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METHODS AND PROTOCOLS

Characterization of the monoclonal antibody against classical swine fever virus glycoprotein E^{rns} and its application to an indirect sandwich ELISA

Ching-Wei Wu · Maw-Sheng Chien · Ting-Yu Liu · Guang-Jan Lin · Wei-Cheng Lee · Chienjin Huang

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Abstract Classical swine fever virus (CSFV) E^{rns} is an envelope glycoprotein possessing RNase activity. The E^{rns}-based enzyme-linked immunosorbent assay (ELISA) has been considered a discriminating diagnostic test for differentiating infected from vaccinated animals. The purpose of this study was to produce a specific monoclonal antibody (MAb) to E^{rns} for further developing an indirect sandwich ELISA. The MAb CW813 was shown to specifically recognize both the monomer and dimer forms of *Pichia pastoris* yeast-expressed E^{rns} (yE^{rns}). The antigenic site recognized by MAb CW813 was mapped to the region of amino acid residues 101–160 of E^{rns} where it was neither a neutralizing epitope nor essential to RNase activity. Furthermore, MAb CW813 was utilized as a capture antibody to develop a yE^{rns}-based indirect sandwich ELISA for detecting swine antibody to E^{rns}. The assay demonstrated a high sensitivity and specificity that may provide an alternative method for developing a diagnostic kit with easy manipulation and low cost.

Keywords Classical swine fever virus · RNase activity · Monoclonal antibody · Sandwich ELISA

C.-W. Wu · T.-Y. Liu · G.-J. Lin · C. Huang (✉)
Graduate Institute of Microbiology and Public Health College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40227, Taiwan, Republic of China
e-mail: cjhuang@dragon.nchu.edu.tw

M.-S. Chien · W.-C. Lee
Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40227, Taiwan, Republic of China

Introduction

Classical swine fever virus (CSFV) belongs to the genus *Pestivirus* of the Flaviviridae family (van Rijn et al. 1996). CSFV infection in swine results in a highly contagious and severe disease characterized by fever and hemorrhages with an acute or chronic course (Lindenbach and Rice 2001). Outbreaks of classical swine fever (CSF) often cause large economic losses in the pig industry worldwide. The positive-stranded RNA genome comprises a single, long open reading frame coding for a polyprotein encompassing all viral proteins. Structural proteins include a nucleocapsid protein C and three envelope glycoproteins, E^{rns}, E1, and E2. Both E^{rns} and E2 are known to induce virus-neutralizing antibodies and mount protective immunity in the natural host (Bouma et al. 2000; Lin et al. 2009; König et al. 1995; van Rijn et al. 1996; Weiland et al. 1992). Vaccination with the live-attenuated CSFV vaccine protects pigs from CSF (Terpstra and Wensvoort 1988). However, these vaccinated animals cannot be serologically distinguished from animals infected with field strains of CSFV, hampering controlled eradication of CSFV (van Rijn et al. 1996). The E2 subunit vaccine recently developed against CSFV has been considered a potential marker vaccine for serological discrimination between vaccinated and infected pigs that can be performed by detecting of antibodies against E^{rns} (Bouma et al. 2000; de Smit 2000; Moormann et al. 2000).

The E^{rns} glycoprotein forms as a disulfide-bonded homodimer with an apparent size of 97 kDa, and about half the molecular mass of the mature E^{rns} glycoprotein is made up of carbohydrates (Meyers and Thiel 1995; Rumenapf et al. 1993). E^{rns} has been identified as an RNase, and two sequence stretches of E^{rns} show sequence homology to members of the $Rh/T_2/S$ RNase superfamily (Schneider et al. 1993; Hulst et al. 1994; Langedijk et al.

2002). E^{rns} lacks a typical transmembrane anchor and is secreted from infected cells. The biological function of E^{rns} RNase activity is not fully understood; however, destruction of the RNase activity by mutations gives rise to viruses that are more cytopathic in culture and attenuated in vivo (Hulst et al. 1998; Meyers et al. 1999).

Expression of E^{rns} in eukaryotic cells infected by recombinant baculovirus (Hulst et al. 1994) or vaccinia virus (König et al. 1995) has been reported. Recently, an active E^{rns} has been produced using the yeast *Pichia pastoris* secreted expression system, and this yeast-expressed E^{rns} (yE^{rns}) demonstrated intrinsic ribonuclease activity with a clear preference for the uridine-rich sequence (Huang et al. 2006). The yE^{rns} also showed correct conformation that could be applied to substitute the E^{rns} antigen in a commercial sandwich blocking enzyme-linked immunosorbent assay (ELISA) kit for detecting specific antibodies against E^{rns}. In the present study, a monoclonal antibody (MAb) which specifically recognized yE^{rns} was established and characterized. This MAb was further utilized as a capture antibody to develop an indirect sandwich ELISA of which the diagnostic specificity and sensitivity for detecting antibody to E^{rns} were evaluated.

Materials and methods

Construction of recombinant plasmid

Plasmid pE23 ml containing the CSFV glycoprotein gene fragment (E^{rns}-E1-E2) of Taiwan isolate p97 was constructed previously (Shiu et al. 1996). The full length of the E^{rns} coding region was amplified by polymerase chain reaction (PCR) using pE23 ml as a template with a specific primer pair corresponding to the E^{rns} sequence (the forward primer F1: 5′-GTGGATCCGAAAATATAACTC-3′; the reverse primer R227: 5′-GTACTCGAGGGCATAGGCACCGA-3′) according to the GenBank accession nos. U43924 and U03290. The primer sequences recognized by the restriction enzyme *Bam*HI or *Xho*I are underlined, and the PCR reaction was carried out as described previously (Huang et al. 2006). The fragment of the E^{rns} gene was gel-purified and digested with restriction enzymes *Bam*HI/*Xho*I followed by cloning into the expression vector pET32a (Novagen) to generate the recombinant plasmid pET32/E0N227. Various DNA fragments encoding a series of C-terminal deletions were prepared by PCR using a primer pair of F1 and appropriate reverse primer (R190: 5′-GTACTCGAG TATGGTATTAGTCATT-3′, R160: 5′-GTACTCGAGG GAAACGTTAAAATTA-3′, R100: 5′-GTACTC GAGGTTCGCTTGGGTTCT-3′, and R50: 5′-GTACTC GAGTTCCGTGTCTGTAGCC-3′) followed by cloning into pET32a to generate pET32/E0N190, pET32/E0N160, pET32/E0N100, and pET32/E0N50, respectively. All the recombinant plasmids were sequenced to confirm the accuracy of the open reading frame of E^{rns} coding sequences.

Expression of E^{rns} in *Escherichia coli* and yeast

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells according to the manufacturer's manual. A single colony of transformants was grown in Luria-Bertani medium containing 50 μg/ml ampicillin at 37 °C until the OD_{600} reached 0.6. Then, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was incubated for an additional 3 h at 37 °C. The cells were harvested by centrifugation and resuspended in 100 mM Tris–HCl (pH 8.0) containing 1 mM EDTA. Cells were broken by sonication; insoluble material was collected by centrifugation at 15,000×*g* for 10 min at 4 °C, and solubilized proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The expressed protein was further identified by recognition of anti-His antibody (Amersham) in Western blotting assay. The expressed protein was further purified by metal affinity chromatography using nickel–nitrilotriacetic acid (Ni–NTA) resin (QIAGEN) according to the manufacturer's manual.

A single colony of recombinant E^{rns}-expressing *P. pastoris* (Huang et al. 2006) was inoculated in 5 ml yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose) medium and incubated at 30 °C in a shaking incubator (250 rpm) overnight. Then, 0.1 ml of the overnight culture was transferred to 50 ml fresh YPD medium in a 250-ml baffled flask and was continuously incubated for 4 days. The supernatants were clarified by centrifugation (20 min, 12,000×*g*, 4 °C), and secreted protein was concentrated by Vivaspin 20 concentration column (Sartorius), followed by dialysis against phosphate-buffered saline (PBS).

Western blotting analysis

Expressed proteins were resuspended in equal volumes of 2× SDS-PAGE sample buffer (125 mM Tris–Cl [pH 6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.25% bromophenol blue). Proteins were separated by 12% SDS-PAGE and transferred by electroblotting onto PolyScreen PVDF transfer membrane (NEN) using semi-dry transfer cell (Bio-Rad) according to the manufacturer's manual. The membrane was then treated sequentially with blocking solution (PBS containing 5% non-fat skim milk), with appropriate dilution of anti-His antibody (Serotec) and with anti-mouse IgG goat antibody conjugated to peroxidase (Zymed). Finally, the membrane was soaked in a chromogen/substrate solution (TMB single solution; Zymed) for color development, or the signal was detected by chemilu-

minescence substrate (ECL reagent, Amersham Pharmacia Biotech) and visualized by autoradiography.

Preparation of the monoclonal antibody against E^{rns}

Five 6-week-old female BALB/c mice were immunized subcutaneously with 0.5 ml of a mixture of 40 μg purified *E. coli*-expressed E^{rns} (N227) and an equal volume of Freund's complete adjuvant (Sigma). A similar immunization was given 2 weeks later using Freund's incomplete adjuvant. After an additional 3 weeks, the immunized mice received intraperitoneally a final booster with 0.5 ml of 40 μg purified E^{rns} without adjuvant. The fusion was carried out 5 days later according to the methods described previously (Huang et al. 1994). The hybridoma secreting specific antibody to E^{rns} was selected by ELISA and further characterized by Western blotting analysis. The subclass of the MAb was determined by Mouse MonoAb-ID kit (Zymed).

Neutralization assay

The neutralizing antibody activity to CSFV was determined as described previously (Terpstra et al. 1984). Replicates of twofold dilutions of MAb (50 μl) were mixed with 200 $TCID_{50}$ CSFV p97 strain (50 μl) in microtitration plate and incubated at 37 °C for 1 h. After adding 1×10^4 PK-15 cells (100 μl) and incubation at 37 °C for 72 h, the cells were subjected to immunofluorescence staining with the MAb WH303 specific to CSFV E2 (Veterinary Laboratory Agency). Neutralizing titers were expressed as the reciprocal of the highest dilution that caused complete neutralization.

RNase activity assay

The RNase activity of expressed E^{rns} degrade polymeric substrate specificity was measured by perchloric acid precipitation as described previously (Windisch et al. 1996) with some modifications. The recombinant E^{rns} protein was incubated with 80 μg of polyU RNA homopolymer in 100 μl renaturing buffer at 37 °C for 60 min. The reaction was stopped by the adding 100 μl of 1.2 M perchloric acid–25 mM lanthanum sulfate. After 1 h incubation on ice and 15 min of centrifugation (15,000×*g*, 4 °C), the A260 of the supernatant was measured by spectrophotometer (Hitachi U-2800A). Each reaction was tested in triplicate, and the optical density (OD) of triplicates was averaged.

Serum samples

Fifty-four swine serum samples were collected from the Animal Disease Diagnostic Center, College of Veterinary Medicine, National Chung Hsing University, including eight SPF, nine baculovirus-expressed E2 vaccine-immunized (BacE2), 19 LPC attenuated vaccine-immunized (LPC), and eight negative (E^{rns}–), as well as ten positive (E^{rns}+) field sera were determined by the commercial E^{rns} blocking ELISA kit (IDEXX). All the SPF and BacE2 sera were classified as negative and those of LPC as positive by the IDEXX ELISA kit.

Indirect sandwich ELISA

ELISA plates (Corning) were coated at 4 °C overnight with 50 μl of 1:2 diluted culture supernatant of MAb against E^{rns} in coating buffer (carbonate buffer, pH 9.6). The capture plate was then thoroughly washed with PBS containing 0.05% Tween-20 (PBST) and blocked with blocking buffer (PBS containing 1% bovine serum albumin) at 37 °C for 1 h. After washing, each well received 50 μl of appropriate dilution of yE^{rns} antigen in blocking buffer and was incubated at 37 °C for 1 h. After washing, each well received 50 μl of appropriately diluted

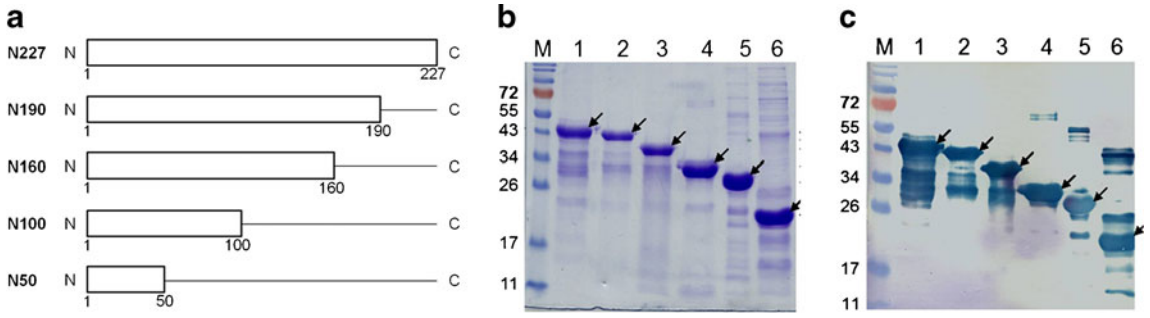

Fig. 1 Expression of E^{rns} and deletion mutants in *E. coli*. **a** Schematic diagrams of CSFV E^{rns} deletion mutants. *Bars* represent expressed E^{rns} sequences and the amino acid residue *numbers* at both termini are indicated. **b**–**c** Characterization of expressed E^{rns} and deletion mutants. Plasmid pET32a (*lane 6*) or recombinant plasmids including pET32/E0N227- (*lane 1*), pET32/E0N190- (*lane 2*), pET32/E0N160- (*lane 3*), pET32/E0N100- (*lane 4*), and pET32/E0N50- (*lane 5*) transformed *E. coli* cells were subjected to induction expression with 1 mM IPTG for 3 h. The cellular lysates were harvested and then purified with Ni–NTA affinity chromatography for SDS-PAGE (14%) analysis (**b**) and Western blotting analysis using anti-His antibody (**c**). The location of each expressed protein is indicated by an *arrow*

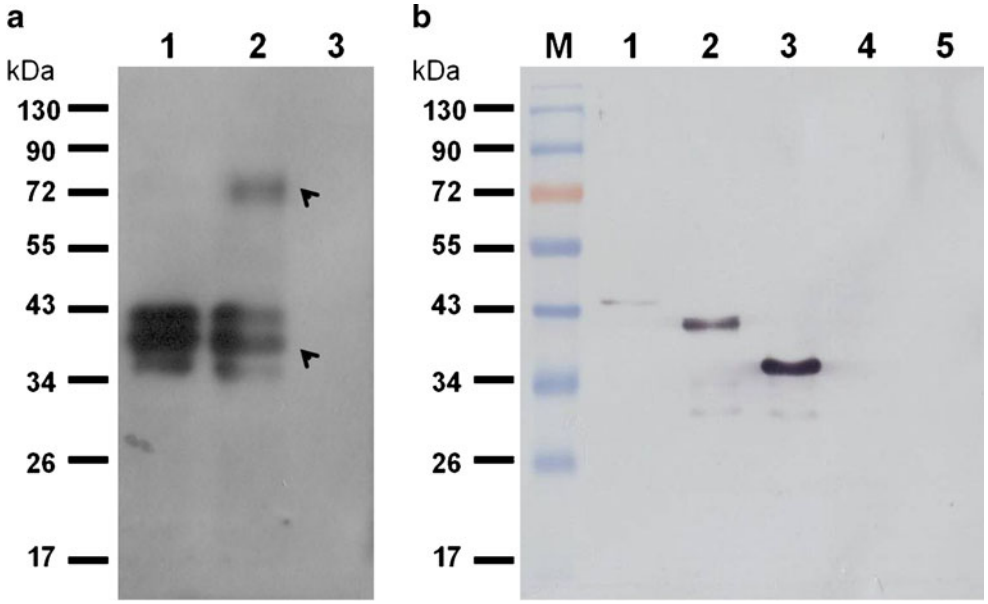

Fig. 2 Characterization of the specificity and epitope mapping of MAb CW813 by Western blotting analysis. **a**Yeast-expressed E^{rns} in the presence of β-mercaptoethanol (*lane 1*) and absent (*lane 2*) were recognized by the MAb CW813. The monomer or homodimer form of yE^{rns} is indicated by an *arrow*. The wild-type yeast-secreted proteins (*lane 3*) were included as controls. **b** The reactivities of MAb CW813 to the full length of E^{rns} N227 (*lane 1*) and deletion mutants N190, N160, N100, and N50 (*lanes 2–5*, respectively) were analyzed for epitope mapping

tested swine serum in blocking buffer and was incubated at 37 °C for 1 h. Subsequently, the plate was washed with PBST thoroughly, and each well received 50 μl of 2,000-fold dilution of goat anti-swine IgG conjugated to peroxidase (Zymed) in blocking buffer at 37 °C for 45 min. Finally, the plate was washed with PBST three times and PBS twice. Then, 50 μl of freshly prepared chromogen/substrate solution (ABTS single solution, Zymed) was added into each well, and the plate was incubated at room temperature for 15 min. The optical density of each well was read at 405 nm using a microplate reader (MRXII, Dynex). Each sample was analyzed in duplicate, and the OD of duplicates was averaged.

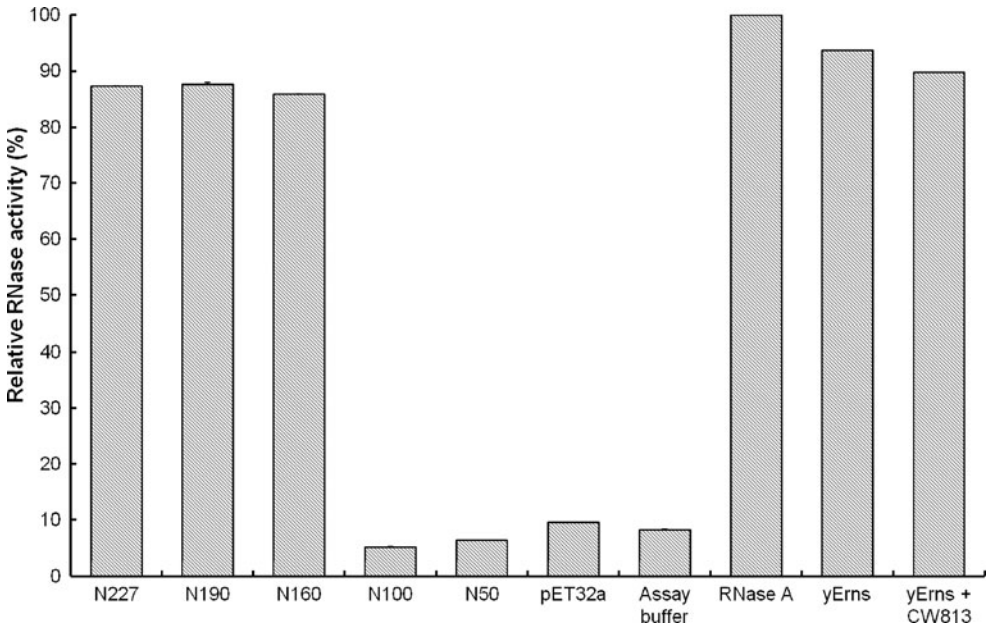

Fig. 3 Analysis of RNase activity of yE^{rns} after reacting with MAb CW813. One microgram of purified yE^{rns} was reacted with MAb CW813 at 37 °C for 1 h and then incubated with 80 μg of poly(U) RNA homopolymer. The RNA-degrading activity was determined by measuring the absorbance at 260 nm due to the release of acid-soluble RNA as described in "Materials and methods". *E. coli*-expressed N227, N190, N160, N100, and N50 were coincidently analyzed to determine their RNase activity. The pET32 tag protein, assay buffer, and RNase A were used as negative and positive controls, respectively. The *bars* show the relative RNase activity as a percentage when using absorbance of RNase A at 100%

Results

Expression of E^{rns} and subunits in *E. Coli*

Recombinant plasmid pET32/E0N227 containing the full length of the coding region of CSFV E^{rns} was constructed to express the E^{rns} protein (N227) for preparation of MAb against E^{rns}. A series of C-terminal deletion mutants encoding the N-terminal 190 (N190), 160 (N160), 100 (N100), and 50 (N50) residues, respectively, were also constructed for further mapping the epitope recognized by the MAb (Fig. 1a). The expressed proteins which fused to a C-terminal His-tag were analyzed by SDS-PAGE (Fig. 1b) and confirmed by Western blotting analysis with anti-His antibody (Fig. 1c).

Characterization of the MAb against the CSFV E^{rns}

A stable hybridoma which secreted antibody reacting specifically with E^{rns} N227 was selected and cloned. The MAb was determined to be the type of IgG1 subclass (data not shown) and denominated CW813. The MAb CW813 could specifically recognize both the monomer and dimer forms of yE^{rns} by the Western blotting analysis (Fig. 2a). In addition, the MAb CW813 could recognize N190 and N160 but not N100 and N50 (Fig. 2b), thus the epitope recognized by the MAb CW813 was mapped to the region of the amino acid residues 101–160. Furthermore, the MAb CW813 neither demonstrated neutralizing antibody activity in the neutralization assay (data not shown) nor blocked the RNase activity of yE^{rns} (Fig. 3).

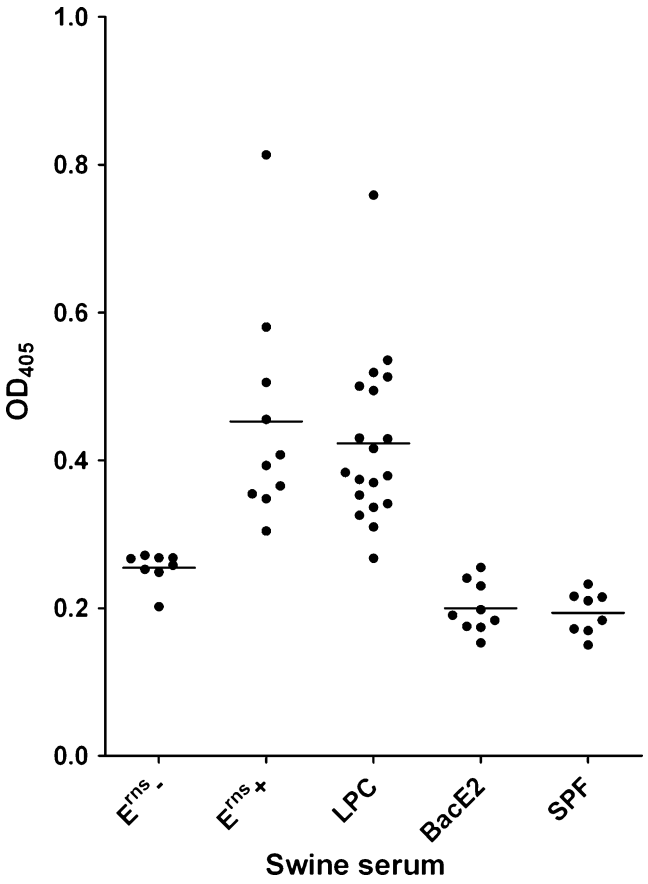

Fig. 4 Reactivities of swine sera from different sources to yE^{rns} in the indirect sandwich ELISA. Twenty-five negative (E^{rns}-, BacE2, and SPF) and 29 positive (E^{rns}+ and LPC) serum samples were tested for reactivity with yE^{rns} as described in "Materials and methods"

Development of the indirect sandwich ELISA

Since E^{rns} is highly glycosylated resulting in inefficient binding to the plate, twofold dilution of MAb CW813 culture supernatant was coated on ELISA plate to capture the antigen yE^{rns}. The optimal concentration of the yE^{rns} was determined by checkerboard titration. The best positive/negative ratio was obtained when the yE^{rns} was diluted at a concentration of 35 μg/ml, and the tested swine serum was diluted at 1:50. Fifty-four swine serum samples were tested by indirect sandwich ELISA, and the results expressed as OD values at 405 nm (OD_{405}) are shown in Fig. 4. Mean OD_{405} of eight SPF sera was 0.1936, and the standard deviation (SD) was 0.0287. When the positive/negative cut-off value was set at mean plus 2 standard deviations (mean+ 2 SD=0.2611), among 25 negative sera including those from SPF, BacE2, and E^{rns}-, 18 were determined as negative and resulted in a specificity of 72% (18/25×100%). However, all the positive sera including those from LPC and E^{rns}+ were positive with the corresponding sensitivities of 100% (29/29×100%). The specificity increased to 100% when the cut-off value was set at mean plus 3 standard deviations (mean+ 3SD=0.2799) with the corresponding sensitivities of 96.6% (28/29×100%). A summary of the cut-off values and sensitivities of the indirect sandwich ELISA to yE^{rns} are shown in Table 1.

Discussion

CSF is an economically important swine disease that has received widespread attention. Detecting antibodies to E^{rns} and E2 in swine serum provides a direct measure of the immune status of animals that had been vaccinated or

Table 1 Summary of cut-off value, specificity, and sensitivity of the indirect sandwich ELISA to yE^{rns}

Cut-off value	OD value	Specificity (%)	Sensitivity (%)
Mean+ 2 SD	0.2611	72 (18/25)	100 (29/29)
Mean+ 3 SD	0.2799	100 (25/25)	96.6 (28/29)

CSFV infected (König et al. 1995; Weiland et al. 1992). Currently, commercially available diagnostic ELISA kits mostly use baculovirus-expressed recombinant E2 or E^{rns} as ELISA antigens (Windisch et al. 1996; Moormann et al. 2000). However, the cost of insect cell culture and time-consumption to purify the expressed product are major concerns. A more economical yE^{rns} recombinant protein has been demonstrated to be a useful antigen in a sandwich blocking ELISA (Huang et al. 2006). In the present study, a yE^{rns}-based indirect sandwich ELISA was established that could offer an inexpensive assay for detecting antibody to E^{rns} with simplicity and easy interpretation. Since yE^{rns} has similar highly glycosylated conformation to native E^{rns}, the binding of yE^{rns} to the ELISA plate was greatly improved by the capture with MAb CW813. Although lectins have been used widely in the capture or detection of glycoproteins (Yue et al. 2009), binding of a specific antigen by the monoclonal antibody is more efficient that nonspecific reactions could be reduced. This MAb was originally screened against *E. coli*-expressed E^{rns} (N227) and further selected for high specificity to yE^{rns} by Western blotting analysis (Fig. 2a). The epitope recognized by CW813 was mapped to the middle region of E^{rns} at amino acid residues 101–160. This was not a neutralizing antigenic site due to the absence of neutralizing activity of CW813.

The CSFV E^{rns} contains RNase activity, which is unique for viral surface proteins (Schneider et al. 1993; Windisch et al. 1996; Langedijk et al. 2002). The role played by E^{rns} in viral pathogenesis is not well characterized. Binding with MAb CW813 did not block the RNase activity of yE^{rns}, suggesting the residues within the epitope recognized by CW813 were not essential to RNase activity. However, *E. coli*-expressed E^{rns} (N227) and two truncation mutants, N190 and N160, also revealed RNase activity (Fig. 3). This result indicates the N-terminal 160 residues of E^{rns} are sufficient for RNase activity, and the region of residues 101–160 besides the unessential epitope of CW813 may contain indispensable sequences contributing to the RNase activity. In addition, glycosylation of E^{rns} is seemingly dispensable for the RNase activity since both *E. coli*-expressed E^{rns} and deglycosylated yE^{rns} (Huang et al. 2006) possess enzymatic activity. The structure and function of E^{rns} and the mechanism of RNase catalysis remain elusive.

Furthermore, the results of yE^{rns}-based indirect sandwich ELISA clearly demonstrate discriminative reactivities between conventional whole live vaccine (LPC) and E2 subunit (BacE2) vaccinated pigs (Fig. 4). Thus, this ELISA may offer a useful diagnostic test to distinguish infected and vaccinated animals. In conclusion, a monoclonal antibody specific to the CSFV E^{rns} was established and applied to develop an indirect sandwich ELISA for detecting the antibody to E^{rns}. This yeast-expressed E^{rns}-based indirect sandwich ELISA demonstrated a high sensitivity and specificity that may provide an alternative method for developing a discriminating diagnostic kit with easy manipulation and low cost.

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