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Differential Diagnosis of Antibody to Classical Swine Fever Virus Field Strain by ELISA with Recombinant E2 Proteins of Various Group CSFV

[1]Qiu Chang-Qing, [1,2]Hu Hui, [1]Cao Xiao-An, [1]Zhou Ji-Zhang and [1]Lin Guo-Zhen
[1]State Key Laboratory of Veterinary Etiological Biology,
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences,
Lanzhou, Gansu 730046, P.R. China
[2]Henan University of Agriculture, Zhengzhou, Henan 450002, P.R. China

Abstract: In this study, an indirect Enzyme-Linked Immunosorbent Assay (ELISA) method for measuring antibodies to Classical Swine Fever Virus (CSFV) was established using recombinant E2 proteins of CSFV C and LT strains, respectively. The optimum dilutions of the E2 antigens and serum were 1:160 and 1:320, respectively. Coating concentration of the E2 antigen was 11.7 μg mL^{-1}; the optimal concentration of enzyme-labeled antibody was 1:800; the optimal reaction times of the first antibody and secondary antibody were 1 h and 45 min, respectively. The tests showed that the E2 antigens were not reacted with both anti-BVD antibody and antibodies against other pathogens which are able to induce abortion of pregnant pigs and death of young piglets. A critical value of the method was 0.3503, namely OD of the tested serum ≥0.35, it was positive for CSF; <0.35, it was negative for CSF. It was found that serum specimens from CSFV LT field strain affected pigs with being diluted to 1:1280 or more could be distinguished by the method, which showed positive in LT strain-E2 ELISA but negative in C strain-E2 ELISA.

Key words: CSFV, recombinant E_2 protein, indirect ELISA, differential diagnosis

INTRODUCTION

At present, acute Classical Swine Fever (CSF) cases occur less but mild CSF or atypical CSF cases are more common clinically (Tu *et al*., 2001; Saatkamp *et al*., 2000) owing to large-scale use of CSF virus C strain vaccine in China. It will be very difficult to confirm a case of atypical CSF only by routine examination and pathological dissection. Now a series of serologic methods for examining CSF were established (Shannon *et al*., 1993; Bunavoglia *et al*., 1989; Colijn *et al*., 1997), for example, an indirect immune fluorescence, indirect ELISA, Dot-ELISA, indirect hemagglutination test, etc. of them, ELISA has very high sensitivity and specificity, which has made it become a more useful tool for diagnosing CSF. However, the antigens used in a conventional ELISA often are concentrated whole CSF viruses particles from CSF virus cell cultures with time-consuming, laborious, expensive and virus culture titer-unstable disadvantages; on the other hand, the diagnosis of CSF might produce false-positive result owing to ruminant pestivirus (bovine viral diarrhea-mucosal disease virus and border disease virus) infection causing interference (Castrucci *et al*., 1973; Carbrey *et al*., 1976; Dahle *et al*., 1987; Edwards *et al*., 1991; Roehe *et al*., 1992; Wentink and Terpstra, 1999; de Smit *et al*., 1999; Kulcsár *et al*., 2001; Wieringa-Jelsma *et al*., 2006).

Therefore, it is a new hotspot that the genetically engineered technique expressing recombinant protein has been used as the antigens of ELISA for diagnosing CSF replacing whole CSF viruses in order to distinguish CSFV from other pestivirus efficaciously (Christmann *et al*., 2001; Dewulf *et al*., 2004; Lin *et al*., 2005; Huang *et al*., 2006; Rau *et al*., 2006; Cho and Park, 2006; He *et al*., 2007; Van Rijn, 2007; Chen *et al*., 2007).

In our laboratory, the expression vector of CSFV E_2 gene was successfully constructed and it was confirmed that the recombinant E2 expression protein has activity of reaction with serum against CSFV (Hu *et al*., 2004; 2005). In present test, the recombinant E_2 proteins from the CSFV C strain and LT field strain will be used as antigens coating 96 wells reaction plate of ELISA and the optimum condition of reaction will be measured to establish an indirect ELISA method for differentially diagnosing CSF.

Corresponding Author: Qiu Chang-Qing, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu 730046, P.R. China

MATERIALS AND METHODS

Antigen: The purified recombinant E2 proteins of CSFV C-strain and LT-strain were prepared according to the references (Hu *et al*., 2004, 2005) and kept at -20°C and will be used as antigen of ELISA in our laboratory.

Preparations of E2 proteins of CSFV C-strain and LT-strain were briefly described as follows:

E2 genes of CSFV of C strain (Chinese lapinized hog cholera virus vaccine strain) and a prevalent field strains LT were amplified by Reverse Transcription (RT) and the nested polymerase chain reaction (nPCR). The amplified E2 fragments of the two strains all were 561bp in length. And they were ligated with plasmid pGEM-T Easy vector. The recombinant plasmids and expression vector pGEX-4T-1 were digested by the same restriction endonucleases. The genes were ligated and transformed into *E. coli*. The insert position, the size and the reading frame all were affirmed by PCR, restriction digestion and sequence analysis. The results showed the prokaryotic expression vectors were constructed successfully. Then the recombinants were transformed into BL21 (DE3) for E2 expression with IPTG inducing. The expressed proteins were measured by SDS-PAGE and western-blotting. The results showed that the E2 genes were able to be expressed successfully in *E. coli*. The western-blotting results indicated that the expressed proteins were recognized by CSFV positive serum. The inclusion bodies in *E. coli* with plasmid pGEX-C and pGEX-LT were obtained by sonication of the bacterial cells, from which the purified activative expressed recombinant E2 proteins were got by urea and TritonX-100 washing and by renaturing, refolding and dialyzing.

Reference sera, viruses, reagents and kits: The positive reference serum and negative serum of CSFV were supplied by Professor SC.Li; positive serum to pig parvovirus, positive serum to pseudorabies virus and positive serum to PRRSV were donated by Professor HC.Chen; positive serum to swine *Chlamydia psittaci* was kept in our laboratory. Neutralization test kit of BVD including BVD virus reference strain Oregon C_{24} V, reference positive serum and negative serum (China Institute of Veterinary Drug Control). HRP labeled rabbits anti-pig IgG (Beijing Jingke Company). OPD, BSA (Shanghai Bioengineering Company). GD3200 Microplate Reader and ELISA plates (Shanghai Bioengineering Company). Other reagents: Na_2CO_3, Tween-20, NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, Citric acid, H_2SO_4 and H_2O_2 (Shanghai Chemistry Reagent Company). Whole CSFV C strain-indirect ELISA kit (20030613) (Lanzhou Veterinary Research Institute of CAAS); Whole CSFV C strain-IHA kit (20030420) (Lanzhou Veterinary Research Institute of CAAS). The CSF diagnostic RT-PCR kit (Lanzhou Veterinary Research Institute of CAAS).

The positive serum to CSFV E_2 antigen was prepared in our laboratory.

Preparation of solutions used in the test: Coating solution: 0.025 mol L^{-1} pH9.6 carbonate buffer: Na_2CO_3 0.4779 g and $NaHCO_3$ 0.4620 g were dissolved in redistilled water and total volume of the coating solution reached 100 mL by adding redistilled water.

Washing solution (PBST): pH7.4 PBS with 0.05% Tween-20: NaCl 8.0 g, Kcl 0.2 g, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g, KH_2PO_4 0.2 g and Tween-20 0.5 mL were fully dissolved in redistilled water and adjusted to pH7.4 and the total volume of the washing solution reached 1000 mL by adding redistilled water.

Blocking solution: PBST with 1% BSA: BSA 1g was fully dissolved in PBST 100 mL and kept at 4°C.

Substrate stock solution (OPD-H_2O_2)

Solution A (0.1 mol L^{-1} citric acid solution): Citric acid 19.2 g was dissolved in redistilled water and total volume of the solution A is 1000 mL. Solution B (0.2 mol L^{-1} $Na_2HPO_4 \cdot 12H_2O$): $Na_2HPO_4 \cdot 12H_2O$ 71.64 g was dissolved in redistilled water and total volume of the solution B is 1000 mL.

Use method: After 4.86 mL Solution A is mixed with 5.14 mL Solution B, 4mg OPD will be fully dissolved in the mixed solution, in which 30% (V/V) H_2O_2 was added for OPD-H_2O_2 substrate work solution.

Stop solution (2M H_2SO_4): 10 mL concentrated H_2SO_4 slowly is added in 60 mL redistilled water with continually stirring and then the total volume of the stop solution will reach 90 mL by adding redistilled water.

Establishment of ELISA method

Selection of the optimal reaction condition for ELISA: The test was carried out by means of the routine checherboard titration (Yin and Liu, 1997). Comparative tests of the coating antigen concentration, serum dilution, optimal work concentration of enzyme and each step reaction times were done using the ratio of positive serum OD value and negative serum OD value (P/N value) as index for ascertaining the optimal conditions for every step reaction.

The operation procedure of an indirect ELISA: Total 96 well reaction plate was coated row-by-row with the recombinant E_2 antigen and blank vector antigen of optimal concentration diluted using 0.05 mol L^{-1} carbonate buffer, 100 μL $well^{-1}$ and then displaced at 4°C over night after placed for 1 h at 37°C. The coating solution in the well of plate was thrown off, the plate was washed 3 times with PBST, 2-3 min per time. And then blocking solution was added at dose of 200 μL $well^{-1}$, the plate was blocked for 2 h at 37°C.Then the plate was washed as above the same method. Serum samples of optimal concentration diluted using block solution were added in wells of the plate, 100 μL $well^{-1}$, each serum sample of same amount being added in three wells and reacted for 1 h. And then the plate was washed as above same method. Then the rabbit-anti-pig IgG enzyme-labeled secondary antibody was added at a dose of 100 μL $well^{-1}$ and the reaction was kept for 45 min. And the plate was washed as above same method and then substrate work solution was added and reacted for 10-15 min avoiding light and then 2 mol L^{-1} H_2SO_4 was added to stop the reaction and OD value of each well was read at 490 nm of ELISA apparatus at once. At the same time, the positive control well, negative control well and blank well were set up in the plate.

Determination of the positive and negative critical values of indirect ELISA: Thirty CSFV antibody-negative sera were determined by the indirect ELISA. Each serum of optimal dilution was added in the plate at a dose of 100 μL $well^{-1}$ and tested by the above procedure of indirect ELISA and OD_{490} values of each well were read. Each serum sample was repeated two wells and the final result was the average of both well OD values. The average (X) of OD values and Standard Deviation (SD) of serum specimen was calculated. On the basis of statistics, when the OD_{490} value of sample was larger than the average (X) of negative sample OD+3×SD, the sample would be judged as CSFV antibody positive on 99.9% level.

Specificity test

Cross reactions: Positive serum to BVDV, positive serum to pseudorabies virus, positive serum to PRRSV, positive serum to *Chlamydia psittaci* and positive serum to pig parvovirus was used in the above ELISA with positive serum to CSFV and negative serum as controls for observing cross reactions.

Blocking test: CSF-positive serum was diluted to the optimal dilution and mixed with same volume of the recombinant CSFV E2 antigen of optimal dilution and reacted for 30 min. And then the mixed solution was added in the plate coated with the antigen for carrying out ELISA and determining OD value. At the same time the controls were set up and the test results were compared and (N-P)/N value would be calculated. If (N-P)/N value was larger than 0.5, blocking test was positive.

(N-P)/N = (not blocked well OD value – blocked well OD value)/ (not blocked well OD value)

Reproducibility test: Random 10 serum samples were determined six times by the above ELISA for calculating the coefficient of its variations and measuring its reproducible property.

Sensitivity test: CSF-positive sera were diluted to 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, respectively and then measured by ELISA with the optimal condition.

Biological sensitivity test: Two CSF-negative pigs were inoculated with C strain vaccine at dose of 1 mL pig^{-1}. CSF antibody titers would be measured on the days 3, day 5, day 7 and day 10 after vaccination with the C-strain E2 antigen ELISA.

Stability test: The recombinant CSFV E2 proteins were kept respectively at 4. -20, -70°C for 6 months, with which the same batch of sera samples was measured using the recombinant CSFV E2 antigen- ELISA for observing its stability.

Comparisons of the above ELISA with whole CSFV-ELISA kit and IHA kit: Total 80 sera specimens of pigs were measured using the recombinant E_2 antigen-ELISA, whole CSFV C strain- ELISA kit and whole CSFV C strain-IHA kit, respectively, for comparing their positive, negative and total coincidence rates.

Differential diagnostic test of serum specimens sampled from CSF-artificialy affected pigs using various E2 antigens ELISA: Two 6 month old pigs negative for CSF antibody were subcutaneously behind the ears infected with CFSV LT field strain at a dosage of 2 mL blood containing CFSV LT strain (virus titer 10^7TCID_{50}/0.2 mL). And the blood specimens were sampled and diagnosed by a CSF diagnostic RT-PCR kit after the pigs became ill. And then serum specimens were sampled from the two affected pigs for measurement by C strain-E2 ELSA and LT strain-E2 ELISA respectively after proper dilution of the tested sera.

RESULTS

The optimal work conditions of ELISA: The optimal coating concentration of E2 antigen and optimal dilution of the positive and negative reference sera.

Table 1: The P/N ratios of checkerboard titration (E2 protein of CSFV C strain)[a]

Dilutions of serum	Dilutions of antigen 1:20	1:40	1:80	1:160	1:320	1:640
1:40	6.53	4.68	6.70	7.48	6.25	4.10
1:80	5.48	4.94	8.36	6.33	5.00	4.32
1:160	7.54	7.79	9.33	6.95	4.52	3.65
1:320	6.61	5.69	6.10	4.84	6.62	4.76
1:640	4.24	5.14	5.32	3.88	3.28	2.76
1:1280	3.46	4.23	2.95	4.44	2.85	0.00

[a]It was calculated as 0.05 that the relative OD_{490} value of negative serum was less than 0.05

Table 2: The P/N ratios of checkerboard titration (E2 protein of CSFV LT strain)[a]

Dilutions of serum	Dilutions of antigen 1:20	1:40	1:80	1:160	1:320	1:640
1:40	9.23	8.68	9.00	8.13	8.35	6.10
1:80	10.18	7.94	8.36	7.63	9.21	7.72
1:160	8.54	9.77	9.84	8.94	6.32	5.65
1:320	7.61	7.69	6.88	11.56	7.92	7.71
1:640	5.24	8.54	9.65	8.75	5.31	6.84
1:1280	8.46	6.23	7.15	6.74	6.35	0.00

[a]It was calculated as 0.05 that the relative OD_{490} value of negative serum was less than 0.05

Purified CSFV E_2 antigens which had been diluted to 1:40, 1:80, 1:160, 1:320, 1:640, respectively were used to coat reaction plate, coating 2 wells using each dilution of E_2 antigen. The checkerboard titration test was performed using CSF-positive and negative reference sera which had been diluted to 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, respectively. The P/N value of checkerboard titration was seen in the Table 1 and 2.

The result of the Table 1 showed the P/N value of CSFV C-strain E2 ranged from 2.76-9.33, when the E_2 antigen dilution was 1:80 and the sera dilution was 1:160, the P/N ratio would reached the largest value, namely 9.33. And the nonspecific reactions of CSF- negative reference serum was not obvious (the corresponding OD_{490} value was 0.09). And the OD_{490} value of CSF-positive reference serum was 0.84. When the antigen dilutions were between 1:80 to 1:160, the corresponding OD_{490} value obviously fell. On the other hand, when the serum dilutions were between 1:160 to 320, the corresponding OD_{490} value visibly decreased too. In a word, the optimal dilutions of the antigen and serum are 1:80 and 1:160, respectively, here coating concentration of the antigen was 8.7 μg mL^{-1}.

The Table 2 showed the P/N ratios of CSFV LT strain E2 ranged from 5.24 to 11.56. When the antigen dilution was 1:160 and the serum dilution was 1:320, the P/N ratio would reached the largest value, namely 11.56. And the nonspecific reactions of CSF- negative reference serum was not obvious (the corresponding OD_{490} value was 0.07), the OD_{490} value of CSF- positive reference serum was 0.81. When the antigen dilutions were between 1:160 to 1:320, the corresponding OD_{490} value obviously fell.

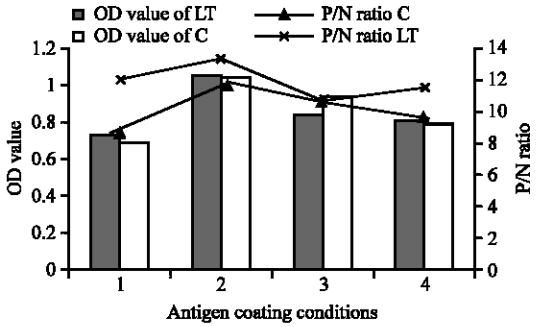

Fig. 1: Coating condition of the recombinant E_2 protein antigen

On the other hand, when the serum dilutions were between 1:320 to 640, the corresponding OD_{490} value visibly decreased too. In a word, the optimal dilutions of the antigen and serum are 1:160 and 1:320, respectively, here the coating concentration of the antigen is 11.7 μg mL^{-1}.

The work concentration of enzyme-labeled antibody: According to the results of the optimal E_2 antigen coating concentration and optimal serum dilution tests, ELISA tests were carried out. And the enzyme-labeled antibody was diluted 1:200, 1:300, 1:400, 1:600, 1:800, 1:1000, 1:1500, 1:2000, respectively with which ELISA tests were carried out and the OD values were measured and P/N ratios were calculated. On the basis of decrease extents of OD values and determinant standard of ELISA, it was thought that optimal concentration of the secondary antibody was 1:800 (i.e. work concentration). The test result was seen in the Table 3.

Coating condition of the recombinant E2 antigen: The purified recombinant E2 proteins were used to coat plates according to 4 various conditions of 37°C 4 h, 37°C 2 h+ 4°C over night, 37°C 1 h + 4°C over night and 4°C over night and then ELISA were carried out using CSF-positive and negative serum for determining the optimal coating condition of antigen. The result of the test was seen in the Fig. 1.

The Fig. 1 showed that when recombinant E_2 protein was used to coat the plate under conditions of 37°C 1 h+ 4°C over night, P/N ratio was largest and the coating effect was best. So it was determined as the optimal coating condition of the recombinant E_2 protein.

Determination of optimal reaction time of serum with HRP labeled anti-porcine IgG: ELISA plate was coated with the recombinant E2 protein of optimal coating concentration at 37°C over night, sera and HRP labeled

Table 3: Determination of the optimal dilution of HRP labeled anti-porcine IgG

Dilutions of the secondary antibody	1:200	1:400	1:600	1:800	1:1000	1:1500	1:2000	Negative serum
OD_{490} of C strain	1.711	1.530	1.222	1.051	0.604	0.527	0.410	0.091
OD_{490} of LT strain	1.520	1.366	1.214	0.931	0.580	0.414	0.377	0.073

Table 4: The results of blocking test

	Block test		Positive control		Negative control	
Items	C	LT	C	LT	C	LT
OD	0.112	0.151	0.930	0.923	0.081	0.073
P/N	1.37	1.88	11.56	13.12		

Table 5: The results of sera-cross test

	E2 protein of C strain as coating antigen					E2 protein of LT strain as coating antigen				
Items	PPV	PRV	Cp	PRRSV	BVD	PPV	PRV	Cp	PRRSV	BVD
OD	0.102	0.153	0.091	0.134	0.157	0.135	0.121	0.103	0.111	0.141
P/N	1.25	1.88	1.13	1.62	1.91	1.86	1.71	1.43	1.57	1.64

Table 6: The results of repeated experiments

	Positive serum				Negative serum				
Repeated times	1	2	3	4	5	6	7	8	9
1	1.016	0.977	1.231	1.172	1.090	0.893	0.081	0.051	0.073
2	1.005	0.910	1.249	1.188	1.114	0.942	0.068	0.054	0.079
3	0.977	0.915	1.250	1.090	1.086	0.867	0.080	0.056	0.080
4	0.930	0.878	1.161	1.245	1.041	0.923	0.076	0.049	0.081
5	1.061	0.912	1.189	1.216	1.119	0.865	0.074	0.052	0.073
6	1.082	0.908	1.200	1.210	1.046	0.865	0.078	0.053	0.080
X[a]	1.012	0.917	1.213	1.187	1.083	0.893	0.076	0.053	0.078
S.D[b]	0.055	0.032	0.036	0.054	0.033	0.034	0.005	0.002	0.004
CV (%)[c]	5.43	3.49	2.97	4.55	3.05	3.81	5.58	3.77	5.13

[a]X means arithmetic mean value; [b]S.D means standard deviation; CV means coefficient of variation

anti-porcine IgG diluted to the optimal dilutions were mixed and reacted for 2, 1.5, 1 and 0.5 h at 37°C, respectively and OD_{490} value was measured and P/N ratio was calculated in order to determinate the optimal duration of reaction of tested sera with the secondary enzyme-labeled antibody. The test results revealed that the optimal reaction durations of the first antibody and second antibody were 1 h and 45 min, respectively.

Determination of the positive and negative critical values of indirect ELISA: Thirty CSFV negative sera were measured by ELISA, which showed that the mean value of the 30 negative sera OD_{490} was 0.1938 and the standard deviation (SD) was 0.05218. Therefore, the critical values of indirect ELISA is 0.3503, namely, when OD_{490} value of tested serum = 0.35, it will be CSF antibody positive; when OD_{490} value of tested serum < 0.35, it will be CSF antibody negative.

Specificity test of ELISA

Blocking test: The OD value of the positive serum blocked with recombinant CSFV E_2 was much less than that which was not blocked and its P/N ratio obviously reduced, too. Here, the (N-P)/N ratio of recombinant E_2 protein of C strain was 0.88; the (N-P)/N ratio of recombinant E2 protein of LT strain was 0.84, all of them were larger than 0.5. The result of test showed that reaction of CSF positive serum with recombinant E2 protein may be blocked with recombinant CSFV E_2 antigen and seen in the Table 4.

Cross test: According to the definitive conditions of ELISA, the reaction plate was coated with recombinant CSFV E_2 protein and positive serum to pig parvovirus, positive serum to pseudorabies virus, positive serum to PRRSV, positive serum to *Chlamydia psittaci* (Cp) and BVD positive serum was used to do ELISA cross test. The results of test showed that all reactions of the five non-CSFV-positive sera with recombinant CSFV E_2 protein were negative in ELISA and their P/N ratios all were less than 1.8. It was confirmed that the recombinant CSFV E_2 protein based ELISA has better specificity. The result of test was seen in the Table 5.

Reproducibility test: The recombinant E2 proteins of C and LT strains were used as coating antigens for coating ELISA plate and then nine serum samples were repeatedly measured 6 time with the ELISA method. The results of test revealed that difference of the OD_{490} values of each time was very little and the coefficient of variation ranged from 2.97-5.58%, which showed that reproducibility of the ELISA method was very ideal. The results of test were seen in the Table 6.

Table 7: The results of sensitivity test

	The dilutions of CSF-positive serum							
Items	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Negative serum
OD of C strain	1.325	1.216	1.071	0.786	0.601	0.340	0.292	0.09
OD of LI strain	1.265	1.117	0.981	0.853	0.701	0.588	0.380	0.07

Table 8: The differential diagnostic test results of serum specimens sampled from CSFV LI-artificialy infected pigs using various E2 antigens ELISA

	C strain-E2 ELISA			LI strain-E2 ELISA				
OD of sera	1:640	1:1280	1:2560	1:5210	1:640	1:1280	1:2560	1:5210	Negative serum
OD of serum I	0.603	0.341	0.293	0.254	0.703	0.637	0.401	0.382	0.09
OD of serum II	0.611	0.343	0.311	0.275	0.732	0.637	0.511	0.408	0.07

Sensitivity test: The reaction plates were coated with the optimal concentration E_2 proteins of C strain and LI strain, respectively and CSF-positive sera were diluted to 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and measured by ELISA with the optimal reaction condition. The results of test were seen in the Table 7.

The result of test (Table 7) revealed that in the wells coated with recombinant E_2 protein of C strain, positive serum was diluted to 1:1280 (well no.6), the OD_{490} was 0.34, which showed negative; in the wells coated with recombinant E_2 protein of LI strain, positive serum was diluted to 1:2560 (well no.7), the OD_{490} was 0.38, showing positive.

Biological sensitivity test revealed that on the day 3 after vaccination the CSF-positive antibody was detected from 1/2 pigs using C-strain E2 ELISA and on the day 5, 7 and 10 the 2 pigs showed positive for CSF antibody and the antibody titers became higher gradually.

Stability test: The purified recombinant E2 proteins kept at 4, -20 and -70°C for six months were used to do ELISA. The results of tests showed that the E2 protein antigen kept at 4°C was degraded, so it was not used to do ELISA. However, the purified recombinant E2 protein antigen kept at -20 or -70°C were steady and effectively used to do ELISA. So keeping time of the E_2 antigen at -20 or -70°C was 6 months or more.

Comparison tests of E_2 antigen- ELISA with whole CSFV C strain antigen- ELISA and IHA kits: Eighty swine serum specimens had been measured with whole CSFV antigen- indirect ELISA, 44 of which were shown as CSF positive and 36 CSF negative. And then the samples were measured for antibodies of CSFV using the E_2 antigen-indirect ELISA and IHA kits, respectively. In 44 CSF-positive serum specimens detected with whole CSFV antigen-indirect ELISA, 41 measured with the E_2 antigen-indirect ELISA were positive; 37 measured with whole CSFV Antigen-Indirect Hemagglutination (IHA) kit were positive. In 36 CSF-negative serum specimens detected with whole CSFV antigen-indirect ELISA, using the recombinant E_2 antigen-indirect ELISA for measurements, 35 samples were negative but one positive; however, by IHA kit, all 36 samples were negative. The results of tests showed that in comparison of the recombinant E_2 antigen-indirect ELISA with whole CSFV antigen-indirect ELISA, the specificity was 97.14% and the sensitivity was 93.18% and both coincidence rate reached 95%. In them there was no significance of difference ($p>0.05$) by statistics. In conclusion, the specificity and sensitivity of the recombinant E_2 antigen-indirect ELISA were higher than that of IHA kit.

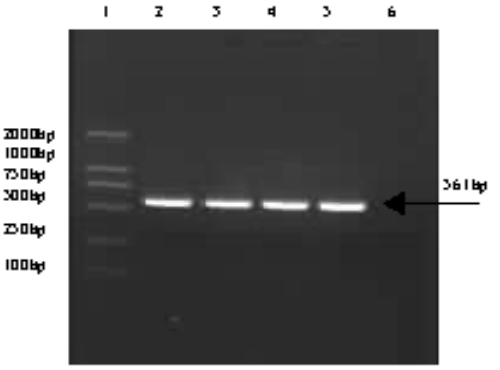

Fig. 2: PCR results of the blood I and II genes, (1) DNA Marker DL 2 000, (2) Amplified result of the blood I, (3) Amplified result of the blood II, (4) Amplified result of the mixed blood I+II, (5) Amplified result of E2 gene of the positive controls, (6) Negative control

Differential diagnostic test of serum specimens sampled from CSFV LT-artificialy infected pigs using various E2 antigens ELISA: Two 6 month old pigs negative for CSF antibody were subcutaneously infected with CFSV LI field strain at a dosage of 2 mL blood containing CFSV LI strain. the two pigs all fell ill showing conspicuous CSF symptoms for example, high body temperatures (39.8 and 40.3°C), anorexia, hyponoia, etc, 10 days post artificial infection and the blood specimens (I and II) were sampled from the affected pigs and diagnosed by a CSF diagnostic RT-PCR kit which were confirmed as CSF (Fig. 2).

The amplified products were 561 bp in size which were in line with the anticipated E_2 DNA fragment of CSFV.

According to the sensitivity test result (Table 7), the 2 serum specimens (serum I and serum II) which were sampled from the two pigs affected with CSFV LT strain were diluted at 1:640, 1:1280, 1:2560 and 1:5120. And then the diluted sera were measured by C strain-E2 ELISA and LT strain-E2 ELISA, respectively. The test results were seen in the Table 8.

It showed that when the serum I and II were diluted to 1:1280 or above the tested result showed CSF negative (the OD_{490} = 0.341 or 0.343 <0.35) in C strain-E2 ELISA but still remained CSF positive (the OD_{490} = 0.637 or 0.658<*f*0.35) in LT strain-E2 ELISA, which revealed if CSF broke out, sera from affected pigs would be diluted to to 1:1280 or above and measured with LT strain-E2 ELISA and the tesed sera showed positive.

DISCUSSION

In this research, the many conditions for using the recombinant CSFV E_2 protein as diagnostic antigen to establish an indirect ELISA for antibody to CSFV were explored and the recombinant CSFV E_2 antigen-indirect ELISA was compared with whole CSFV C strain antigen-indirect ELISA kit and IHA kit. Glucoprotein E_2 is the most important immune-predominant protein of four structural components (C, E_0, E_1, E_2) of CSFV (Summerfield *et al*., 1998; Koing Lengsfeld, 1998; Behrens *et al*., 1998). We applied recombinant E_2 protein expressed in *E. coli* to replace entire CSFV antigen for establishing ELISA method for diagnosis of CSF, which overcomes many shortcomings including high costing, alive CSFV diffusing and cross reaction, etc. In this test, GST-E_2 fusion protein was used, through Western-blotting and ELISA-determination test, it was showed that there was no influence of existence of GST label on crude activity of the expressed recombinant E_2 protein in GST-E_2 fusion protein.

About the optimum work conditions of the indirect ELISA, various coating times may obviously affect the results of test in the coating process, by comparisons it was found that the optimum coating condition was 37°C 1 h + 4°C over night. According to the characters of the established ELISA method, the reaction periods of both the first antibody and the second antibody were determined, which confirmed that the optimal time of the first antibody reaction was 1 h and the secondary antibody 45 min. It was very important for successful experiments that the optimal work concentration of enzyme conjugate was determined. If the concentration of enzyme conjugate was much low, the sensitivity of ELISA would be reduced, but much high, false- positive results would be produced. In addition, the unlinked enzyme conjugate must be rinsed cleanly from wells of the plates before substrate will be added, otherwise false-positive reactions will be produced also.

There were differences in the results tested with the recombinant E_2 protein antigen-indirect ELISA and whole CSFV antigen- indirect ELISA kit because of dissimilitude of the coating antigens used in both ELISA. In the recombinant E_2 antigen-indirect ELISA, the used antigen was recombinant CSFV E_2 protein but in ELISA kit, the used antigen was entire CSFV particles, so if antibodies to E_0 or E_1 were more but antibodies to E_2 were less, the detectable rate of the recombinant E_2 protein antigen-indirect ELISA might decrease. In 80 serum samples, 3 were diagnosed as negative in the recombinant E2 antigen-indirect ELISA but positive in whole CSFV antigen- indirect ELISA kit.

In the sensitivity test, the result of test (Table 7) revealed that in the wells coated with recombinant E2 protein of C strain, positive serum was diluted to 1:1280 (well no.6), the OD_{490} was 0.34, which showed negative; in the wells coated with recombinant E_2 protein of LT strain, positive serum was diluted to 1:2560 (well no.7), the OD_{490} was 0.38, showing positive. This status may be associated with differences of both strains E_2 genes (Guo H and Qiu C, 2003). In the CSFV LT strain artificial infection test, it showed that when the serum I and II were diluted to 1:1280 or above the tested result showed CSF negative (the OD_{490}= 0.341 or 0.343 <0.35) in C strain-E2 ELISA but still remained CSF positive (the OD_{490}= 0.637 or 0.658<*f*0.35) in LT strain-E2 ELISA, the test result would denote CSF occurred. So we think the method is valuable for diagnosis of atypical CSF incorporating clinical symptoms of affected pigs. We know that C strain belongs to CSFV group 1 and LT-strain belongs to CSFV group2 (Guo *et al*., 2003) and the homology of C strain E_2 gene and LT strain E_2 gene was 88.6% and the homology of amino acid of C strain E_2 and LT strain E_2 was 82.4% (Guo *et al*., 2003), so we think it may be possible that differences between C strain E_2 and LT strain E_2 were utilized to develop an ELISA method for differential diagnosis of CSFV field strains group 2 infected antibody. Atypical CSF has been more frequent and epidemic CSFV group2 are predominate in China (Han *et al*., 1999; Tu *et al*., 2001; Guo *et al*., 2003), so developing a diagnosis method for rapid detecting CSFV field strains infected antibody will be very important for control/eradication of CSF in China.

The recombinant E_2 protein was used as antigen of indirect ELISA for measuring antibody to CSF in the test and it was proved that this method had very strong

specificity and very high sensitivity without any cross reactions with many viral diseases including BVD. In this research, a part of the E2 gene including A, B, C and D antigen epitopes was expressed (Hu *et al*., 2004, 2005), which had kept away from high homology domain with BVDV (Van Rijn, 2007). Using the CSFV C strain E2 protein and TL strain E2 protein expressed as antigens to detect the positive sera to BVDV by the indirect ELISA, respectively, the values of OD490 were 0.157and 0.141, respectively, which all were smaller than 0.35 and showed no cross-reaction between BVDV and the target protein, so the expressed proteins could be used as coating antigen to develop an ELISA kit for detecting anti-CSFV antibody. Of course, the expressed CSFV C strain E2 protein and TL strain E2 protein may be also used to prepare monoclonal antibodies against CSFV for studying a differential technique of distinguishing C strain vaccination antibody from CSFV infection antibody but need a lot of money. However, in this research, the tested pig sera were diluted to 1:1280 or above which maybe avoided C-strain antibody disturber and measured by the ELISA and discriminated each other, which was very simple and convenient as well as convenient. Of course, this discovery was finally accepted though measurements of a lot of samples. So far it was not reported that the method was applied to differentiate atypical CSF from C-strain vaccinated antibody.

The low-cost expression system selected in the test had advantages of economic costs, operation simpleness and short cycle and used to produce a lot of diagnostic antigens of CSF. Successful establishment and application of the recombinant E_2 protein antigen-indirect ELISA will supply a strong tool of diagnosis and monitoring of CSF with pig industry.

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