Development and application of an indirect ELISA for the detection of antibodies against encephalomyocarditis virus

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Abstract. Encephalomyocarditis virus (EMCV) can cause acute myocarditis in young pigs or reproductive failure in sows. It has been recognized worldwide as a pathogen infecting many species and causes substantial economic losses. In the present study, an indirect ELISA was developed for the detection of antibodies to EMCV. The VP1 gene of EMCV was amplified by reverse transcription-quantitative polymerase chain reaction and expressed in *Escherichia coli* with 49.3 kDa under the condition of isopropyl-β-d-thiogalactoside. Following this, the authors obtained the recombinant protein VP1 as a coating antigen. The antigen concentration and serum dilution were optimized using a checkerboard titration. Compared with viral neutralization tests, the sensitivity and specificity of the indirect ELISA was 95.7% and 92.9%, respectively. A total of 265 clinical swine serum samples from different pig farms in China were used to a serological survey. The seropositive rate of the serum samples was 81.9%. In conclusion, the developed indirect ELISA assay is sensitive and specific, which will be useful for large-scale serological survey in EMCV infection and monitoring antibodies titers against EMCV.

Introduction

Encephalomyocarditis virus (EMCV), a member of the genus Cardiovirus in the family *Picornaviridae*, is a single-stranded positive-sense RNA virus of ~7.8 kb in length with a large open reading frame (ORF) (1). It contains a 5' untranslated region (5' UTR) and a 3' untranslated region (3' UTR). The ORF encodes for 11 proteins, including four structural proteins (VP1, VP2, VP3, VP4), seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) and a leader protein. The VP1 region, which is highly variable, is an important protective antigen of EMCV (2). It can stimulate the body to produce neutralizing antibodies. It is also the important region for researching gene vaccine (3).

EMCV has been recognized worldwide as a pathogen infecting many species, including pigs, cattle, rodents, raccoons, elephants, marsupials and primates such as baboons, chimpanzees, monkeys, and even humans (4-7). The virus, which currently affects the global swine industry, can cause myocarditis, reproductive failure and high mortality in pregnant sows, fetuses and weaning piglets. In China, it had been confirmed that EMCV infection occurs in many pig farms by the methods of etiology and serology (8).

In the current study, the authors used the reverse transcription-quantitative polymerase chain reaction method to successfully amplify the full-length VP1 coding sequence. They subsequently produced the recombinant gene product in the *Escherichia coli* expression system, which is commonly used to produce adequate amounts of protein suitable for the intended application. *E. coli* is easy to transform, grows quickly in simple media, and requires inexpensive equipment for growth and storage. The produced recombinant VP1 was then used to develop an indirect ELISA assay with sensitivity and specificity comparable to virus neutralization tests.

Materials and methods

RNA isolation. EMCV strain BD2 (KF709977) was isolated from three clinically ill newborn pigs, which exhibited anorexia, rapid breathing and acute myocarditis, from a commercial pig farm in Hebei, China, as described previously (9). BHK-21 cells were grown in 75 cm^2 plastic flasks, inoculated with 1 ml of virus supernatant from positive cell cultures. After an extensive cytopathic effect was observed, frozen and thawed three times and centrifuged at 13,400 x g for 10 min at 4˚C.

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Abbreviations: EMCV, encephalomyocarditis virus; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-d-thiogalactoside

Key words: encephalomyocarditis virus, indirect ELISA, VP1, antibodies

Genomic RNA of EMCV BD2 was extracted from the cultural supernatant of infected BHK-21 cells using the EasyPure Viral DNA/RNA kit (Beijing Transgen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Amplification of the EMCV VP1 gene. The VP1 gene was amplified by RT-qPCR using the template from the last step. The genome sequence of the BD2 strain EMCV-VP1 has been uploaded to the NCBI (accession no. KF709977.1).

The forward primer contained a BamHI restriction site at the 5'-end of the sequence coding for VP1, while the reverse primer contained an XhoI site positioned after the stop codon at the 3'-end. The sequences of the primers were as follows: forward, 5'-CGCGGATCCGGAGTAGAAAACGCTGAAAAAG-3' and reverse, 5'-CCGCTCGAGCTCTAGCATCAAGACTCCA GCT-3'. Both primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and used without further purification. The PCR reaction mixture included 1 μg DNA template, 1 μl PrimeSTART HS DNA Polymerase (Takara Biotechnology Co., Ltd., Dalian, China), 10 μl 5X PrimeSTART buffer (Beyotime Institute of Biotechnology, Haimen, China), all four dNTPs at final concentrations of 0.2 mM each, primers at a final concentration of 0.2 μM each, and ddH_2O in a total volume of 50 μl. The cycling conditions included an initial denaturation step at 98˚C for 30 sec, and then 30 cycles consisting of 30 sec denaturation at 98˚C, 30 sec annealing at 56˚C, and 50 sec extension at 72˚C, followed by a final extension for 10 min at 72˚C. The amplification product was purified on a 1% (w/v) agarose gel in TAE buffer, stained with ethidium bromide, and extracted using a commercial kit (QIAquick Gel Extraction kit; Sangon Biotech Co., Ltd.), according to the manufacturer's instructions.

Construction of recombinant proteins. Following extraction, the DNA product and the expression vector pET32a plasmid were each digested with BamHI and *XhoI* using standard molecular biology protocols. The VP1 DNA insert (55 ng) and linearized pET32a (20 ng) were incubated at 16˚C overnight in the presence of 1 μl T4 DNA ligase in a total volume of 10 μl according to the manufacturer's instructions.

The entire ligation reaction was transformed into Trans1-T1 chemically competent cells (Beijing Transgen Biotech Co.). Several colonies were screened for the presence of EMCV VP1 DNA by colony PCR; colonies that were positive were grown in liquid culture and plasmids were purified using a TIANprep Mini Plasmid kit (Tiangen Biotech Co., Ltd., Beijing). The presence of EMCV VP1 DNA was confirmed by restriction digests of the purified plasmids, and plasmids were sequenced to confirm that the correct sequence had been inserted. In addition, the specificity of VP1 was confirmed via cross-reactivity using ELISA kits for classical swine fever virus (cat. no. SL0002Po; Sunglong Biotech Co., Ltd., Hangzhou, China), porcine respiratory and reproductive syndrome virus (cat. no. KA2120; Abnova, Taipei, Taiwan), pseudorabies virus (cat. no. PRV041117A; Zhejiang Gloria Bioscience, Co., Ltd., Hangzhou, China) and porcine circovirus type 2 virus (cat. no. AE-200150-2; Alpha Diagnostic International, Inc., Texas, USA).

The expression plasmid was then transformed into BL21(DE3) chemically competent cells (Takara Biotechnology, Co., Ltd.). Colonies that were positive were grown in liquid culture. Following confirmation of the correct sequence, the bacteria were expanded on a large scale and preserved with 50% glycerinin normal saline at -20˚C.

Protein expression and analysis. A small culture (15 ml) of LB media containing 100 μg/ml ampicillin was inoculated with 150 μl preserved bacteria. After shaking at 37˚C for 2-3 h, the optical density (OD) of the culture at 600 nm reached 0.6-0.8. At this point, 1ml of the culture was collected before addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Then expression was measured for several hours at 37˚C with shaking, and cultures were harvested at every hour. The control was the non-carrier of pET32a that was induced by IPTG for 5 h. All of the cultures were centrifuged for 3 min at 9,000 x g. Following centrifugation, the supernatant was discarded. Pellets were resuspended in 50 μl 2X SDS buffer and degenerated at 100˚C for 10 min. Following centrifugation for 3 min at 9,000 x g, the supernatant were run on a 12% SDS-PAGE gel. Finally, the recombinant protein activity was analyzed by western blotting. For western blotting, the cells were rinsed three times with PBS, lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and incubated on ice for 30 min. The lysates were then centrifuged at 13,400 x g for 20 min at 4˚C and the supernatant extracts were quantified for the total protein using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) with bovine serum albumin as the standard. Aliquots from each protein lysate sample were separated in 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer (TBS and 5% skimmed milk) for 1 h at room temperature and incubated overnight in the presence of rabbit anti-foot and mouth disease virus polyprotein (VP1 protein) primary antibody (1:800; cat. no. PL0301542; PL Laboratories, Inc., Port Moody, Canada) at 4˚C. The membrane was washed three times with TBS containing 0.05% Tween-20 (TBST) and subsequently reacted with anti-rabbit immunoglobulin G (IgG; H + L) conjugated to horseradish peroxidase (HRP; 1:800; cat. no. E030120; EarthOx, LLC, Millbrae, CA, USA) for 1 h at room temperature. Following thorough washing with TBST, the immunoreactive bands were developed using enhanced chemiluminescence detection reagents (EMD Millipore, Billerica, MA, USA). The OD of the bands on the films was analyzed using imaging software (ImageQuant Las 4000, version 1.2; GE Healthcare Life Sciences, Chalfont, UK).

Recombinant protein (VP1) purification and analysis. According to the optimal condition, a small culture (10 ml) of LB media containing 100 μg/ml ampicillin was inoculated with 10 μl preserved bacteria. After shaking overnight at 37˚C, the small culture was added to 500 ml LB media (with antibiotics at the concentrations used for the small culture) and incubated at 37˚C with agitation until the OD of the culture at 600 nm reached 0.6-0.8. At this point, the culture was allowed to add IPTG to a final concentration of 1 mM. Expression was allowed for several hours at 37˚C with shaking, and cultures were harvested at the optimal time by centrifugation for 20 min at 9,000 x g. The pellets were stored at -80˚C until

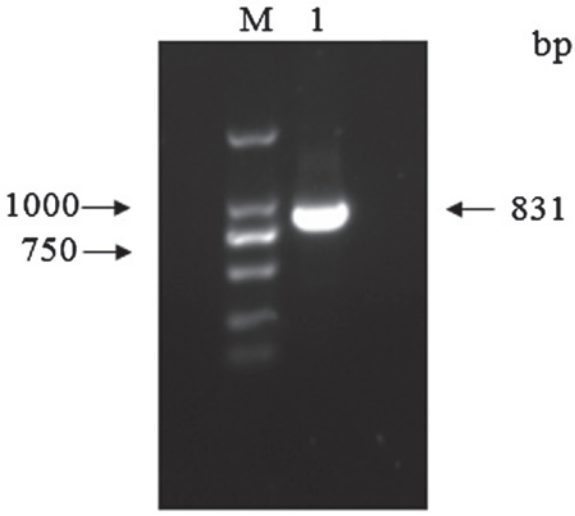

Figure 1. Data graph of VP1 amplification from encephalomyocarditis virus by reverse transcription-quantitative PCR. Lane 1, VP1 gene. The approximate size of the PCR product (right) corresponds to the projected size of 831 bp. M, 2,000 bp DNA Marker. PCR, polymerase chain reaction.

purification took place. In addition, the purification of VP1 was conducted under a low temperature environment, a condition of non degeneration.

The pellets (~1.0 g wet weight, representing 500 ml liquid culture) were thawed on ice and resuspended completely in 20 ml lysis buffer (Beyotime Institute of Biotechnology). The cells were sonicated on ice using 10 cycles of the following sequence: 1 sec on, 1 sec off for 20 sec; rest on ice for 40 sec. The lysate was centrifuged at 13,400 x g for 15 min at 4˚C, and the pellet was collected. The solid white pellet was gently and completely resuspended on ice with 10 ml binding buffer [20 mMTris(2-carboxyethyl)phosphine-HCl pH 7.9, 5 mM imidazole, 0.5 M NaCl, 8 M urea]. Then centrifuged at 13,400 x g for 20 min at 4˚C. Following centrifugation, the supernatant was collected. The solution was added to a column containing 1 ml complete His-Tag Purification resin (CWBIO, Beijing, China) and washed extensively with binding buffer containing 5 mM imidazole. EMCV VP1 protein was eluted in elution buffer (Beyotime Institute of Biotechnology); two elution buffers (10 ml each, containing 200 and 500 mM imidazole, respectively) were added sequentially to the column, and the elute was collected in 5 ml fractions. Fractions were then run on a 12% SDS-PAGE gel.

Development of the indirect ELISA-VP1. Optimal dilutions of VP1 and sera were determined by a checkerboard titration test with EMCV positive and negative sera previously analyzed by virus neutralization tests. The antibody of VP1 has neutralizing activity, and it was confirmed that the virus did not exist in the negative sera. The antigen was coated in 96-well ELISA plates and diluted in 0.05 mol/l carbonate buffer (pH 9.6) to 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200. Reference positive and negative sera were both diluted in 1:10, 1:20, 1:40 and 1:80, respectively, and tested to determine the optimal serum dilution. The dilutions that gave the maximum difference in absorbance at 450 nm between the positive and the negative sera (P/N) were selected to test the sera panel. The working dilution of rabbit anti-swine horseradish peroxidase (HRP)-IgG (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), the reaction temperature, time and other conditions also were optimized.

Carbonate buffer solution (0.05 mol/l, pH 9.6) served as coating buffer. The antigen was diluted to 0.605 μg/ml and coated on 96-well microtiter plates with 100 μl, overnight at 4˚C. The plates were washed three times for 5 min with PBS and 0.5% Tween-20 (PBS-T). Then, the plates were blocked with blocking buffer [Tris-buffered saline (TBS) and 5% skimmed milk] at 37˚C for 1 h followed by a washing step. Subsequently, 100 μl sera was diluted (1:40) in dilution buffer and incubated at 37˚C for 1 h followed by a washing step. A total of volume of 50 μl HRP-labeled rabbit-anti-swine protein IgG (1:800; cat. no. E030110; EarthOx, LLC) was added to all wells and incubated at 37˚C for 1 h. After washing again, 100 μl freshly prepared substrate solution (1 mg/ml tetramethylbenzidine that contained 3 μl 30% hydrogen peroxide) was dispensed into each well. Color development was in the dark for 15 min, and the reaction was stopped by addition of 50 μl 2 M sulfuric acid. The absorbance values were read at 450 nm using an absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

To determine the cut-off value of the ELISA-VP1, 20 samples of SPF swine serum was obtained and the dilution was 1:40. The test for indirect ELISA is done in the optimal conditions. The result was analyzed by statistics to achieve the average value, X, and the standard deviation, S. The samples with OD450≥X +3S were positive. The samples with OD450<X+ 3S were negative.

Comparison of the indirect ELISA with the virus neutralization tests. A total of 120 sera samples were obtained from different swine herds in Baoding, China. They were tested to evaluate the correlation between the neutralization test and the indirect ELISA.

Quality assurance and repeatability of ELISA. The repeatability (intra-plate variability) was assessed by the same analyst who tested five sera samples, four times, on the same plate, on the same day. The mean and coefficients of variation (CVs) were computed using Excel 2003 (Microsoft Corporation, Redmond, WA, USA).

The reproducibility (inter-plate variability) of the test results was assessed by testing five sera samples 10 times. The results were obtained by use of distinct lots, on different days, by different analysts. The mean and CVs were computed.

Field application of VP1-ELISA in clinical samples. During 2013 and 2014, a total of 265 clinical swine serum samples were collected from pig farms in surrounding of Baoding, China. According to the established indirect ELISA method, they were detected for the EMCV antibody.

Results

PCR amplification and sequencing of the EMCV VP1 gene. The VP1 DNA was amplified with primers designed to insert a BamHI restriction site at the 5'-end of the sequence and an *Xho*I site at the 3'-end. Analysis of the reaction products by agarose

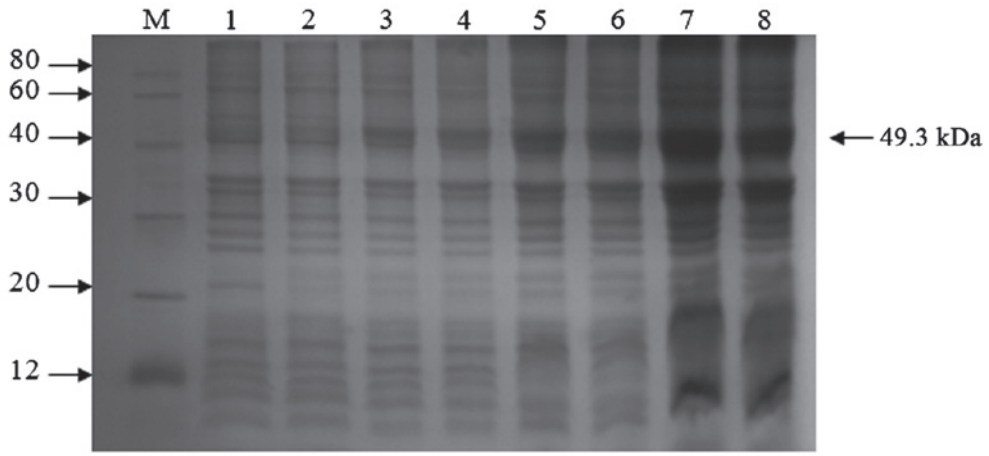

Figure 2. SDS-PAGE analysis of recombinant VP1 protein expressed in pET32a. Lane 1, pET32a/BL21 induced with IPTG(5h); lane 2, pET32a- VP1/BL21 without induction with IPTG; lanes 3-8, pET-32a-VP1/ BL21 induced with IPTG for 1, 2, 3, 4, 5 and 6 h, respectively. M, molecular weight marker; IPTG, isopropyl-β-d-thiogalactoside.

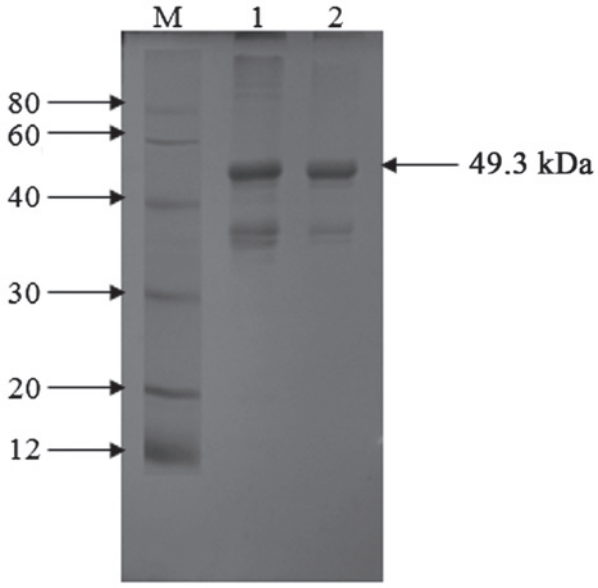

Figure 3. Samples from Ni-NTA purification run on SDS-PAGE (12%). M, molecular weight marker; lane 1, eluate using 200 mM imidazole; lane 2, eluate using 500 mM imidazole.

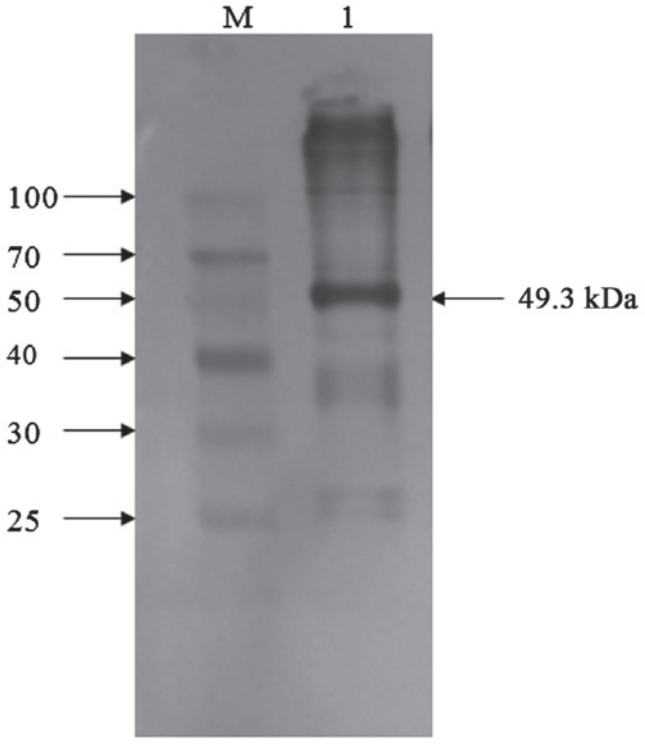

Figure 4. Western blot analysis of recombinant VP1 protein expressed in pET-32a. M, prestained molecular weight marker; lane 1,recombinant VP1 protein.

gel electrophoresis and ethidium bromide staining revealed a specific amplimer of the expected size, ~831 bp (Fig. 1).

Expression and purification of VP1. Following digestion, ligation and sequencing, the plasmid was transformed into BL21(DE3) chemically competent cells for expression. Expression was induced at 37˚C and was allowed to continue for 6 h. SDS-PAGE analysis of the samples indicated that the control of pET32a induced for 5 h and the non-induced recombinant VP1 protein did not have target bands. The cultures that were induced by IPTG every hour show that a target band appeared and the optimal expression time of VP1 protein is 5 h (Fig. 2). Following purification using Ni-NTA, fractions were run on a 12% SDS-PAGE gel, where the majority of pure VP1 protein was found to elute at 500 mM imidazole (Fig. 3). Western blotting with an anti-EMCV VP1 monoclonal antibody revealed a specific band of the same size (Fig. 4). By purifying, 0.484 mg/ml recombinant VP1 protein was obtained then stored in -80˚C. The positive immunoblotting results suggested that could recombinant EMCV VP1 expressed and purified in *Escherichia coli* could be used to develop an indirect ELISA test.

ELISA-VP1. By using checkerboard titration tests, the OD value gave the maximum difference between the positive serum and negative serum (P/N value of 5.62) when the dilutions of antigen and serum were 1:800 and 1:40, respectively (Table I). Therefore, the final concentration of coating antigen was 0.605 μg/ml by calculation, and the optimal dilution of the HRP-IgG was 1:800 (Table II). A total of 20 samples of

Table I. Determination of the coating concentration of antigen and the dilution of serum samples.

	OD of positive serum with different coating antigen dilutions of recombinant VP1					
Serum dilution	1:100	1:200	1:400	1:800	1:1,600	1:3,200
1:20						
P	1.465	1.351	1.280	1.120	0.982	0.991
N	0.442	0.402	0.339	0.320	0.252	0.252
P/N	3.31	3.36	3.78	3.50	3.90	3.93
1:40						
P	1.301	1.230	1.185	1.063	0.895	0.792
N	0.377	0.334	0.299	0.189	0.170	0.154
P/N	3.45	3.68	3.96	5.62	5.26	5.14
1:80						
P	1.086	0.982	0.931	0.883	0.802	0.647
N	0.319	0.276	0.251	0.228	0.175	0.148
P/N	3.40	3.56	3.71	3.87	4.58	4.37
1:160						
P	0.902	0.852	0.780	0.670	0.602	0.591
N	0.288	0.226	0.208	0.190	0.148	0.138
P/N	3.13	3.77	3.75	3.53	4.07	4.28

OD, optical density; P/N, ratio between positive and negative sera.

Table II. Determination of the optimal working concentration of HRP-labeled rabbit anti-swine IgG.

HRP-labeled dilution	Positive serum(Ab)	Negative serum(Ab)	P/N value
1:200	1.327	0.276	4.81
1:400	1.269	0.214	5.93
1:800	1.108	0.179	6.19
1:1600	0.719	0.160	4.49

HRP, horseradish peroxidase; P/N, ratio between positive and negative sera; Ab, antibody; IgG, immunoglobulin G.

Table III. Presence of antibodies against EMCV as determined by iELISA and neutralization test of 120 swine serum samples from pig farms.

	Neutralization test		
iELISA[a]	Positive (+)	Negative (-)	Total (n)
Positive (+)	88	2	90
Negative (-)	4	26	30
Total	92	28	120

[a]Relative sensitivity of iELISA Neutralization test=88 of 92, 95.7%; relative specificity of iELISA Neutralization test=26 of 28, 92.9%. EMCV, encephalomyocarditis virus; iELISA, indirect ELISA.

negative swine serum were tested under optimal conditions by indirect ELISA. The highest OD450 was 0.194 and the lowest OD450 was 0.103. The average value is 0.142 and the standard deviation is 0.02463. X +3S=0.22. Therefore, the cut-off value is 0.22. A serum sample was considered positive if its P/N value was ≥0.22. At this value, the highest efficiency of sensitivity and specificity was achieved. With this value, four of the 92 positive samples determined by neutralization test were negative by indirect ELISA, and two of the 28 negative sera by neutralization test were tested positive by indirect ELISA. The ELISA gave 95.7% (88/92) sensitivity and 92.9% (26/28) specificity, respectively (Table III). The result for 265 clinical swine serum samples from Baoding demonstrated that 217 samples were positive. The antibody positive rate against EMCV was 81.9%. It indicated that swine in Baoding infected with EMCV is a serious issue.

Discussion

EMCV mainly causes piglet encephalitis, myocarditis and sudden mortality. Many countries and regions have reported an outbreak of the disease in swine herds. For the first time in 2005 in China, EMCV was isolated in dead piglets and aborted fetuses (10). A serological survey indicated that EMCV has become a danger to the healthy development of China's pig industry (8). This research using the prokaryotic expression vector of pET32a constructed EMCV VP1 gene recombinant expression plasmid pET32a-VP1, and in *E. coli* BL21 (DE3) strains successfully expressed protein VP1. Although the prokaryotic expression system cannot be modified, it is often used to express high quantities of foreign proteins at low cost. The current study used the prokaryotic expression system to

demonstrate that, following induction by IPTG, recombinant VP1 protein is highly expressed. The results indicated that the accumulation of VP1 protein in the cytoplasm may have been due to an imbalance between the rapid expression of exogenous protein and its removal from the cell. The cultivation conditions and availability of the amino acids components of VP1 may have also been influential factors regarding the level of VP1 expression (11). Busuttil *et al* previously indicated that the soluble protein ratio may be increased in the following ways: Reduction in temperature; co-expression with a molecular chaperone; and addition of a chemical reagent in the process of induction (12). Meanwhile, other experiments by our group attempted to change several conditions, including temperature, speed of the shaking table, and the dosage and frequency of IPTG administration; however, the majority of the recombinant protein was present in inclusion bodies (data not shown). To improve the expression of soluble protein, other methods should be trialed in future studies.

In addition, the present study also identified that recombinant VP1 was a high activity protein in terms of sensitivity and specificity. This may form a basis for the development of clinical diagnostic kits and genetic engineering vaccines.

EMCV is a pathogen of zoonosis and can be diagnosed by RT-qPCR (13,14). This method has high sensitivity, strong specificity and a simple operation. It has been widely used in the diagnosis of diseases of pigs. Nevertheless, this ELISA provided an alternative, inexpensive and rapid serological detection method that would be suitable for screening for anti-EMCV antibodies titers on a large scale. In the current study, the authors established an indirect ELISA to detect the antibodies against EMCV with the recombinant pET32a-VP1 epitope protein in swine. By using checkerboard titration tests, the optimal antigen concentration and the dilutions of serum was obtained. Meanwhile, the dilution of the secondary antibody is important as well.

EMCV infections are a worldwide issue. In Panama, a strain of EMCV was isolated from sick pigs in 1958 for the first time. By the 1970s, many countries, including Australia, Greece, Belgium, South Africa, Italy and Japan had reported the disease (15-17). The positive rate of neutralizing antibody in serum ranges between 2 and 87% (18). In the Netherlands in 2006, 3,237 sera from 277 pig breeding farms were analyzed to identify antibody levels. 9.3% of the serum samples were positive, 58.8% of the sow was positive (19). In Tianjin, 295 serum samples from 66 pig breeding farms in seven counties were measured with indirect ELISA for the antibody levels and 84.75% of the serum were positive (20). In 13 provinces of China, 3,250 serum samples from the 46 farms were measured with this method for the antibody levels. The rate of antibody positive is 72% (21).

In conclusion, the indirect ELISA assay was developed successfully for the detection of antibodies to EMCV with high levels of sensitivity and specificity. The assay will be useful test for large-scale serological survey in EMCV infection and monitoring antibodies titers against EMCV.

Acknowledgements

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