribonucleoprotein particle native antigen purified from FMDV-producing cultures is commonly used but since this cannot be properly inactivated it is therefore associated with potential risks of reactivation in susceptible livestock[6]. It would be highly beneficial therefore if, instead of the whole protein, only a epitope representing the antibody-binding site involved in the recognition could be used. The use of peptide epitopes as diagnostic antigens may allow a focus on relevant single peptide specificities and avoid the diagnostically unimportant epitopes present in complex antigens. Some studies have shown that mimotopes selected antibodies against pathological antigens can be important probes for the detection of antibodies produced during infection [4, 14, 15, 16, 25].

Here we demonstrated that GST-fused B cell epitopes within VP1 of type O FMDV can react specifically with pig antiserum to type O FMDV, without cross-reactivity with antisera against FMDV of other types or SVDV. The recombinant VP1 of FMDV strain Tibet/CHA/99 also showed no cross-reactivity with other serotypes; considering the existence of many subtypes within serotypes, we decided that, instead of using the full length of VP1, to use the epitopes by deleting the VP1 sequence upstream epitopel and that between epitope1 and epitope2 for antibody detection, may avoid the probably non-specific reactions. Although some studies have identified the epitope2 sequence as a real epitope, since it only comprises 14 amino acids, the possibility exists of non-specific reactivity against GST. Therefore, another approach based on recombinant DNA techniques was used in our study to construct proteins with tandem repeat epitopes. The fused epitopes of GST-E1, GST-E2 (2+) and GST-E1-2 were used as antigen for ELISA. The experiment results were shown to correlate well with the VP1-ELISA and the LPB-ELISA, and high sensitivities were achieved by using the GST-E1 (93.3%) and GST-E2 (+2) (95.0%). Combination of two epitopes (GST-E1-2) increased the sensitivity (100%), indicating that an individual serum sample is likely to have higher titer antibody to one epitope than to the other; each different epitope will therefore recognize a different subset of antibodies. An effective diagnostic method would be achieved by combinationally using as many of type-specific epitopes within all FMDV SPs so as to recognize every FMDV-positive sample, which would increase the sensitivity of detection.

Acknowledgments

The work is supported by National key Technology R&D Program (2006BAD06A14).

References

1. **Alexandersen S, Zhang Z, Donaldson A I, *et al.*** 2003. The pathogenesis and diagnosis of foot-and-mouth disease. **J Comp Pathol,** 129 (1): 1-36.
2. **Brown F.** 2003. The history of research in food-and-mouth disease. **Virus Res,** 91 (1): 3-7.
3. **Cao Y M, Lu Z J, Liu Z X, *et al.*** 2007. Comparison of Three ELISA Kits for the Differentiation of Foot-and-mouth Disease Virus-infected from Vaccinated Animals. **Virol Sin,** 22 (1): 74-79.
4. **Casey J L, Coley A M, Anders R F, *et al.*** 2004. Antibodies to malaria peptide mimics inhibit Plasmodium falciparum invasion of erythrocytes. **Infect Immun,** 72 (2): 126-1134.
5. **DiMarchi R, Brooke G, Gale C, *et al.*** 1986. Protection of cattle against foot-and-mouth disease by a synthetic peptide, **Science,** 232 (4750): 639-641.
6. **Doel T R.** 2003. FMD vaccines. **Virus Res,** 91 (1): 81- 99.

7. **Domingo E, Baranowski E, Escarmís C, *et al.*** 2002. Foot-and-mouth disease virus. **Comp Immunol Microbiol Infect Dis,** 25 (5-6): 297-308.
8. **Dus Santos M J, Wigdorovitz A, Trono K, *et al.*** 2002. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. **Vaccine,** 20 (7-8): 1141-1147.
9. **Ferris N P, Dawson M.** 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. **Vet Microbiol,** 16 (3): 201-209.
10. **Golding S M, Hedger R S, Talbot P.** 1976. Radial immuno-diffusion and serum neutralisation techniques for the assay of antibodies to swine vesicular disease. **Res Vet Sci,** 20 (2): 142-147.
11. **Grubman M J, Baxt B.** 2004. Foot-and-mouth disease. **Clin Microbiol Rev**, 17 (2): 465-93.
12. **Hamblin C, Barnett I T R, Hedger R S,** 1986a. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. **J Immunol Methods,** 93 (1): 115-121.
13. **Hamblin C, Barnett I T R, Hedger R S,** 1986b. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. II. Application. **J Immunol Methods,** 93 (1): 123-129.
14. **Kaikkonen L, Lankinen H, Harjunpää I, *et al.*** 1999. Acute-Phase-Specific Heptapeptide Epitope for Diagnosis of Parvovirus B19 Infection. **J Clin Microbiol,** 37 (12): 3952-3956.
15. **Kouzmitcheva G A, Petrenko V A, Smith G P.** 2001. Identifying diagnostic peptides for Lyme disease through epitope discovery. **Clin Diagn Lab Immunol,** 8 (1): 150-160.
16. **Lundin K, Samuelsson A, Jansson M, *et al.*** 1996. Peptides isolated from random peptide libraries on phage elicit a neutralizing anti-HIV-1 response: analysis of immunological mimicry. **Immunology**, 89 (4): 579-586.
17. **Mackay D K J, Bulut A N, Rendle T, *et al.*** 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. **J Virol Methods,** 97 (1-2): 33-48.
18. **Parry N, Fox G, Rowlands D, *et al.*** 1990. Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. **Nature,** 347 (6293): 569-572.
19. **Rémond M, Kaiser C, Lebreton F.** 2002. Diagnosis and screening of foot-and-mouth disease. **Comp Immunol Microbiol Infect Dis,** 25 (5-6): 309-320.
20. **Roeder P L, Le Blanc Smith P M.** 1987. Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. **Res Vet Sci,** 43 (2): 225-232.
21. **Schiappacassi M, Rojas E R, Carrillo E, *et al.*** 1995. Response of foot-and-mouth disease virus C3 Resende to immunological pressure exerted in vitro by antiviral polyclonal sera. **Virus Res,** 36 (1): 77-85.
22. **Sobrino F, Sáiz M, Jiménez-Clavero M A, *et al.*** 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. **Vet Res,** 32 (1): 1-30.
23. **Song H, Zhou L, Fang W, *et al.*** 2004.High-level expression of codon optimized foot-and-mouth disease virus complex epitopes and cholera toxin B subunit chimera in Hansenula polymorpha. **Biochem Biophys Res Commun,** 315 (1): 235-239.
24. **Sorensen K J, Madekurozwa R L, Dawe P.** 1992. Foot-and-mouth disease: detection of antibodies in cattle sera by blocking ELISA. **Vet Microbiol,** 32 (3-4): 253-265.
25. **Sun T，Lu P, Wang X.** 2004. SerologicaLocalization of infection-related epitopes on the non-structural protein 3ABC of foot-and-mouth disease virus and the application of tandem epitopes tests are particularly useful for the surveillance following an outbreak to identify silent infections in species like sheep and goat which show little or no signs of disease. **J Virol Methods**, 119 (2): 79-86.
26. **Wang G H, Du J Z, Chang H Y, *et al.*** Establishment of Indirect ELISA Diagnose Based on the VP1 Structural Protein of Foot-and-mouth Disease Virus (FMDV) in Pigs. **Chin J Biotechnol,** 2007, 23 (5): 961-966.
27. **Wong H T, Cheng S C, Xie Y, *et al.*** 2000. Plasmids encoding foot-and-mouth disease virus VP1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. **Virology,** 278 (1): 27-35.
28. **Zamorano P I, Wigdorovitz A, Perez Filgueira D M, *et al.*** 1998. Induction of anti foot and mouth disease virus T and B cell responses in cattle immunized with a peptide representing ten amino acids of VP1. **Vaccine,** 16 (6): 558-563.