INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–141/2020/23–1–68–74
DOI: 10.17957/IJAB/15.1259
http://www.fspublishers.org

Full Length Article

Development of an Indirect ELISA Based on VP1-CRT Fusion Protein for Detection of FMDV-O in Swine

Chang Liu[1,2†], Hua Feng[2†], Yunchao Liu[2], Yumei Chen[3], Suzhen Yang[2], Qingxia Lu[2], Lili Feng[4], Ruiguang Deng[2] and Gaiping Zhang[1,2,3,5,*]

[1]*College of Veterinary Medicine, Jilin University, Changchun 130033, Jilin, China*
[2]*Key Laboratory of Animal Immunology of the Ministry of Agriculture, Henan Provincial Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences, Zhengzhou 450002, Henan, China*
[3]*School of Life Sciences, Zhengzhou University, Zhengzhou 450001, Henan, China*
[4]*Institute of Agricultural Economics and Information, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China*
[5]*College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, Henan, China*
[*]For correspondence: zhanggaip@126.com
[†]Contributed equally to this work and are co-first authors
Received 09 August 2019; Accepted 28 August 2019; Published 08 January 2020

Abstract

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. The open reading frame (ORF) of VP1 codes the most important structure protein of foot and mouth disease virus (FMDV). To develop a highly sensitive and specific indirect ELISA method for detecting FMDV-O antibody, the prokaryotic optimized gene *vp1* was fused with truncated *calceticulin* (rT5V) and co-expressed with trigger factor (Tf16) which could assist soluble expression of antigenicity polymers in *Escherichia coli*. The rT5V was purified using size-exclusion chromatography with the purity about 80%. The rT5V-ELISA method was developed using the purified rT5V as coating antigen for FMDV-O antibody detection. The sensitivity and specificity of the rT5V-ELISA were 84.2 and 100%, respectively. The comparative test between rT5V-ELISA and the liquid-phase blocking ELISA kit (LPB-ELISA) with 376 clinical samples showed the coincidence rate was 84.04%. In conclusion, the rT5V was achieved in soluble polymers by co-expressing with Tf16 in *E. coli*. The rT5V-ELISA was developed as a highly sensitive and specific method for FMDV-O antibody monitoring after infection and/or vaccination.

Keywords: FMDV-O VP1; Fusion protein; Polymers; Prokaryotic expression; Indirect ELISA

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease among cloven-hoofed animals, which is characterized by vesicular lesions on tongue, udder or teats, feet and myocarditis among young animals (Charleston *et al.* 2011). This disease is induced by foot-and-mouth disease virus (FMDV), which including seven immunologically distinct serotypes: O, A, C, Asia 1, and the South African Territories (SAT) serotypes SAT1, SAT2, SAT3 (Carrillo *et al.* 2005). Among them, FMDV serotype O (FMDV-O) is the mostly pandemic and severely impacts swine industry and food security (OIE/FAO).

The conventional diagnostic methods of FMD mainly include virus neutralization test (VNT) (An *et al.* 2000), RT-PCR (Dill *et al.* 2017), liquid-phase blocking ELISA (LPB-ELISA) (Araujo *et al.* 1996) and solid-phase competition ELISA (SPC-ELISA) (Chenard *et al.* 2003). However, the intact virus involved in these methods leads to the risk of incomplete inactivation and virus escape from laboratory. In recent years, ELISAs methods based on non-structural proteins (NSPs) and structural proteins (SPs) of FMDV are safe, reliable and easy-to-use for laboratory diagnosis. NSPs-based ELISA methods have been developed for differentiating infected animals from vaccinated ones, while SPs-based methods are normally used to detect antibody levels of animals no matter they are infected (Ma *et al.* 2011) or vaccinated (Alexandersen *et al.* 2003). VP1 , as the most important protective SPs, contains some critical epitopes, which are responsible for the induction of neutralizing antibodies (Haydon *et al.* 1998) and binding of virus receptors (Wang *et al.* 2003). Therefore, this antigenic protein is usually used as target protein for FMDV-O diagnosis and vaccine development.

Calreticulin (CRT) is a Ca^{2+}-binding glycoprotein in the endoplasmic reticulum of eukaryotic cells. Recombinant

proteins fused with truncated CRT could self-polymerize and exhibited strong immunogenicity *in vivo* (Hong *et al.* 2010; Huang *et al.* 2013). However, it is rarely reported that VP1 polymers formed by truncated CRT were used as detector for diagnosis. Therefore, it is reasonable and effective to develop a SPs-based detection method by using high molecular of VP1 polymers.

In present study, an indirect ELISA method based on CRT-fused VP1 protein was developed for massive screening antibodies after FMDV-O infection or vaccination. Soluble VP1 protein fused with truncated CRT (150-230 aa) (rT5V) under the assistance of trigger factor 16 (Tf16) was obtained in *Escherichia coli*. And the rT5V could self-polymerize and exhibited a good reactogenicity with clinical FMDV-O positive serum and anti-FMDV monoclonal antibodies (mAbs), which was further used for developing rT5V-ELISA method. Furthermore, the sensitivity and specificity of rT5V-ELISA were evaluated, and its comparative trial was carried with commercial LPB-ELISA kit.

Materials and Methods

Serum samples

Twenty-two positive swine sera collected from FMDV-O recovered pigs, while 79 positive swine sera sourced from herds immunized with commercial inactivated FMDV-O vaccines. Newborn piglet's sera with no pathogens and 160 negative swine sera were sampled from FMDV-free and non-vaccinated farms. Furthermore, 376 unidentified field serum samples was also collected from 12 farms in different districts of Henan Province and other neighboring cities.

Positive sera of other swine pathogens: 3 positive sera for each of Swine classic fever virus (SCFV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circovirus virus type 2 (PCV2), Swine vesicular stomatitis virus (SVSV), Porcine epidemic diarrhea virus (PEDV), Porcine parvovirus virus (PPV), Swine influenza virus (SIV), 34 positive sera for FMDV serotype A/Asia1 were purchased from China Institute of Veterinary Drug Control. All these sera were preserved by Henan Provincial Key Laboratory of Animal immunology.

Expression and purification of the rT5V

The complete *vp1* gene from FMDV O/mya-98 strain (GenBank Acc. **No. DQ164925**) fused with truncated (150-230aa) calreticulin (GenBank Acc. **No. EU639407**) at C terminal by using 5×GGGGS linker (Fig. 1a). The recombinant fragment named as rT5V (GenBank Acc. **No. MK409983**) was synthesized after codon optimization by Genscript, then the fused fragment was inserted into plasmid pEG-28a (GE Healthcare, Sweden) between *Bam*HI and *Xho*I sites and transformed into *E. coli* BL21 (DE3) competent cells with a Tf16 plasmid according to the manufacturer's protocol (TaKaRa, China). The positive clone was selected and cultured in Luria-Bertani (LB) medium with 50 mg/L kanamycin and 34 mg/L chloromycetin, and the target protein and Tf16 were induced by 0.1 m*M* IPTG and 0.5 g/L L-arabinose at the same time, respectively. The harvest cells were suspended in lysis buffer (20 m*M* PB, 150 m*M* NaCl, 5% *(w/v)* Glycerol, 5% *(w/v)* Triton X-100, 2 mM EDTA, 2 mM DTT, pH 9.0) and lysed by sonication (99 cycles of 2 s On/5 s Off, Amp 25%). The soluble rT5V was purified using size-exclusion chromatography with Superdex 200 pg (26/60) gel filtration column (GE Healthcare, U.S.A.). The cross-flows were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot with anti-His mAbs. The target bands were visualized by AEC Peroxidase Substrate Kit (Solabio, U.S.A.) following manufacture manual.

Characterization of rT5V

Dynamic light scattering (DLS) was used to evaluate the hydrodynamic diameter of purified rT5V, the number-based size distribution and the polydispersity index for multimeric protein.

The reactivity of purified rT5V with various antibodies was tested by indirect enzyme-linked immunosorbent assay (ELISA). The rT5V coated 96-well microtiter plates overnight at 4°C. Then all plates were blocked with 5% *(w/v)* skimmed milk in PBST for 2 h at room temperature. Mouse anti-FMDV-O mAbs 2D2 (Abcam, U.S.A.), anti-pep mAbs 3D-A11 (anti-pep VP1 (141-160 aa)) (preparation by laboratory) were added as prime antibody and incubated for 1 h at 37°C, respectively. After the incubation with Horseradish Peroxidase (HRP) conjugated goat anti-mouse IgG (HRP-IgG) for 1 h at 37°C, chromogenic reaction was produced by 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (Sigma, U.S.A.). The optical density values at absorption of 450 nm (OD_{450}) were measured in Infinite 200 PRO microplate reader.

Development of rT5V-ELISA operation procedure

The rT5V-ELISA was carried out as the conventional indirect ELISA method by using the rT5V as candidate detective antigen. The indirect ELISA conditions were optimized by using 2-fold serial dilutions of rT5V as coating antigen, and 2-fold serial dilutions of positive or negative swine sera as probe and HRP-IgG as secondary antibody. Briefly, the rT5V antigen was 2^4 to 2^{-7} mg/L serially diluted with different coating buffers including 0.05 M CBS (pH 9.6), 0.01 *M* Tris (pH 8.0), 0.02 M PBS (pH 7.2) and coated with 96-well microtiter plates overnight at 4°C. For swine serum, 12 concentration gradients from 1:25 to 1:51200 was set up using blocking buffer, while the HRP-IgG was also differently diluted with blocking buffer at 1:500, 1:1000, 1:2000, 1:3000, 1:4000 and 1:5000, respectively. The reaction time of them was controlled at 15, 30, 45, 60, 75

and 90 min, respectively. After washed by PBST, plate was incubated with substrate for 3, 5, 7, 10, 15 and 20 min at room temperature. The sera from animals immunized with inactivated virus vaccine were used as positive samples and the newborn piglet's sera as negative samples. The ratio of signal to noise was calculated to decide the optimal reaction conditions for the ELISA. Signal to Noise Ratio (P/N) calculation = Mean OD_{450} of positive sample/Mean OD_{450} of negative sample.

Calculation of cut-off value in rT5V-ELISA

A random selection of 60 out of 160 negative swine sera were tested using rT5V-ELISA method in triplicate. The average negative OD_{450} ($\overline{X}$) and standard deviation (SD) were calculated, and $\overline{X}$ +3SD was regarded as the cut-off value for detecting serum. $OD_{450} \geq \overline{X}$ +3SD could be considered as positive and $OD_{450} < \overline{X}$ +3SD as negative.

Sensitivity, specificity and repeatability of the of rT5V-ELISA

Twenty-two positive sera from FMDV-O infected swine and 79 sera from swine vaccinated by inactivated FMDV-O vaccine were used to identify the sensitivity of rT5V-ELISA with the established cut-off value. The other 100 out of 160 negative sera, 55 positive sera of other pathogens including SCFV, PRRSV, PCV2, SVSV, PEDV, PPV, SIV and FMDV-A/Asia 1 were used to analyze the specificity of rT5V-ELISA. For calculating the intra-assay coefficient, five positive serum samples were randomly selected and tested in quadruplicate with same batch of rT5V coated plate; while four different batches of rT5V coated plate were used for calculating the inter-assay coefficient of variation in the same way.

Comparative trial between rT5V-ELISA and LPB-ELISA

Three hundred and seventy-six swine clinical sera from 12 farms around Henan Province were collected in order to estimate the antibody levels using rT5V-ELISA method. The results were evaluated and compared with the LPB-ELISA kit (Lanzhou Veterinary Research Institute of Chinese Academy of Agricultural Sciences).

Statistical methods

All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SEM

Results

Soluble expression, purification and western blot confirmation of rT5V

The obtained rT5V was went through Superdex 200 pg (26/60) gel filtration column and the target protein was presented as the first and highest peak, which indicated that rT5V could form into some kind of high-molecular-weight polymers (Fig. 1b). Under the assistance of Tf16 (56 kDa), the recombinant Soluble CRT-fused VP1 protein (rT5V) was expressed as a soluble 38 kDa His-tagged protein corresponding to the expected size. In addition, the purity of rT5V was about 80% (Fig. 1c). Western blot analysis confirmed that the purified rT5V was able to react to anti-His mAbs (Fig. 1d). Besides, the third peak was also recognized by anti-His mAbs, which indicated that the rT5V might also exist in form of monomer (Fig. 1b, c and d).

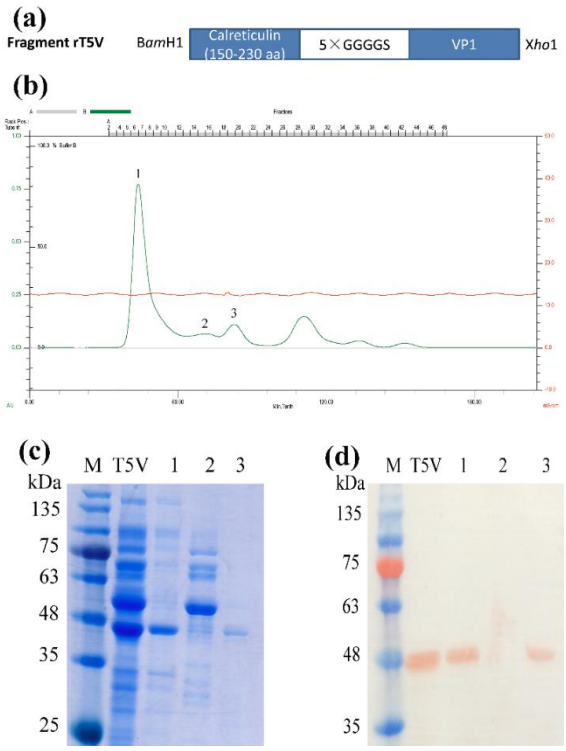

Fig. 1: Construction and purification of rT5F. (**a**) The schematic structure of the CRT-VP1 fusion protein rT5F. Blue squares represent completely *vp1* of FMDV-O and truncated calreticulin (150-230aa). GGGGS in grey are linkers between *vp1* and calreticulin. The fragment is encoded by B*am*HI and X*ho*I. (**b**) Graphic description of size exclusion chromatography of rT5F. Arabic numerals represent absorption peak numbers. SDS-PAGE of size exclusion chromatography (**c**) and Western-Blot analyze of purified rT5F (**d**). M: Pre-stained Protein Ladder; 1. Supernatant of pET-28a-rT5V with Tf16; 2. The first absorption peak; 3. The second absorption peak; 4. The third absorption peak

Characterization of rT5V

DLS showed that the average diameter of purified rT5V was about 100nm (Fig. 2a). Overall antigenic analysis showed that the recombinant CRT-fused VP1 protein was able to

specifically recognize anti-FMDV mAbs (2D2 and 3D-A11). Furthermore, the antigenic reaction to anti-FMDV mAbs 2D2, indicated the polymers might have the similar immunogenicity as intact virus particle (Fig. 2b, c).

Development of rT5V-ELSIA

The purified rT5V was used as candidate for detecting antibody of FMDV-O. As shown in Fig. 3-5, the optimal operational conditions for rT5V-ELISA were as follows: 1 mg/L of rT5V protein (Fig. 3a) in coating buffer (CBS, pH 9.6) coated plates for overnight (Fig. 3b), 1:256 diluted swine serum (Fig. 4a) in blocking buffer was incubated for 45 min (Fig. 5a) and of 1:2000 diluted HRP-IgG (Fig. 4b) in blocking buffer was incubated for 30 min (Fig. 5b); finally, the best substrate action time was 15 min (Fig. 5c).

The standards of rT5V-ELISA

The average OD_{450} ($\overline{X}$) of 60 negative sera of FMDV was 0.149 with the standard deviation (SD) at 0.050. So, the value for discrimination positive or negative was set at 0.299 following the formula $\overline{X}$ +3SD.

Sensitivity, specificity and repeatability of rT5V-ELISA

Clinical sera from infected, vaccinated and non-vaccinated healthy swine, as well as the positive sera for SCFV, PRRSV, PCV2, SVSV, PEDV, PPV, SIV were tested by the constructed rT5V-ELISA to assess the sensitivity and specificity of this method, respectively. The sensitivity of rT5V-ELISA for FMDV-O positive sera was 84.2%, while no nonspecific reaction with FMDV-O negative sera and the positive sera from other disease, indicating the specificity of the ELISA is 100% (Table 1). The reproducibility test showed the intra and inter-assay coefficient of variation (CVs) ranged from 4.040–7.019% and 4.480–10.549%, respectively (Table 2).

Comparative trial between rT5V-ELISA and LPB-ELISA

Three hundred and seventy-six sera of swine from 12 farms around Henan Province were tested by rT5V-ELISA and LPB-ELISA kit. One hundred and seventy-nine positive sera and 137 negative sera were equally identified by both methods, while only 60 samples were inconsistent. Thus, compared to LPB-ELISA, the relative sensitivity and specificity of rT5V-ELISA were 83.26% (179/215) and 85.09% (137/161) (Table 3). The total coincidence rate of the two methods was 84.04% (316/376) (Table 3).

Discussion

Rapid diagnostic methods to monitor FMDV infection are critical to swine industry since the incidence of FMDV has greatly increased in China since 2010 (OIE/FAO). The

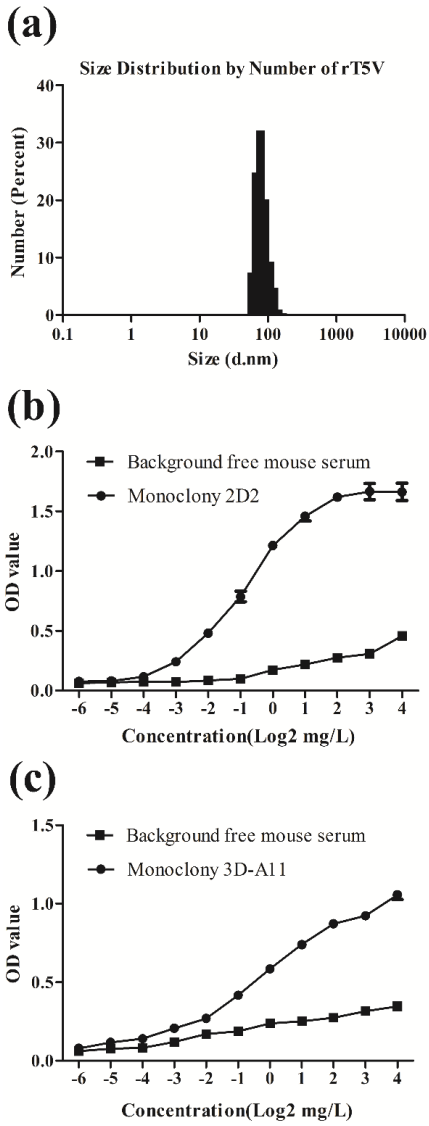

Fig. 2: Characterizing of rT5F. (**a**) Size distribution of purified rT5V whose diameter was around 100 nm. Antigenic characterizations of purified rT5V using anti-FMVDV-O mAbs 2D2 (**b**), anti-pep (VP1 141-160aa) mAbs 3D-A11 (**c**) by indirect ELISA, and the results are expressed as mean OD value ± SEM

present study successfully expressed the soluble CRT-fused VP1 (rT5V) in form of high-molecular-weight polymers, which was applied for constructed a rT5V based novel indirect ELISA method for detecting FMDV-O antibodies. The constructed rT5V-ELISA showed a high sensitivity, specificity for the detection and diagnosis of FMDV-O specific antibodies.

SPs-based ELISA methods were introduced in the laboratory for FMD diagnosis to avoid the threatened of bio-safety. Among FMDV viral genes, the capsid protein VP1-encoding gene is widely studied because of its significance for protective immunity. It has been

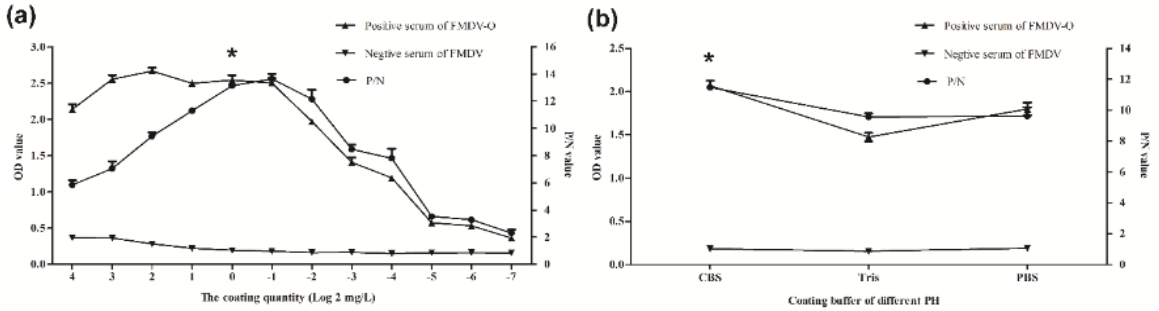

Fig. 3: The optimal concentration of coating antigen (**a**) and the suitable PH (**b**) in rT5V-ELISA. The curves show the OD value (left) and Signal to Noise Ratio (P/N) (right) per sample. * Represents the suitable P/N ratio from this study

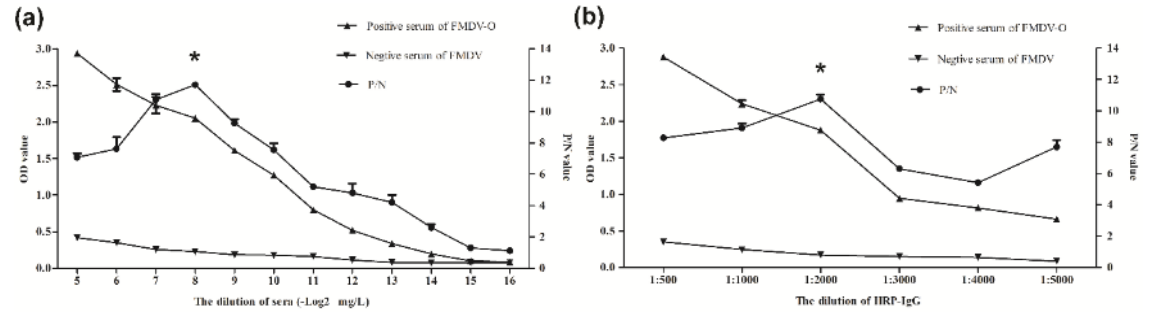

Fig. 4: The optimal dilution of positive/negative serum (**a**) and IgG-HRP (**b**) in rT5V-ELISA. The curves show the OD value (left) and Signal to Noise Ratio (P/N) (right) per sample. * Represents the suitable P/N ratio from this study

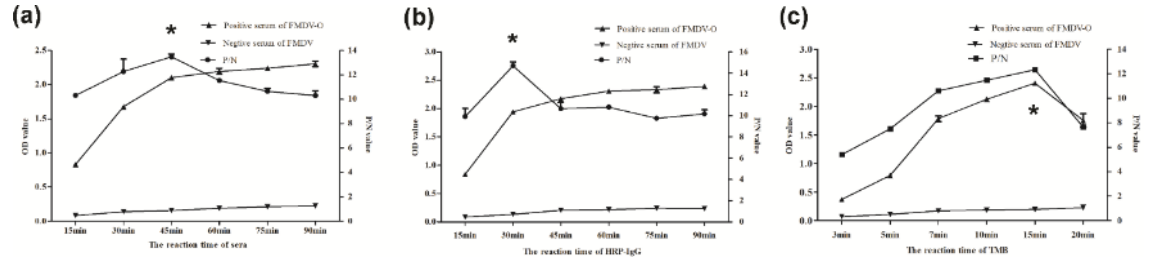

Fig. 5: The optimal reaction time of positive/negative serum (**a**), IgG-HRP (**b**) and substrate (**c**) in rT5V-ELISA. The curves show the OD value (left) and Signal to Noise Ratio (P/N) (right) per sample. * Represents the suitable P/N ratio from this study

demonstrated that VP1 possessed critical epitopes responsible for induction of neutralizing antibodies and virus receptors binding (Bittle *et al.* 1982). Thus, some ELISA methods based on these epitopes were developed to detect viral antibodies (Gao *et al.* 2010). However, the use of those epitopes as diagnostic antigens limits the method to diagnose distinctive epitopes on VP1 proteins. LPB-ELISA is still widely used in the market as a persuasive method for screening antibodies of FMDV. In our study, rT5V-ELISA had a high coincidence rate with the commercial LPB-ELISA kit. Besides, the recombinant CRT-fused VP1 protein could be prepared in any laboratory without infectivity that could threat any susceptible animals. It could avoid limitation of LPB-ELISA, such as variable stability of inactivated antigens, hazardous virus production and handling. Thus, it is better to replace the inactive whole FMDV with recombinant FMDV VP1 as the detector in indirect ELISA tests, which suggests that rT5V-ELISA could be a promising tool for monitoring FMDV-O infection and antibody condition in swine herd.

A number of expression systems have been used to produce recombinant VP1 protein of FMDV (Andrianova *et al.* 2011; Peralta *et al.* 2013), prokaryotic expression system was widely used as it is ease-of-use, ideal target yield and suitable for scale preparation (Lee *et al.* 2009). However, recombinant protein of VP1 produced by *E.coli* often leads into biologically inactive aggregates known as inclusion bodies (IBs) (Suryanarayana *et al.* 1999). Trigger factor (Tf16) is one of the most important molecular chaperones and co-expression with it has successfully enhanced yield and biological activity of some recombinant proteins in *E.coli* (Nishihara *et al.* 2000). Therefore, chaperone co-expression strategies have been successfully used for facilitating soluble expression of different type's

Table 1: Specificity and sensitivity test of rT5V-ELISA

	Total (%)	Serum samples	rT5V-ELISA (+)	rT5V-ELISA (-)
Sensitivity	84.2 (85[a]/101)	Infection	20	2
		Vaccination	65	14
Specificity	100 (155[b]/145)	Healthy and non-vaccinated	0	100
		SCFV	0	3
		PRRSV	0	3
		PCV2	0	3
		SVSV	0	3
		PEDV	0	3
		PPV	0	3
		SIV	0	3
		FMDV serotype A/Asia1	0	34

a. Number of positive serum samples; b. Number of negative serum samples

Table 2: Repeatability and reproducibility analysis of rT5V-ELISA

Sample	Repeat	Intra-assay variability		Batches	Inter-assay variability	
		Mean	CVs		Mean	CVs
No.1	4	1.327 ± 0.078	5.953	4	1.294 ± 0.116	8.964
No.2	4	1.782 ± 0.072	4.040	4	1.741 ± 0.078	4.480
No.3	4	0.983 ± 0.069	7.019	4	0.948 ± 0.088	10.549
No.4	4	1.215 ± 0.063	5.185	4	1.252 ± 0.067	5.351
No.5	4	1.932 ± 0.131	6.782	4	1.859 ± 0.091	4.895

Table 3: Comparison results between rT5V-ELISA and LPB-ELISA

		LPB-ELISA		
		Positive	Negative	Total
rT5V-ELISA	Positive	179	24	203
	Negative	36	137	173
	Total	215	161	376
	Total (%)	83.26 (179/215)	85.09 (137/161)	84.4 (316/376)

recombinant proteins. In this study, it was the first try to produce recombinant VP1 fused with truncated CRT protein (rT5V) in soluble form under the assisting of Tf16, which suggested the co-expression with Tf16 strategy could be a useful tool for maximally maintaining the natural structure and reactivity of rT5V.

For the protocol, the rT5V were in the saturated state with no trend to descend when the coating quantity was in a range of 2^3 to 2^{-2} mg/L, so 1mg/L was chosen to be the best coating quantity as convenient to calculate. The sensitivity (84.2%) and specificity (100%) of rT5V-ELISA indicated that it would be a reliable test to identify infected or immunized herds. The coefficient of variation within the batches was less than 8% (4.040–7.019%) and between batches was less than 11% (4.480–10.549%), which indicated that the same serum sample had a small coefficient of variation in the plate coated with rT5V prepared in the same or different batches (Table 2). The developed rT5V-ELISA method was reliable based on the stable expressed rT5V.

Conclusion

In conclusion, we demonstrated that truncated CRT-fused VP1 of FMDV-O could specifically react with swine antiserum to FMDV-O. The development process highlighted the importance of accurate selection of experimental conditions. The constructed rT5V-ELISA was a highly sensitive and specific method that could be used for screening for FMDV-O infection or vaccination and monitoring antibody titers against FMDV-O.

Acknowledgments

This work was funded by grants from the National Key Research and Development Program of China (2016YFD0501503 and 2016YFD0500704).

References

Alexandersen S, Z Zhang, AI Donaldson, AJM Garland (2003. The pathogenesis and diagnosis of foot-and-mouth disease. *J Compar Pathol* 129:1–36

An K, AQ Paulsen, MB Tilley, RA Consigli (2000). Use of electron microscopic and immunogold labeling techniques to determine polyomavirus recombinant VP1 capsid-like particles entry into mouse 3T6 cell nucleus. *J Virol Meth* 90:91–97

Andrianova, EP, SR Krementsugskaia, NN Lugovskaia, TK Mayorova, VV Borisov, MA Eldarov, NV Ravin, AS Folimonov, KG Skryabin (2011). Foot and mouth disease virus polyepitope protein produced in bacteria and plants induces protective immunity in guinea pigs. *Biochemistry* 76:339–346

Araujo JP, HJ Montassier, AA Pinto (1996). Liquid-phase blocking sandwich enzyme-linked immunosorbent assay for detection of antibodies against foot-and-mouth disease virus in water buffalo sera. *Amer J Vet Res* 57:840–843

Bittle JL, RA Houghten, H Alexander, TM Shinnick, JG Sutcliffe, RA Lerner, DJ Rowlands, F Brown (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* 298:30–33

Carrillo C, ER Tulman, G Delhon, Z Lu, A Carreno, A Vagnozzi, GF Kutish, DL Rock (2005). Comparative genomics of foot-and-mouth disease virus. *J Virol* 79:6487–6504

Charleston B, BM Bankowski, S Gubbins, ME Chase-Topping, D Schley, R Howey, PV Barnett, D Gibson, ND Juleff, ME Woolhouse (2011). Relationship between clinical signs and transmission of an infectious disease and the implications for control. *Science* 332:726–729

Chenard G, K Miedema, P Moonen, RS Schrijver, A Dekker (2003). A solid-phase blocking ELISA for detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. *J Virol Meth* 107:89–98

Dill V, M Beer, B Hoffmann (2017). Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses. *J Virol Meth* 246:58–64

Gao SD, JZ Du, HY Chang, GZ Cong, JJ Shao, T Lin, S Song, QG Xie (2010). B cell epitopes within VP1 of type O foot-and-mouth disease virus for detection of viral antibodies. *Virol Sin* 25:18–26

Haydon D, S Lea, L Fry, N Knowles, AR Samuel, D Stuart, ME Woolhouse (1998). Characterizing sequence variation in the VP1 capsid proteins of foot and mouth disease virus (serotype 0) with respect to virion structure. *J Mol Evol* 46:465–475

Hong C, X Qiu, Y Li, Q Huang, Z Zhong, Y Zhang, X Liu, L Sun, P Lv, XM Gao (2010). Functional analysis of recombinant calreticulin fragment 39-272: implications for immunobiological activities of calreticulin in health and disease. *J Immunol* 185:4561–4569

Huang SH, LX Zhao, C Hong, CC Duo, BN Guo, LJ Zhang, Z Gong, SD Xiong, FY Gong, XM Gao (2013). Self-oligomerization is essential for enhanced immunological activities of soluble recombinant calreticulin. *PLoS One* 8:1-11

Lee CD, YP Yan, SM Liang, TF Wang (2009). Production of FMDV virus-like particles by a SUMO fusion protein approach in *Escherichia coli*. *J Biomed Sci* 16:69

Ma LN, J Zhang, HT Chen, JH Zhou, YZ Ding, YS Liu (2011). An overview on ELISA techniques for FMD. *Virol J* 8:419

Nishihara K, M Kanemori, H Yanagi, T Yura (2000). Overexpression of trigger factor prevents aggregation of recombinant proteins in Escherichia coli. *Appl Environ Microbiol* 66:884–889

Peralta A, GA Maroniche, V Alfonso, P Molinari, O Taboga (2013). VP1 protein of Foot-and-mouth disease virus (FMDV) impairs baculovirus surface display. *Virus Res* 175:87–90

Suryanarayana VV, S Viswanathan, G Ratish, P Bist, K Prabhudas, MR Gajendragad, C Natarajan (1999). *E. coli* expressed proteins as diagnostic reagents for typing of foot-and-mouth disease virus. *Arch Virol* 144:1701–1712

Wang JH, CM Liang, JM Peng, JJ Shieh, MH Jong, YL Lin, M Sieber, SM Liang (2003). Induction of immunity in swine by purified recombinant VP1 of foot-and-mouth disease virus. *Vaccine* 21:3721–3729