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Secretory Expression of E2 Main Antigen Domain of CSFV C Strain and the Establishment of Indirect ELISA Assay*

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Abstract: The sequence encoding an E2 main antigen glycoprotein of the C strain of classical swine fever virus (CSFV) was highly expressed in the host cell *E. coli* BL21–CodonPlus (DE3)–RIL using the pGEX-4T-1 expression vector and the soluble recombinant product was purified with Glutathione Sepharose TM^{4B} by centrifugation. The soluble recombinant protein showed good immune reactions and was confirmed by Western blot using anti-CSFV-specific antibodies. Then an indirect ELISA with the purified E2 protein as the coating antigen was established to detect antibody against CSFV. The result revealed that the optimal concentration of coated antigen was 0.6 μg/well and the optimal dilution of serum was 1:80. The positive cut-off value of this ELISA assay was $OD_{\text{tested serum}} / OD_{\text{negative serum}} \geqslant 2.1$. The E2-ELISA method was evaluated by comparison with the indirect hemagglutination test (IHAT). When a total of 100 field serum samples were tested the sensitivity and specificity were 90.3% and 94.7% respectively. Specificity analysis showed that there were no cross-reactions between BVD serum and the purified E2 protein in the E2-ELISA.

Key words: CSFV; C strain; E2 gene; Indirect ELISA; Secretory Expression

Classical Swine Fever (CSF) is one of the most important infectious diseases of swine which can spread in an epizootic form as well as establishing enzootic infections in domestic and wild pig populations and cause significant economic losses to the pig industry all over the world (5). Classical swine fever virus (CSFV) belongs to the genus Pestivirus of the Flaviviridae family along with bovine viral diarrhoea virus (BVDV) (15).

Detection of virus-specific antibodies is a prerequisite for aiding epidemiological surveys in tracing the spread of the virus and for monitoring in an eradication program. The neutralization test (NT) has been described for detection of specific antibodies to CSFV (18) but while it is reliable and sensitive, it is not readily applicable for large-scale screening in animal husbandry production because it is time-consuming. Several recombinant antigen ELISA have been developed (3, 11), but a more sensitive as well as

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specific and rapid indirect ELISA for screening of a large number of serum samples during an outbreak is still needed. Therefore, a CSFV indirect ELISA method using a recombinant E2 protein as antigen was developed in this study.

MATERIALS AND METHODS

Strain and serum

CSFV C strain, *E. coli* BL21–CodonPlus(DE3)–RIL, positive sera and negative sera to CSFV were all kept in the State Key Laboratory Of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute. Sera to BVDV was purchased from the China Institute of Veterinary Drug Control (Beijing, China). Plasmid pGEM-E2 had been constructed in our previous study.

Reagents

Glutathione Sepharose TM^{4B} was purchased from Xinjingke Biotechnology Co., Ltd (Beijing, China); X-gal、IPTG and penicillin were all purchased from Sangon (Shanghai, China); rabbit against swine HRP-IgG (H+L) was purchased from BioDov-Tech (Beijing, China); DAB was purchased from Amresco (Massachusetts, USA); CSFV IHA kit was supplied by Lanzhou Veterinary Research Institute. The rest of the reagents were all purchased in China.

Construction of the expression plasmid

The 558 bp gene fragment, amplified from Plasmid pGEM-E2 using the primers F (5'-GAAGATTACAG GTACGCA-3') and B (5'-ACCTTTCACACATGTC CA-3') and located at the N-terminal 2 456 to 3 013 in the E2 gene sequence, was subcloned in frame into the *Bam*H I and *Xho* I restriction sites of the plasmid pGEX-4T-1 and transformed into *E coli* strain BL21-–CodonPlus (DE3) –RIL, following the standard procedure for DNA manipulation (13). A clone containing the inserted aim DNA in the correct orientation was designated pGEX-E2.

Recombinant protein expression, purification and Western blot analysis

The positive single clone described above was inoculated with 5 mL LB medium with 100 μg/mL Amp and was incubated overnight at 37℃ with 230 r/min, and 1 mL cultures were inoculated with 1000 mL LB medium and incubated at 37℃ with 230 r/min until an OD_{600} value 0.6-1.0 was measured, then the cultures were induced for 18 h at 16℃ with 0.1 mmol/L IPTG. At the same time, pGEX-4T-1 was transformed into BL21—CodonPlus (DE3)–RIL and induced according to the above conditions (9).

1000 mL cell cultures were centrifuged for 10 min at 5000 r/min, and the pellets harvested were resuspended with buffer (137 mmol/L NaCl，2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4，1 mmol/L EDTA，pH 8.0) after being washed one time with PBS (pH 7.4), then lysozyme was added to a final concentration of 100 μg/mL. The cells were then broken up by ultrasound and centrifuged at 4℃ for 15 min at 12 000 r/min, and the supernatant and deposition were collected. The target proteins in supernatant and deposition were both analyzed by SDS-PAGE. The recombinant strain BL21/pGEX-4T-1 was processed in the same manner (4).

The soluble target protein and GST protein in the supernatant were purified with Glutathione Sepharose TM^{4B} by centrifugation. The solution protein purified was filtered through a 0.45 nm filter, and concentrated into 0.5-2.0 mL and the purity and concentration were confirmed by measuring OD_{260} and OD_{280} values. Finally, the recombinant protein concentrated was

analyzed by Western blot (10).

Development of the iE2-ELISA

A checkerboard titration was performed for optimization of working dilution of antigen, serum and HRP-IgG (second antibody marked with enzyme) on a 96-well ELISA plate. The antigen and serum dilutions that gave maximum difference in absorbance at 490 nm between positive and negative (P/N) were selected for testing the serum samples on larger scales. Test sera also included standard controls such as positive, negative and blank samples. At the same time, the reaction temperature, time and other conditions were optimized by index of P/N value (1).

After the optimization tests above, the indirect ELISA was carried out using purified recombinant protein as coating antigen diluted (0.6 μg of antigen/ well) in 0.05 mol/L carbonate/bicarbonate buffer (pH 9.6) in 96-well polystyrene microtitre plates overnight at 4 ℃ after incubation at 37 ℃ for 1 h. Plates were washed three times with washing buffer PBST (0.002 mol/L PBS containing 0.05% Tween 20) to remove unbound antigen and then the remaining sites in each well were blocked with 50 μL of blocking buffer (PBST containing 1% bovine serum albumin). After incubation and washing of the plate, serum diluted in blocking buffer was added in 50 μL volume and incubated at 37℃ for 1 h The horseradish peroxidase labeled rabbit-anti-pig IgG (HRP-IgG) diluted in blocking buffer was added (50 μL/well) and incubated for 1 h at 37℃. After incubation, a washing step was performed as described before. Substrate solution freshly prepared (OPD 1 mg/ mL containing 4 μL 3% H_2O_2) was added (50 μL/well) in each well and the colour reaction was developed in the dark for 10 min before stopping the reaction with 2 mol/L H_2SO4 (25 μL/well). The absorbance values were measured at a wavelength of 490 nm using an ELISA reader (Model 680, BIO-RAD, USA). Controls included blanks (PBS only), known negative sera (negative control), Positive sera (Cpositive contral) and GST protein as coating antigen as above.

Then, samples of known negative sera were used to determine cut-off value. For this purpose, 300 CSFV-negative serum samples and the standard negative control were tested to obtain 300 values of *OD* the negative serum/*OD* the negative control (P/N). A mean of P/N with a standard deviation (SD) was calculated as performance of the serological assays is reported to be improved by adding 2 to 3 times the SD to the mean P/N value (7).

Comparison iE2-ELISA with CSFV IHAT

Anti-CSFV antibodies of 100 sera samples were titrated with iE2-ELISA and indirect haemaggluti- nation tests (IHAT) as the pertinent manipulation instructions (14). The proportions of positive and negative samples detected, randomly from pigs in endemic areas in China, were taken as the sensitivity and specificity of the assay, respectively.

Testing of cross-reactivity

8 known positive sera to BVDV were measured by putative iE2-ELISA of CSFV recombinant protein antigen for testing cross-reaction.

RESULTS

Recombinant protein expression, purification and Western blot analysis

The recombinant expressed protein was 46.65 kDa in size including GST protein (26.0 kDa in size) and target protein (20.65kDa in size) as estimated, and could be observed by SDS-PAGE analysis both in supernatant and deposition. The ratio was approxi-

mately 1:4 as measured by lamina-scan ananlysis (Fig.1). After the recombinant protein in supernatant was purified with Glutathione Sepharose TM4B, the value of OD_{280}/OD_{260}, concentration and purity were 1.63, 4.752 mg/mL and 80%, respectively, and for the GST protein, were 1.80, 3.502 mg/mL and 90%, respectively (Fig.2).

Western blot analysis, revealed the band of the expressed protein was ~46.65 kDa in size but no band corresponding to GST protein was observed (Fig.3), indicating that the target protein could react with anti-CSFV serum.

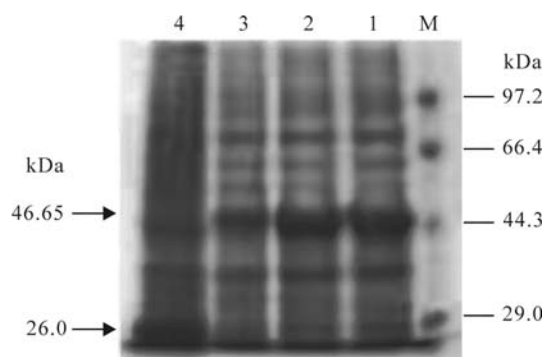

Fig.1. SDS-PAGE analysis of expression products of BL21/pGEX-E2. M, Low molecular weight protein Marker; 1-2, Expression products in deposition of BL21/pGEX-E2; 3, Expression products in supernatant of BL21/pGEX-E2; 4, Expression products of BL21/pGEX-4T-1.

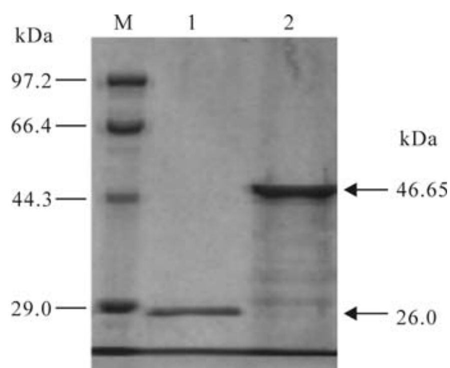

Fig.2. SDS-PAGE analysis of the protein purified. M, Low molecular weight protein Marker; 1, GST protein purified; 2, Target protein purified.

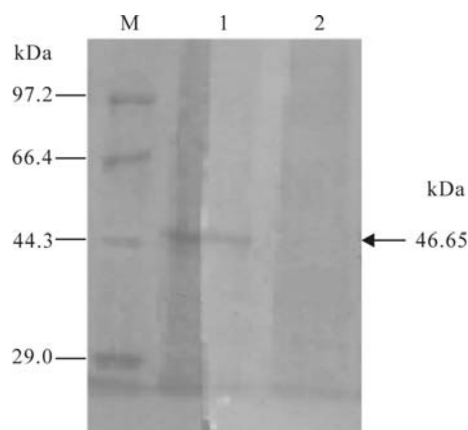

Fig. 3. Western blot analysis of the protein purified. M, Low molecular weight protein Marker; 1, Target protein purified; 2, GST protein purified.

iE2-ELISA establishment

By checkerboard titration tests, the concentration of coating antigen was 0.6 μg/well, and the dilutions of the sera and HRP-IgG were 1:80 and 1:2000 respectively. A mean P/N of 1.461 with a standard deviation (SD) of 0.196 was obtained from 300 CSFV-negative sera, so the cut-off value was adjusted to 2.1 (mean+3 SD). Namely, 2.1 times the OD_{490} value obtained from reference negative serum was set as the criteria. A sample was considered positive if its OD_{490} value was over or equal to the criterion.

Comparison iE2-ELISA with CSFV IHAT

To determine the specificity and sensitivity of the iE2-ELISA, 100 serum samples were tested and compared with the results obtained with CSFV IHAT (Table 1). Of the 62 positive serum samples in the CSFV IHAT, 6 were negative in iE2-ELISA. For the

Table 1. Comparison of the iE2-ELISA with the whole Ag IHA

iE2-ELISA	Whole Ag IHA		
	Positive	Negative	Total
Positive	56	2	58
Negative	6	36	42
Total	62	38	100

Table 2. The result of cross-reaction test

Anti-BVDV sera	1	2	3	4	5	6	7	8
Value of OD_{490}	0.275	0.331	0.309	0.286	0.294	0.316	0.285	0.290

OD_{490} value from the reference negative serum was 0.173, so the cut-off was 0.363 (0.173×2.1).

corresponding negative serum samples, 2 out of 38 tested positive in iE2-ELISA. The comparison of the tests gave a calculated sensitivity of 90.3% and a specificity of 94.7%.

Testing of cross-reactivity

In this test, no cross-reaction with known positive sera to bovine viral diarrhoea virus (BVDV) was seen in the iE2-ELISA using E2 recombinant antigen, giving values well below the defined cut-off (Table 2).

DISCUSSION

Cloned genes often can be expressed highly in *E.coli*, but many proteins expressed are inclusion body and need to be recovered, and much protein is lost during the process, so it is important to optimize expression conditions and obtain soluble protein (2). At present, some evidence indicates that the inclusion body can be expressed in *E.coli* due to overlap of the protein (12). If the *E.coli* transformed expression vector is cultured at lower temperature, the amount of inclusion body expressed will be reduced (6). In this research, when the recombinant *E.coli* BL21–Codon Plus (DE3) –RIL was cultured for 18 h at 16℃, and 2.908 mg purified protein was expressed with a purity above 80%.

iE2-ELISA was developed in this study after Western blot analysis showed that anti-CSFV serum could react with E2 recombinant protein but not with GST protein. A crucial point in the establishment of serological tests is the differences occurring between individual sera due to varying antibody titres, leading to potential false negative or positive results (17). To overcome this, a serial dilution ELISA was used. A mean P/N of 1.461 with a standard deviation (SD) of 0.196 was obtained from 300 CSFV-negative sera, so the cut-off value was adjusted to 2.1 (mean+3 SD), which would serve as a threshold between the positive and the negative serum samples.

In this study, the performance of iE-ELISA in terms of relative sensitivity and specificity was compared with that of IHAT of whole Ag using a two-sided contingency table. It was showed a high degree of specificity (94.7%) and sensitivity (90.3%). But on the other hand, the positive results would be also obtained using positive sera to BVDV when the pig herds were infected by CSFV, because there are some same antigen between BVDV and CSFV (18). Therefore, part of the E2 gene including A, B, C and D antigen epitopes located in 2 456 to 3 013 in the E2 gene sequence was expressed, which define a highly conserved epitope among different strains of CSFV but not among different pestivirusesm, such as BVDV (8, 16). The validity of the approach was proven by the absence of cross-reactions with known positive sera to bovine viral diarrhoea virus (BVDV) in the cross-reaction tests using iE2-ELISA.

In conclusion, the iE2-ELISA provides an alternative, inexpensive and rapid serological diagnostic tool and is suitable for CSFV screening, especially in species that harbour BVDV. It can be used in the diagnosis

and serological epidemiological investigation of CSFV in China.

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