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Development and evaluation of indirect enzyme-linked immunosorbent assay for a screening test to detect antibodies against classical swine fever virus

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Abstract

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for a screening test to detect antibodies against classical swine fever virus (CSFV). Viral glycoproteins, which were purified from swine kidney cells infected with CSFV ALD/A76 strain by the immunoaffinity purification using monoclonal antibody against E2 protein, were adsorbed on a microtiter plate as the antigen for the antibody detection. Each antibody titer of serum sample was expressed as a sample per positive value calculated with optical absorbance of each sample and that of a positive control. The advantage of this ELISA is its higher sensitivity: most sera containing more than 4 neutralization titers were determined to be positive. This ELISA is unable to discriminate between antibodies against CSFV and those against other ruminant pestiviruses, therefore positive sera in this ELISA should be evaluated by a cross-neutralization test using CSFV, bovine viral diarrhea virus, and border disease virus. Taken together, the indirect ELISA developed in this study is useful screening tool to detect antibodies against CSFV for the large-scale monitoring of classical swine fever.

Key words: antibody, classical swine fever, ELISA, pestivirus

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Introduction

Classical swine fever (CSF) is an economically important, highly contagious disease of pigs caused by classical swine fever virus (CSFV). The virus belongs to the genus *Pestivirus* of the family *Flaviviridae*, together with bovine viral diarrhoea virus (BVDV) and border disease virus (BDV)⁹. CSFV possesses a single-stranded positive-sense RNA genome of approximately 12.3 kb with one large open reading frame flanked by a 5' and 3' untranslated region and coding for a polyprotein of approximately 4,000 amino acids⁹. Co- and posttranslational processing of the polyprotein by cellular and viral proteases yields the 12 cleavage products N^{pro}, C, E^{ms}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B⁹. The structural components of the CSFV virion include the capsid (C) protein and the glycoproteins E^{ms}, E1, and E2, and others are the non-structural proteins^{2,9}.

Since CSF was epidemic and caused severe economic damage before the 1990s, live attenuated GPE⁻ vaccine²¹ have been used widely in Japan. The final outbreak of CSF in Japan was in 1992 and no CSF case has been reported in the last 20 years. In 2007, Japan was designated a CSF-free country based on the regulations of the World Organization for Animal Health. As CSF causes severe loss to industries, various countries have striven to eradicate CSF. The basic principal of CSF eradication is a "check and slaughter" policy, and antigen detection is the highest priority in diagnosis. However, CSF also runs a chronic or inapparent course, like typical acute and sub-acute courses⁸. This variety of symptoms is dependent on the virulence of the virus and the time of infection^{18,22}. Adult pigs usually display less severe signs of disease than young piglets and stand a better chance of survival. For these reasons, serological methods are also valuable for monitoring and prevalence studies, and are essential if a country wish to be internationally recognized as being free from the disease in the absence of vaccination. In the Netherlands, 10,000 serum

samples from slaughterhouses have been examined each year for such monitoring⁴.

The virus neutralization test (VNT) has been used as the golden standard of antibody test¹⁴. The immunofluorescence test, neutralizing peroxidase-linked assay¹⁴, and the exaltation of Newcastle disease virus (END) method^{6,10} have been used as VNTs. However, these methods have common defects: one is that these methods require BVDV antibody free serum, and the other is that these methods require a staining procedure or superinfection with other viruses because the usual CSFV is non-cytopathogenic. A novel virus neutralization method, the cytopathogenicity-neutralization test (CP-NT), was developed in CPK-NS and FS-L3 cells that are able to grow in serum-free culture medium^{19,20}, and has been used in local veterinary service in Japan. Since this CP-NT method is based on the cytopathic effect (CPE) caused by the infection of the CSFV GPE⁻ strain¹ in CPK-NS and FS-L3 cells, it is a safe and easy routine test of antibody detection in veterinary service. Although these VNTs are very sensitive and specific, they are complicated and time consuming, require several days to get final results, and also require considerable effort in keeping the virus strains and the cultured cells in good condition. Furthermore, a high biosecurity level facility is necessary to avoid release of infectious viruses into the environment.

In present study, CSFV envelope glycoproteins were purified from a micro-carrier culture of SK-L cells infected with CSFV ALD/A76. By using these purified proteins containing E2 glycoprotein as major component, an indirect enzyme-linked immunosorbent assay (ELISA) was developed and its utility is described here.

Materials and Methods

Viruses and cells: CSFV strains ALD/A76⁵ and GPE⁻²¹, and BDV 87/6¹⁷ were propagated in the stable swine kidney cell line, SK-L²⁰. BVDV genotype-1 Nose¹¹ and genotype-2 KZ-91NCP¹²

were propagated in primary bovine fetal muscle (pBFM) cells. SK-L and pBFM cells were grown in Eagle's MEM, supplemented with 0.295% tryptose phosphate broth, 2.292mg/ml L-glutamine, and 10% horse serum, adjusted at pH 6.8-7.2 with sodium bicarbonate. CPK-NS cells were grown in serum-free medium comprising with Eagle's MEM, 0.295% tryptose phosphate broth, 0.5% bacto peptone, 10 mM N,N-bis (2-hydroxymethyl)- 2-aminoethane sulfonic acid, 0.292 mg/ml of L-glutamate, and 2.25 mg/ml of sodium bicarbonate¹⁹). Hybridoma cells producing monoclonal antibody A301 to the E2 protein of CSFV¹³) were grown in RPMI 1640 medium containing 10% fetal calf serum.

Virus neutralization test (VNT): Antibody titer against CSFV was assayed with a CP-NT as follows using CPK-NS cells grown in the serum-free medium described above¹⁹). Each equal volume of sample serum and 200 TCID₅₀ of the CSFV strain GPE⁻ was mixed and incubated at 37°C for 1 hr. This mixture and a CPK-NS cell suspension were inoculated into a microtiter plate and incubated for 7 days. The neutralizing antibody was titrated by using CPE as an infectious marker. Antibody titers against BVDV and BDV were determined by immunoperoxidase staining in pBFM and SK-L cells, respectively. Each equal volume of sample serum and 200 TCID₅₀ of the BVDV Nose, KZ-91NCP, or BDV 87/6 strains was mixed and incubated at 37°C for 1 hr. This mixture and a cell suspension were inoculated into a microtiter plate and incubated for 4 days at 37°C and 5% CO₂. After 4 days of incubation, viral NS3 was detected by immunoperoxidase staining using monoclonal antibody 46/1²²).

Preparation of ELISA antigen: The large-scale cultivation of SK-L cells was carried out in a suspension culture using micro-carriers (JNC Co., Tokyo, Japan). CSFV strain ALD/A76 was inoculated after the generation of confluent SK-L cellular sheets on micro-carriers. After cultivation for 4 days at 37°C, the micro-carriers were collected

on a stainless mesh and then washed with phosphate buffered saline (PBS) three times. The micro-carriers were suspended and allowed to stand for 2 hr in 0.05 M Tris-HCl (pH 7.15) supplemented with 1% polyoxyethylene (9)-*p*-*t*-octylphenyl ether (Tween 20) in order to solubilize the cell membrane and virus. The debris was removed with centrifugation at 10,000 g for 10 min. This lysate was supplemented with 1/10 volume of 0.05 M Tris-HCl (pH 7.15) containing 10% Tween 20 and 1.5 M sodium chloride and then applied on an affinity column immobilized with the monoclonal antibody A301. After washing with the same buffer several times, the A301 affinity column was eluted with the 0.1 M glycine buffer (pH 2.5). The eluted fractions were neutralized with saturated Tris-HCl and purified protein fractions were stocked as the ELISA antigen at -20°C before use. Purified protein was analyzed by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)⁷) under nonreducing and reducing conditions using 2-mercaptoethanol and stained by Silver stain kit Wako (Wako Pure Chemical, Osaka, Japan).

Indirect ELISA: The antigen stock was diluted with 64 mM sodium carbonate buffer (pH 9.6) containing 0.001% Tween 20 and 0.05% sodium azide, and then the diluted antigen solution was added into odd numbered wells of a 96-well immunoplate Maxisorp (Thermo Fisher Scientific, MA, USA). The carbonate buffer without the antigen was added into the even numbered wells of the plate. The plate was incubated for 48 hrs at 4°C for antigen coating, and then washed with purified water three times. Blocking of each well with 0.01 M sodium phosphate buffer (pH 7.2) containing 1% skim milk and 0.05% sodium azide was then carried out at 4°C for 24 hrs. The plates were washed again and stocked in a refrigerator with the PBS containing 0.05% Tween 20 and 0.05% sodium azide. Each 50 µl of the sample serum, inactivated at 56°C for 30 min, was diluted with 450 µl of the serum dilution buffer (5% skim milk, 1% Tween

20, and 0.05% sodium azide in PBS). Each diluted 100 μ l sample, the negative control and the positive control were dispensed into each antigen negative and positive wells. Nothing was added to the blank wells and the plate was incubated at 37°C for 1hr. After the incubation, all liquid was removed and each well was washed 4 times with 300 μ l of wash solution comprising PBS with 0.05% Tween 20. All wells were then filled with 100 μ l of the enzyme conjugate solution (rabbit anti-pig IgG antibodies labeled with horseradish peroxidase) diluted in the PBS containing 1% Tween 20. After the incubation at 37°C for 30 min, all liquid was removed and each well was washed with the wash solution in the same manner as above. All wells were filled with 100 μ l of enzyme substrate solution containing 1.32% 3, 3', 5, 5'-tetramethylbenzidine (TMB, Wako Pure Chemical) and 0.003% hydrogen peroxide in 0.05 M sodium acetate buffer (pH 5.5). The plate was incubated at room temperature for 13 min and 50 μ l of stop solution (2 N sulfuric acid) was dispensed in all wells. The optical absorbance at 450 nm was determined in each well with an adjustment of the optical absorbance. The sample per positive (S/P) value of each well was calculated according to the following formula. $S/P \text{ value} = \{S_{Ag+} - S_{Ag-}\} / \{PC_{Ag+} - PC_{Ag-}\}$, S_{Ag+} : optical absorbance of samples in an odd numbered well coated with the antigens, S_{Ag-} : optical absorbance of sample in an even numbered well not coated with the antigens. PC_{Ag+} : average optical absorbance of positive controls in the wells coated with the antigens, PC_{Ag-} : average optical absorbance of positive controls in the wells coated without the antigens.

Serum samples in the field: In order to examine the sensitivity and specificity of ELISA, 813 serum samples were collected from feeders, parent stocks, or slaughterhouses in Japan. As a field trial, we also examined the following three kinds of farms: 93 samples from finished pigs without CSF vaccination (Farm 1), 56 samples from finished pigs with CSF vaccination (Farm

2), 55 samples from pigs of convalescent stage in CSF endemic area in Rachaburi province, Thailand (Farm 3) were kindly provided by the Livestock Hygiene Service Center of Tochigi and Chiba Prefecture, Japan and National Institute of Animal Health, Bangkok, Thailand, respectively.

Experimental infection of pigs with CSFVs, BVDVs, and BDV: In order to assess the reactivity of the antibodies against CSFV, BVDV, and BDV in this ELISA, 1 ml of cell culture supernatant containing $10^{7.0}$ TCID₅₀ of each virus was inoculated intramuscularly into 2-month-old female SPF miniature pigs (*Sus scrofa domestica*, NIBS line; Nippon Institute for Biological Science, Yamanashi, Japan). At 32 or 33 days post inoculation (dpi), pigs were euthanized by pentobarbital (100 mg/kg, intravenous administration) after collecting blood sample. Virus isolation was carried out from each whole blood sample in SK-L or pBFM cells. Neutralizing antibody titers of each serum sample against CSFV GPE⁻, BVDV Nose, BVDV KZ-91NCP, and BDV 87/6 were titrated as described above. Before VNT, sera were mixed with an equal volume of diethyl ether (Wako Pure Chemical) to inactivate infectious virus and the supernatant was collected for further steps. The institutional animal care and use committee of the Graduate School of Veterinary Medicine, Hokkaido University, authorized this animal experiment and all experiments were performed according to the guidelines of the committee.

Immunization of pigs with commercial vaccines: Commercial vaccines for transmissible gastroenteritis virus (The Chemo-therapeutic Research Institute, Kumamoto, Japan), porcine epidemic diarrhea virus (Nippon Institute for Biological Science, Tokyo, Japan), pseudorabies virus (Kyoritsu Seiyaku co., Tokyo, Japan), swine influenza virus (Kyoto Biken Laboratories, Kyoto, Japan), *Erysipelothrix rhusiopathiae* (Scientific feed Laboratory, Tokyo, Japan), combined vaccines for Japanese encephalomyelitis virus, porcine getah virus, porcine parvo virus (Kyoto Biken Laboratories),

and combined vaccines for *Bordetella bronchiseptica* and *Pasteurella multocida* (Scientific feed Laboratory) were inoculated into two 40-day-old SPF pigs (JA Azumino, Nagano, Japan). No antibodies against these pathogens and CSFV were detected from these pigs before the experiment. Two months after the final vaccination, sera were collected for further experiments. The institutional animal care and use committee of the National Institute of Animal Health authorized this animal experiment and all experiments were performed according to the guidelines of this committee.

Results

Purification of viral glycoproteins

To elucidate the protein component in the eluate after purification using immunoaffinity column immobilized with monoclonal antibody A301, 5 μ l of elution fractions were analyzed by 10% SDS-PAGE under nonreducing and reducing conditions and stained by silver staining kit (Fig. 1). Two bands were detected at 65 kDa and 90 kDa under the nonreducing condition (Fig. 1, lane 1). These proteins were separated into 4 bands at 33 kDa, 50 kDa, 80 kDa, and 95 kDa under the reducing condition (Fig. 1, lane 2). The results of immunoblotting with specific antiserum against ALD/A76 suggested that all bands were derived from viral proteins (data not shown). Based on the molecular weights, the 33 kDa, 50 kDa, 80 kDa and 95 kDa bands were identified as E1 monomer, E2 monomer, E1-E2 heterodimer, and E2 homodimer, respectively.

Establishment of indirect ELISA

Purified antigen was coated onto plastic plates and the reaction condition was evaluated according to the standard positive and negative sera. The antibody titer of the serum sample in ELISA was expressed as an S/P value calculated using the optical absorbance of the test sample and that of the positive control. Finally, samples whose S/P values were larger than 0.1 were

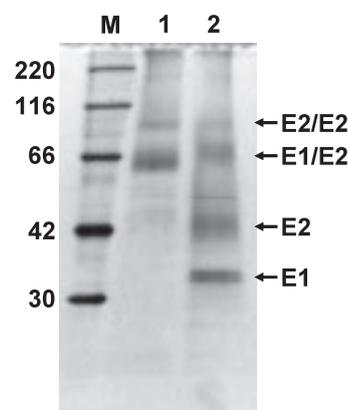


Fig. 1. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified fraction in the immunoaffinity column with A301, monoclonal antibody against classical swine fever virus (CSFV) E2 protein. Purified fraction as antigen of enzyme-linked immunosorbent assay (ELISA) was separated by 10% SDS-PAGE either under nonreducing conditions (Lane 1) or reducing conditions (Lane 2). E1 monomer, E2 monomer, E1-E2 heterodimer, and E2 homodimer were detected as major bands at the estimated molecular weights under reducing conditions (Lane 2). M, molecular marker.

evaluated as positive, those from 0.1 to 0.05 were evaluated as gray zone, and those under 0.05 were evaluated as negative. The reproducibility of antigen preparation and reactivity of ELISA were confirmed in several lots produced independently. The stability of this kit was also confirmed for the ELISA plates stocked in a refrigerator at 4°C for 5, 9, and 13 months (data not shown).

Sensitivity and specificity of indirect ELISA

Eight hundred and thirteen sera, of which neutralization titers were already known, were examined with this ELISA. Table 1 shows a comparison of the results with both methods. All sera samples by VNT except one were negative and one sample was gray zone in ELISA. Most sera containing more than 4 neutralization titers were determined to be positive by ELISA. The relative sensitivity and relative specificity were calculated as 95.5% and 99.8%, if the number in the gray zone was included as positive.

Table 1. Comparison of antibody detection between virus neutralization test and enzyme-linked immunosorbent assay (ELISA)

VNT titer	Total	Sample numbers classified with ELISA		
		Positive ^{a)}	Gray zone ^{b)}	Negative ^{c)}
<1	456	0	1	455
1	16	2	5	9
2	10	2	2	6
4	13	9	4	0
8	13	10	2	1
16	26	26	0	0
32	36	36	0	0
64	82	82	0	0
128	74	74	0	0
256	42	42	0	0
512	24	24	0	0
1024	13	13	0	0
2048	8	8	0	0
Total	813	328	14	471

^{a)}Positive: sample per positive (S/P) value ≥ 0.1 .

^{b)}Gray zone: $0.1 > \text{S/P value} \geq 0.05$.

^{c)}Negative: S/P value < 0.05 .

Abbreviation: VNT, virus neutralization test.

Table 2. Specificity of indirect ELISA for swine sera prepared from pigs experimentally infected with classical swine fever viruses, bovine viral diarrhoea viruses, and border disease virus.

Pig ID	Inoculated virus	Days post inoculation	Virus Recovery (TCID ₅₀ /ml)	Antibody detection				CSFV indirect ELISA (S/P value)
				VNT titer				
				CSFV GPE ⁻	BVDV Nose	BVDV KZ-91NCP	BDV 87/6	
#1	CSFV ALD/A76	33	10 ^{5.6}	2	NT	NT	NT	Positive (0.191)
#2	CSFV GPE ⁻	33	—	128	2	<2	4	Positive (0.711)
#3	CSFV Kanagawa/74	32	—	1024	16	<2	64	Positive (0.899)
#4	BVDV Nose	32	—	2	128	<2	<2	Positive (0.126)
#5	BVDV KZ-91NCP	33	—	<2	32	128	<2	Positive (0.490)
#6	BDV 87/6	32	—	<2	4	<2	32	Positive (0.393)

Abbreviations: CSFV, classical swine fever virus; BVDV, bovine viral diarrhoea virus; BDV, border disease virus; VNT, virus neutralization test; S/P, sample per positive.

Detection of antibodies against CSFV and other related pathogens by indirect ELISA

CSFV and other pestiviruses were inoculated into miniature pigs (#1 ~ 6) and sera at 32-33 days post infection were evaluated for the specificity of ELISA (Table 2). The pig inoculated with CSFV ALD/A76 (#1) showed diarrhoea,

cyanosis, weight loss, severe leukocytopenia, and thrombocytopenia. High virus titers were still recovered in the blood at 33 dpi and the titer of VNT was 2. The pigs inoculated with CSFV GPE⁻, Kanagawa/74, BVDV Nose, KZ-91NCP, and BDV 87/6 (#2-6) showed only transient leukocytopenia and no infectious virus was detected from blood

Table 3. Specificity of CSFV ELISA for swine sera immunized with various commercial vaccines.

Pig ID ^{a)}	Antibody titers											CSFV ELISA (S/P value)
	CSFV ^{b)}	TGEV ^{b)}	PEDV ^{b)}	PrV ^{b)}	JEV ^{c)}	PGV ^{c)}	PPV ^{c)}	SIV ^{c)}		<i>Erysipelothrix</i> <i>rhusiopathiae</i> ^{d)}	<i>Bordetella</i> <i>bronchiseptica</i> ^{d)}	
								H1N1	H3N2			
#7	<2	2048	64	40	10	80	320	128	32	16	20	Negative (0.003)
#8	<2	2048	256	40	10	40	320	64	32	32	40	Negative (-0.012)

^{a)}Each pig was inoculated with various commercial vaccines for TGEV, PEDV, PrV, JEV, PGV, PPV, SIV, *E. rhusiopathiae* and *B. bronchiseptica*.

^{b)}Antibody was titrated in serum neutralization test.

^{c)}Antibody was titrated in haemagglutinin inhibition test.

^{d)}Antibody was titrated in agglutination test.

Abbreviations: CSFV, classical swine fever virus; TGEV, transmissible gastroenteritis virus; PEDV, porcine epidemic diarrhea; PrV, Pseudorabies virus; JEV, Japanese encephalitis virus; PGV, porcine getah virus; PPV, porcine parvovirus; SIV, swine influenza virus; S/P, sample per positive.

A			B			C					
		VNT						VNT			
		+	-			+	-				
ELISA	+	0	0	ELISA	+	51	0	ELISA	+	54	0
	+/-	0	0		+/-	0	0		+/-	1	0
	-	0	93		-	0	5		-	0	0

Fig. 2. Correlation of antibody detections of field samples with indirect ELISA and virus neutralization test (VNT). Anti-CSFV antibodies were detected with indirect ELISA and VNT from field sera from a non-vaccinated farm in Japan (A), vaccinated farm in Japan (B), and a farm with an outbreak of classical swine fever in Thailand (C). Numbers in each column are the sample number evaluated with each method. + : Positive [sample per positive (S/P) value ≥ 0.1 for ELISA, ≥ 2 for VNT], +/- : Gray zone ($0.1 > \text{S/P value} \geq 0.05$ for ELISA), - : Negative (S/P value < 0.05 for ELISA, < 2 for VNT).

samples at 32-33 dpi. Antibody titers of each serum against homologous viruses in VNT were 128, 1024, 128, 128, and 32, respectively. The VNT titers against homologous viruses were always higher than those against heterologous viruses. In indirect ELISA, all serum samples against CSFVs, BVDVs, and BDV were positive, indicating that antibodies against CSFV, BVDV, and BDV were bound to the purified CSFV glycoproteins.

In contrast, serum samples of 2 pigs (#7 and 8), which were immunized with various commercial vaccines, were negative on ELISA, indicating that antibodies against other swine viral and bacterial pathogens had no impact on the binding to the CSFV glycoproteins (Table 3).

Field trials of ELISA

As field trials of ELISA, we selected 3 farms: a non-vaccinated farm in Japan (Fig. 2A), a farm

vaccinated with CSFV GPE⁻ strain in Japan (Fig. 2B), and a farm with a CSF outbreak in Thailand (Fig. 2C) to evaluate the usefulness of ELISA for antibody monitoring of CSF. All sera from the non-vaccinated farm were negative on VNT and ELISA (Fig. 2A). In the vaccinated farm, both methods showed high correlation for positive and negative samples. Five samples that were negative samples with both methods may have had no immunoresponse due to an inadequate vaccination procedure (Fig. 2B). CSF was epidemic in Thailand; and a CSF outbreak occurred 2 month ago in Rachabri province and serum samples of pigs in the convalescent stage were collected for the evaluation of ELISA. The pathogenicity of isolated CSFV was moderate and most fattening pigs except piglets survived the infection (personal communication). All these sera from the convalescent stage were also positive or in the gray zone on ELISA (Fig. 2C).

All sera were also positive in VNT and antibody titers of each serum were higher than those from the vaccinated healthy farm (data not shown). The results of the field trials indicated that this ELISA is applicable for detecting antibody against CSFV for samples in various situations.

Discussion

The golden standard of diagnosis for CSF is antigen detection from pigs showing clinical signs in typical acute and sub-acute courses. However, it is also recognized that moderate or low pathogenic CSFV does not show typical clinical signs of CSF and only death of piglets, stillbirth, and persistent infection are observed in pigs infected with these strains⁸. To detect the pigs infected with moderate or low pathogenic CSFV in non-vaccinated situations, serological surveillance is recognized as powerful monitoring tool¹⁴. Several ELISA kits to detect antibodies against CSFV are commercially available outside of Japan, especially in Europe, since CSF outbreaks still occur in Europe and vaccination is prohibited for domestic pigs^{3,4,16}. In Japan, vaccination of CSF was stopped in 2000 and CSF-free status was granted in 2007, and development of a sensitive and timesaving ELISA is an urgent issue for the monitoring of CSFV antibody. This report describes the development and evaluation of an indirect ELISA as a screening test to detect antibodies against CSFV, CSFV Ab ELISA Kit (JNC Corporation, Tokyo, Japan), which has been routinely used in veterinary service in each prefecture in Japan.

The advantage of this indirect ELISA is higher sensitivity. In general, VNT is the most reliable and sensitive method for the detection of antibody against CSFV¹⁴. However, VNT requires a facility to handle infectious viruses according to the biosafety regulations, and so the laboratories suitable for VNT are limited. In this study, glycoproteins of CSFV ALD/A76 strain were purified from swine kidney cells by an affinity

column immobilized with the monoclonal antibody against E2 protein. In addition, it was confirmed that the infectious virus was inactivated and removed completely from the final purification product (data not shown). The E2 is immunodominant protein and the major target of virus-neutralizing antibodies compared with other immunogenic proteins (i.e., E^{ms} and NS3)⁹, suggesting that the purified glycoproteins containing E2 protein are thought to contribute to the higher sensitivity of this ELISA. In fact, this indirect ELISA detected antibodies containing 1, 2, 4, 8, and 16 VNT titers, although the detection limit of Ceditest CSFV E2 ELISA (Cedi-Diagnostics, Lelystad, The Netherlands), one of the common ELISA kits for CSF, is 25 VNT titer³.

One of the greatest difficulties in the diagnosis of CSF is infection of BVDV and BDV in pigs⁸. The original host of BVDV is ruminants, mainly cattle. However, interspecies transmission of BVDV to pigs may occur when pigs and cattle are kept in the same barn. During the outbreak of CSF in the Netherlands in 2007, discrimination of BVDV infection in pigs was important issue⁴. Interspecies transmissions of BVDV and BDV to pigs were reported mainly in Europe, and no isolation of BVDV and BDV from pigs has been reported in Japan. Since sequence homology between CSFV and other ruminant pestiviruses is 60–70% on amino acids of E2 protein and the antigenicity of glycoproteins of CSFV is related to those of BVDV and BDV, antibodies against BVDV and BVD could react to the glycoproteins of CSFV. To solve this cross reactivity, Ceditest CSFV E2 ELISA was modified as a competitive ELISA using monoclonal antibodies to the E2 glycoprotein³. It is, however, risky to use a monoclonal antibody as a competitor because novel genotypes of CSFV, such as CSFV genotype-3 have antigenic differences and some common epitopes of CSFV genotype-1 and genotype-2 are not conserved in CSFV-3¹⁵. In addition, a competitive ELISA does not have higher sensitivity than the indirect ELISA developed in this study,

as mentioned above. Taken together, we conclude that the ELISA developed in this study is useful screening tool to detect antibodies against CSFV with high sensitivity and a positive sample in this ELISA should be evaluated by cross VNT using CSFV, BVDV, and BDV at a reference diagnosis center. Further study and development of ELISA with high sensitivity and specificity will be necessary to improve the diagnosis of CSF.

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References

- 1) Aoki, H., Sakoda, Y., Nakamura, S., Suzuki, S. and Fukusho, A. 2004. Cytopathogenicity of classical swine fever viruses that do not show the exaltation of Newcastle disease virus is associated with accumulation of NS3 in serum-free cultured cell lines. *J. Vet. Med. Sci.* **66**: 161-167.
- 2) Behrens, S. E., Grassmann, C. W., Thiel, H. J., Meyers, G. and Tautz, N. 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. *J. Virol.* **72**: 2364-2372.
- 3) Colijn, E. O., Bloemraad, M. and Wensvoort, G. 1997. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. *Vet. Microbiol.* **59**: 15-25.
- 4) de Smit, A. J., Eble, P. L., de Kluijver, E. P., Bloemraad, M. and Bouma, A. 2000. Laboratory experience during the classical swine fever virus epizootic in the Netherlands in 1997-1998. *Vet. Microbiol.* **73**: 197-208.
- 5) Komaniwa, H., Fukusho, A. and Shimizu, Y. 1981. Micro method for performing titration and neutralization test of hog-cholera virus using established porcine kidney-cell strain. *Natl J Anim Health Q* **21**: 153-158.
- 6) Kumagai, T., Shimizu, T., Ikeda, S. and Matumoto, M. 1961. A new in vitro method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle disease virus in swine tissue culture. I. Establishment of standard procedure. *J. Immunol.* **87**: 245-256.
- 7) Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
- 8) Le Potier, M.-F., A. Mesplede, and P. Vannier. 2006. Classical swine fever and other pestiviruses. in: Diseases of Swine, 9th ed., pp. 309-322, Straw, B. E., Zimmerman, J. J., D'Allaire, S., and Taylor D. J. eds., Blackwell Pub., Ames, Iowa.
- 9) Lindenbach, B. D., Thiel, H. J. and Rice, C. M. 2007. *Flaviviridae*: the viruses and their replication. In: Fields Virology, 5th ed., pp. 1101-1152, Knipe, D. M. and Howley, P. M. eds., vol. 1. Lippincott-Raven Publishers, Philadelphia, Philadelphia
- 10) Matumoto, M., Kumagai, T., Shimizu, T. and Ikeda, S. 1961. A new in vitro method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle disease virus in swine tissue culture. II. Some characteristics of END method. *J. Immunol.* **87**: 257-268.
- 11) Nagai, M., Sakoda, Y., Mori, M., Hayashi, M., Kida, H. and Akashi, H. 2003. Insertion of cellular sequence and RNA recombination in the structural protein coding region of cytopathogenic bovine viral diarrhoea virus. *J. Gen. Virol.* **84**: 447-452.
- 12) Nagai, M., Sato, M., Nagano, H., Pang, H., Kong, X., Murakami, T., Ozawa, T. and Akashi, H. 1998. Nucleotide sequence homology to bovine viral diarrhoea virus 2 (BVDV 2) in the 5' untranslated region of BVDVs from cattle with mucosal disease or persistent infection in Japan. *Vet. Microbiol.* **60**: 271-276.
- 13) Nishimori, T., Yamada, S. and Shimizu, M. 1996. Production of monoclonal antibodies against classical swine fever virus and their use for antigenic characterization of Japanese isolates. *J. Vet. Med. Sci.* **58**: 707-710.
- 14) OIE 2011, Classical swine fever in: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. <http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/>.
- 15) Parchariyanon, S., Inui, K., Damrongwatanapokin,

- S., Pinyochon, W., Lowings, P. and Paton, D. 2000. Sequence analysis of E2 glycoprotein genes of classical swine fever viruses: identification of a novel genogroup in Thailand. *Dtsch. Tierarztl. Wochenschr.* **107**: 236–238.
- 16) Paton, D. J. and Greiser-Wilke, I. 2003. Classical swine fever--an update. *Res. Vet. Sci.* **75**: 169–178.
- 17) Roehe, P. M., Woodward, M. J. and Edwards, S. 1992. Characterisation of p20 gene sequences from a border disease-like pestivirus isolated from pigs. *Vet. Microbiol.* **33**: 231–238.
- 18) Ruggli, N., Summerfield, A., Fiebach, A. R., Guzylack-Piriou, L., Bauhofer, O., Lamm, C. G., Waltersperger, S., Matsuno, K., Liu, L., Gerber, M., Choi, K. H., Hofmann, M. A., Sakoda, Y. and Tratschin, J. D. 2009. Classical swine fever virus can remain virulent after specific elimination of the interferon regulatory factor 3-degrading function of Npro. *J. Virol.* **83**: 817–829.
- 19) Sakoda, Y., Hikawa, M., Tamura, T. and Fukusho, A. 1998. Establishment of a serum-free culture cell line, CPK-NS, which is useful for assays of classical swine fever virus. *J. Virol. Methods* **75**: 59–68.
- 20) Sakoda, Y., Yamaguchi, O. and Fukusho, A. 1998. A new assay for classical swine fever virus based on cytopathogenicity in porcine kidney cell line FS-L3. *J. Virol. Methods* **70**: 93–101.
- 21) Shimizu, Y., Furuuchi, S., Kumagai, T. and Sasahara, J. 1970. A mutant of hog cholera virus inducing interference in swine testicle cell cultures. *Am. J. Vet. Res.* **31**: 1787–1794.
- 22) Tamura, T., Sakoda, Y., Yoshino, F., Nomura, T., Yamamoto, N., Sato, Y., Okamatsu, M., Ruggli, N. and Kida, H. 2012. Selection of classical swine fever virus with enhanced pathogenicity reveals synergistic virulence determinants in E2 and NS4B. *J. Virol.* In press.