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Enhanced expression of the E^{rns} protein of classical swine fever virus in yeast and its application in an indirect enzyme-linked immunosorbent assay for antibody differentiation of infected from vaccinated animals

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A B S T R A C T

Classical swine fever (CSF), caused by classical swine fever virus (CSFV), is a devastating disease of swine worldwide. Although a mandatory vaccination with the modified live vaccine C-strain has been implemented in China for decades, CSF remains a serious threat to the swine industry. To facilitate the control and eradication of CSF in China, the E2-based marker vaccine rAdV-SFV-E2, an adenovirus-delivered, alphavirus replicon-vectored vaccine, has been developed. Accordingly, an accompanying discriminatory test that allows differentiating infected from vaccinated animals (DIVA) is required. Here, the enhanced expression of E^{rns} protein of CSFV was achieved in the methyltropic yeast *Pichia pastoris* by codon-optimization of the E^{rns} gene, and an indirect enzyme-linked immunosorbent assay (iELISA) based on the yeast-expressed E^{rns} (yE^{rns}) was developed and evaluated. The optimized iELISA was able to detect CSFV-specific antibodies in the serum samples from the CSFV-infected pigs as early as 6 days post-infection, and discriminate the CSFV-infected pigs from those vaccinated with rAdV-SFV-E2. The iELISA was evaluated using a panel of swine sera, and showed comparable sensitivity (94.6%) and specificity (97.1%), and the consistence rates with the virus neutralization test were 96.8% for CSFV-infected swine sera, 83.3% for C-strain-vaccinated swine sera, and 95.0% for field swine sera. In addition, the iELISA showed higher sensitivity (90.4%) compared with PrioCHECK CSFV E^{rns} (59.6%). Taken together, the yE^{rns}-based iELISA is specific and sensitive, representing a promising DIVA test for E2-based marker vaccines against CSF.

1. Introduction

Classical swine fever (CSF), caused by classical swine fever virus (CSFV), is an economically important, highly contagious and often fatal disease of pigs worldwide (Edwards et al., 2000). CSFV, together with bovine viral diarrhea virus 1 (BVDV-1), BVDV-2 and border disease virus (BDV), belongs to the genus *Pestivirus* within the family *Flaviviridae* (Meyers and Thiel, 1996). CSFV is a small enveloped virus with a single-stranded, positive-sense RNA genome of approximately 12.3 kb in length. The CSFV genome contains a single open reading frame encoding a polyprotein of 3898 amino acids that undergoes co- and post-translational processing by cellular and viral proteases, giving rise to four structural proteins C, E^{rns}, E1 and E2, and seven non-structural proteins N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A and NS5B (Rümenapf et al., 1993; Meyers and Thiel, 1996; Tautz et al., 1997). Upon CSFV infection, antibodies are developed against the structural proteins E2, E^{rns}

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and the non-structural protein NS3. Neutralizing antibodies that confer protective immunity are induced by E2 and E^{rns} (König et al., 1995). Therefore, the serological diagnosis of CSF is mainly based on the detection of E2- or E^{rns}-specific antibodies (Moser et al., 1996; Colijn et al., 1997; Moormann et al., 2000; Clavijo et al., 2001).

So far, there are two types of commercially available vaccines for CSF, modified live vaccines (MLV) and E2 subunit vaccines. MLV is mainly based on the Chinese lapinized vaccine strain (C-strain) of CSFV (Qiu et al., 2006; Luo et al., 2014). Although C-strain has been proven to be safe and efficacious, antibodies against MLV do not allow the differentiation of infected from vaccinated animals (DIVA). E2 subunit vaccines (Ahrens et al., 2000; Moormann et al., 2000) comply with the DIVA principle, but show suboptimal efficacy compared with MLV (Dewulf et al., 2001). Currently, CSF remains a serious threat to the swine industry worldwide (www.oie.int). Although a mandatory vaccination with MLV has been implemented in China for decades, CSF has not been completely controlled and occurs occasionally in many regions (Luo et al., 2014).

To facilitate the control and eradication of CSF in epidemic or endemic areas, there is an urgent need for improved DIVA vaccines against CSF. During the past years, several groups have developed different DIVA vaccines, including Flc9 and Flc11 (van Gennip et al., 2000), CP7_E2alf (Reimann et al., 2004) and rAdV-SFV-E2 (Sun et al., 2011, 2013). rAdV-SFV-E2 is an adenovirus-delivered, alphavirus replicon-vectored vaccine expressing the E2 protein of CSFV developed in our group. The vaccine has been shown to be able to induce sterilizing immunity and complete protection comparable with C-strain (Sun et al., 2011, 2013), and is a promising DIVA vaccine with potential to be included in CSF eradication in China.

E^{rns}-based enzyme-linked immunosorbent assays (ELISAs) have been developed for the evaluation of CSF DIVA vaccines (de Smit, 2000; Floegel-Niesmann, 2001). However, the specificity of the assays was too low to reliably discriminate vaccinated from CSFV-infected pigs (Floegel-Niesmann, 2001). Recently, a novel DIVA ELISA accompanying the marker vaccine CP7_E2alf was developed based on the *Escherichia coli*-expressed recombinant E^{rns} proteins from three pestivirus species, CSFV, BVDV and BDV (Aebischer et al., 2013). The ELISA showed high sensitivity and specificity, but the procedure includes two indirect ELISAs (one for screening, the other one for confirmation purpose). Currently, commercially available DIVA-ELISA kits include the Chekit CSF-Marker (IDEXX Laboratories, USA) and PrioCHECK CSFV E^{rns} (Prionics, USA). The former is not available in China; the latter is less sensitive than the E2-ELISAs and not recommended (Schroeder et al., 2012; Aebischer et al., 2013).

Here, to develop an accompanying DIVA ELISA for the marker vaccine rAdV-SFV-E2, an indirect ELISA (iELISA) was established using the methyltropic yeast *Pichia pastoris*-expressed recombinant E^{rns} protein of CSFV and evaluated for its sensitivity, specificity and DIVA potential.

2. Materials and methods

2.1. Expression vector, yeast strain and serum samples

The yeast expression vector pPIC9K and the yeast *P. pastoris* GS115 strain were purchased from Invitrogen (Carlsbad, USA).

The following serum samples were tested in this study: (1) 254 swine serum samples confirmed to be free of CSFV antibodies using the virus neutralization test (VNT) and the HerdChek CSFV Ab ELISA kit (IDEXX, USA) for cutoff value determination; (2) sequential serum samples from 9 pigs upon vaccination with CSFV C-strain or marker vaccine rAdV-SFV-E2, and subsequent challenge with CSFV virulent Shimen strain; (3) a panel of swine sera for comparison of yE^{rns}-iELISA with VNT, including 63 sera from pigs experimentally infected with the CSFV Shimen strain, 18 C-strain-vaccinated swine sera, 34 rAdV-SFV-E2-immunized swine sera and 101 clinical swine serum samples.

Other serum samples included 3 sera from pigs experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV), 3 sera from pigs experimentally infected with pseudorabies virus (PRV), 3 sera from pigs infected with porcine circovirus type 2 (PCV2), 3 sera from pigs experimentally infected with porcine epidemic diarrhea virus (PEDV), 19 sera from pigs experimentally infected with BVDV (4 sera from the EU Reference Laboratory for CSF, Hannover, Germany) and 4 sera from pigs experimentally infected with BDV (from the EU Reference Laboratory for CSF).

2.2. Construction of the recombinant expression plasmid and transformation of yeast

The E^{rns} coding region of the CSFV Shimen strain was optimized based on the synonymous codon bias of *P. pastoris*. Thereafter, the codon-optimized E^{rns} gene with the introduced *Eco*RI and *Not*I sites and the coding sequence for the C-terminal 6×His tag was synthesized and cloned into the pMD18-T vector (TaKaRa, Japan) to generate the recombinant plasmid pMD-E^{rns} (Invitrogen, Shanghai, China). Subsequently, the E^{rns} gene with the coding sequence for the 6×His tag was subcloned into the *Eco*RI cloning site of the yeast expression vector pPIC9K to construct the expression plasmid pPIC9K-E^{rns}. All constructs were verified by sequencing.

The recombinant expression plasmid pPIC9K-E^{rns} was linearized and transformed into *P. pastoris* GS115 strain by electroporation according to the manufacturer's instructions (Invitrogen), with the empty vector as a background control for expression. The transformants with high copies were screened by geneticin and the inserted E^{rns} gene was confirmed by PCR using the primers specific to the E^{rns} gene, forward primer, 5′-AAT GGA ACT TGT CTG ATA ACG G-3′, and reverse primer, 5′-GAT GAT GAT GAT GAT GAG CGT A-3′.

2.3. Expression of the E^{rns} protein in yeast

A single colony of the recombinant *P. pastoris* containing the E^{rns} gene was inoculated into 50 ml of Buffered Glycerol-complex Medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) in a 500-ml baffled flask and incubated at 30 °C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600nm} of 2.0–6.0 (approximately 16–18 h). Meanwhile, GS115 transformed with the empty vector was included as a background control for expression. The cells were harvested by centrifuging at 1500 × *g* for 5 min at room temperature. To induce expression, the supernatant was decanted and the cell pellet was resuspended in Buffered Methanol-complex Medium (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol) using 1/5 of the original culture volume (approximately 10 ml). The cell suspension was transferred into a 100-ml baffled flask and incubated for 5 days, and methanol was added every 12 h during the incubation, to a final concentration of 0.5% to maintain induction. One milliliter of the culture was harvested every day to analyze the expression level of the E^{rns} protein and determine the optimal time post-induction to harvest.

The culture was centrifuged at maximum speed for 2–3 min at room temperature, and the supernatant and cell pellets were stored in separate tubes at −80 °C. The supernatants and cell pellets were analyzed for protein expression by SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) and Western blotting or dot blotting analysis.

2.4. Dot blotting and Western blotting analysis

The culture supernatant and the supernatant of ultrasonicated cell pellet suspended in phosphate buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) were analyzed by dot blotting. The samples to be detected is applied directly on the nitrocellulose membrane, which was subsequently blocked with blocking solution (phosphate-buffered saline (PBS) containing 5% skim milk) for 2 h at room temperature. Thereafter, the membrane was incubated at room temperature for 2 h with anti-His tag monoclonal antibody (MAb) (Invitrogen, USA) with 2000-fold dilution in PBS. After washing 6 times with PBS containing 0.05% (v/v) Tween-20 (PBST), the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, USA) diluted in PBST containing 5% skim milk at room temperature for 1 h. Finally, the membrane was soaked in 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (Pierce, Thermo Scientific, USA) for signal development.

The positive samples screened by dot blotting analysis were separated by 12% SDS-PAGE (Laemmli, 1970) and transferred onto nitrocellulose membrane by using semi-dry transfer cell (Bio-Rad, USA) according to the manufacturer's manual. The membrane was also treated sequentially with blocking solution (PBS containing 5% skim milk), anti-His tag MAb of 2000-fold dilution, and IRDye800CW goat anti-mouse IgG (LICOR odyssey, USA) of 10,000-fold dilution. Finally, the fluorescence signal was detected with an Infrared Fluorescence Imaging System (LI-COR, Odyssey).

2.5. Purification of the E^{rns} protein

The recombinant E^{rns} protein was purified through Ni^{2+} affinity chromatography according to the manufacturer's guidelines (GE Healthcare, USA). The purified proteins were resolved by 12% SDS-PAGE, and the protein concentration was determined using a modified Lowry protein assay kit (Pierce, Thermo Scientific, USA).

2.6. Optimization of the iELISA based on yeast-expressed E^{rns} (yE^{rns})

To develop an efficient iELISA, different assay parameters and the concentrations of component reagents of the iELISA were optimized. The optimal concentrations of the coating antigen (purified yE^{rns}) and dilutions of sera and secondary antibodies (horseradish peroxidase-conjugated goat anti-pig IgG, Sigma, USA) were determined by the checkerboard titration method (Reed and Muench, 1938).

MaxiSorp Nunc-Immuno microplates (Nunc, Denmark) were coated overnight at 4 °C with 100 μl of the purified yE^{rns} (2.5 μg/ml diluted in 0.05 M carbonate–bicarbonate buffer, pH 9.6). After washing twice with PBST, the wells were blocked with 5% skim milk in PBS (pH 7.2) at 37 °C for 1 h. Thereafter, the plates were incubated at 37 °C for 1 h with 100 μl of serum samples diluted at 1:100 in PBST containing 5% skim milk. After washing 6 times with PBST, the plates were incubated with horseradish peroxidase-conjugated goat anti-pig IgG (Sigma, USA) diluted in PBST containing 5% skim milk at 37 °C for 1 h. The bound-antibodies were detected by dispensing a TMB substrate solution (Sigma, USA). The reaction was stopped by the addition of 100 μl of 2 M H_2SO_4 and the optical density (OD) values were measured at 450 nm by a microplate reader (Corona Electric, Japan). Each sample was analyzed in duplicate. The cutoff value was defined as the mean plus 3 standard deviations of the $OD_{450\,nm}$ values of 254 CSFV-negative sera confirmed by VNT and the HerdChek CSFV Ab ELISA kit.

2.7. Evaluation of the iELISA

The specificity of the assay was tested by using 63 sera of pigs experimentally infected with CSFV and 254 sera of non-infected pigs, as confirmed using VNT and the HerdChek CSFV Ab ELISA kit (IDEXX, USA). In addition, the sera of pigs experimentally infected with PRRSV, PRV, BVDV, BDV, PCV2 or PEDV were used to evaluate the cross-reactivity with the assay.

A panel of swine sera was tested in parallel with both yE^{rns}-iELISA and VNT, including 63 sera from pigs experimentally infected with CSFV Shimen strain, 18 C-strain-vaccinated swine sera, 34 rAdV-SFV-E2-immunized swine sera and 101 clinical swine serum samples. VNT was performed as described previously (Li et al., 2009).

2.8. Comparison of yE^{rns}-iELISA with PrioCHECK CSFV E^{rns}

Sixty-six clinical swine serum samples, which have been determined positive or negative by VNT, were tested in parallel with both yE^{rns}-iELISA and PrioCHECK CSFV E^{rns}.

3. Results

3.1. Enhanced expression of CSFV E^{rns} in P. pastoris

To improve the expression level of the protein in yeast, the E^{rns} coding region of CSFV was optimized according to the codon usage bias of *P. pastoris* (Fig. S1). The E^{rns} gene was integrated into the genome of *P. pastoris* GS115 strain by transformation with the plasmid pPIC9K-E^{rns}, as confirmed by PCR targeted to the E^{rns} gene and sequencing (data not shown).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.05.006

Intracellular expression of the yE^{rns} protein was examined by dot blotting (Fig. 1A) and Western blotting analysis (Fig. 1B) using anti-His tag MAb compared with *P. pastoris* GS115 transformed with the empty vector (background control), indicating a band of approximately 37 kDa (Fig. 1B), and almost no target protein was secreted into the culture supernatant (data not shown). The optimal harvest time was determined to be 96 h post-induction, when the expression of the E^{rns} protein reached the peak level (data not shown). The cell lysate containing the E^{rns} protein was further purified with Ni–agarose affinity chromatography (Fig. 1C). According to the output of the purified yE^{rns} protein the expression level was estimated to be 500 mg/L of the culture.

3.2. Optimization of the yE^{rns}-based iELISA

To develop an iELISA, the conditions of the assay were optimized, resulting in the concentration of the coating antigen as 2.5 μg/ml, the dilution of the serum samples as 1:100, and that of the secondary antibody as 1:10,000 (Fig. 2).

3.3. Performance of the yE^{rns}-based iELISA

To validate the potential of the yE^{rns}-based iELISA for detection of specific antibodies against CSFV, the assay was evaluated using 254 CSFV-free swine serum samples and 63 sera of pigs experimentally infected with CSFV. As expected, only CSFV-infected sera showed OD values higher than the cutoff ($OD_{450\,nm} > 0.2$). In addition, no reaction was observed in sera of pigs experimentally infected with PRRSV, PRV, BDV, PCV2, or PEDV; most sera from pigs experimentally infected with BVDV also showed negative reaction, only 1 sample showed weak cross-reaction (with an $OD_{450\,nm}$ value of 0.226, very close to the threshold of

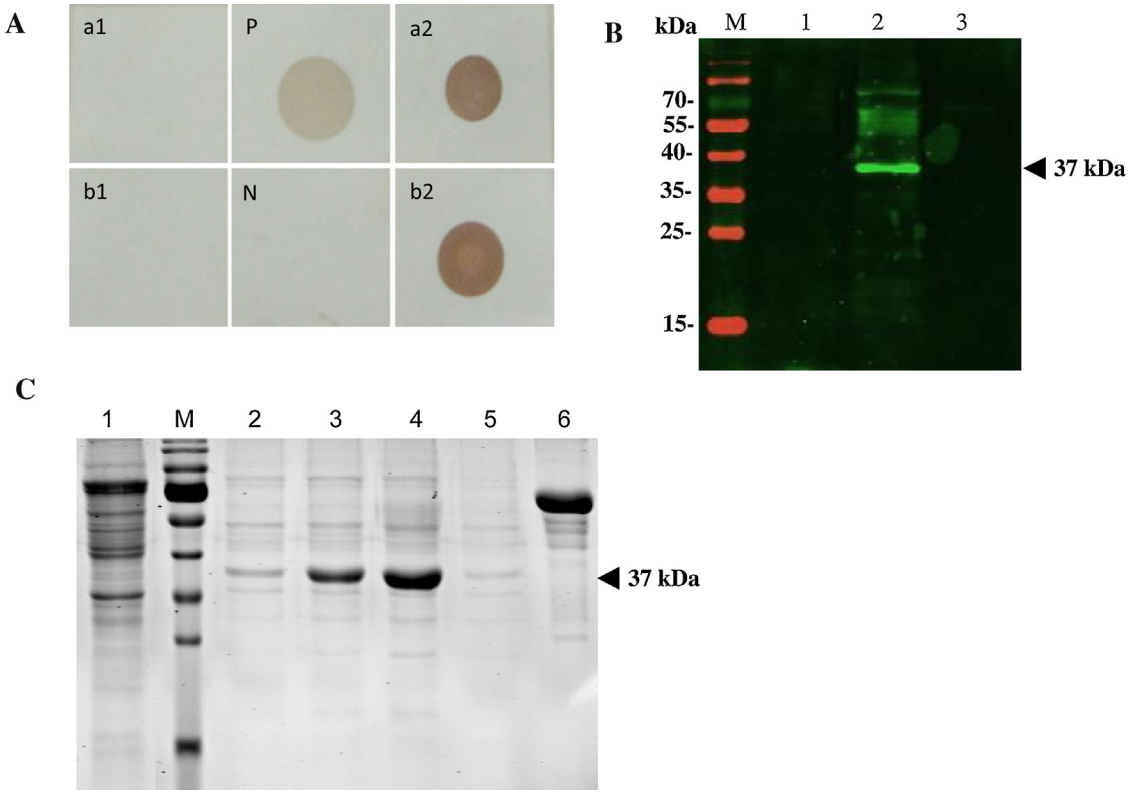

Fig. 1. Characterization of recombinant E^{rns} protein expressed in the yeast *P. pastoris* GS115 strain. (A) Dot blotting analysis of the yeast-expressed E^{rns} protein (yE^{rns}) using anti-His tag monoclonal antibody (MAb). a1 and b1, pre-induction cell lysate of recombinant yeast clones 1 and 2, respectively; a2 and b2, cell lysate of recombinant yeast clones 1 and 2 collected at 72 h post-induction; P, His-tagged E2 protein expressed in yeast; N, cell lysate of *P. pastoris* GS115 transformed with the empty vector. (B) Western blotting analysis of the yE^{rns} protein using anti-His tag MAb. Lane M, protein marker; lane 1, pre-induction cell lysate of recombinant yeast; lane 2, cell lysate of recombinant yeast collected at 72 h post-induction; lane 3, cell lysate of *P. pastoris* GS115 transformed with the empty vector. (C) SDS-PAGE analysis of the purified yE^{rns} protein. Lane M, protein marker; lanes 1–5, 25 mM, 50 mM, 100 mM, 200 mM and 500 mM imidazole eluted protein, respectively. Lane 6, 500 μg/ml BSA standard. The arrow indicates the yE^{rns} protein.

the assay) (Fig. 3). The results indicate that the developed E^{rns}-based iELISA is specific and suitable for sero-surveillance of CSFV infection. The E^{rns}-specific antibodies were detected in the sera from pigs experimentally infected with the CSFV Shimen strain as early as 6 days post-infection (Fig. 4). In addition, the iELISA showed a good reproducibility with the E^{rns} protein of the different batches, with the variable coefficients ranging from 0.78% to 7.18%.

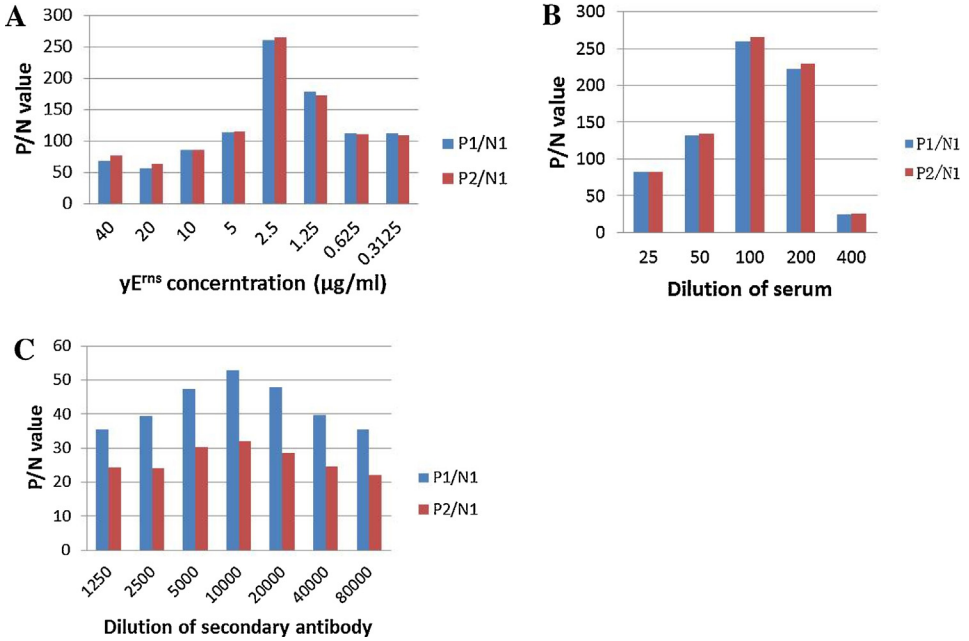

Fig. 2. Optimization of the iELISA based on yeast-expressed E^{rns} (yE^{rns}). (A) Optimization of the coating antigen. (B) Optimization of the serum dilution. (C) Optimization of the dilution of the secondary antibody. P1 and P2, sera from pigs experimentally infected with CSFV; N1, CSFV negative sera.

Table 1
Comparison of the yE^{rns}-based iELISA with the virus neutralization test for the detection of a panel of swine serum samples.

Swine sera	yE^{rns}-iELISA		Virus neutralization test (VNT)		Agreement rates (%)
	+	–	+	–	
CSFV-challenged	52	11	50	13	96.8
C-strain-immunized	8	10	11	7	83.3
Field	46	55	51	50	95.0
rAdV-SFV-E2-immunized	0	34	14	20	Not applicable

Note: +, positive; –, negative; VNT-positive, titer > 5; VNT-negative, titer ≤ 5.

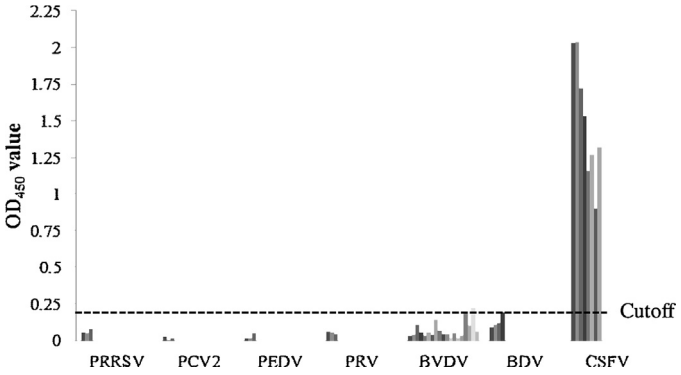

Fig. 3. Specificity of the yE^{rns}-based iELISA. The specificity of the assay was validated using a panel of swine antisera against major swine viruses, including PRRSV (n = 3), PCV2 (n = 3), PEDV (n = 3), PRV (n = 3) and CSFV (n = 8), and closely related BVDV (n = 19) and BDV (n = 4).

To evaluate the DIVA potential of the iELISA, serial serum samples from pigs upon vaccination with CSFV C-strain or marker vaccine rAdV-SFV-E2, and subsequent challenge with CSFV virulent Shimen strain were used to validate the assay. As shown in Fig. 4, the ELISA detected CSFV E^{rns}-specific antibodies in the sera from the pigs infected with wild-type CSFV or vaccinated with C-strain, but not in those from the pigs vaccinated with rAdV-SFV-E2, indicating the DIVA potential of the assay for discriminating CSFV-infected pigs from those vaccinated with marker vaccines.

3.4. Agreement between yE^{rns}-iELISA and VNT

The yE^{rns}-iELISA was compared with VNT for the detection of diverse swine serum samples. The iELISA displayed high sensitivity

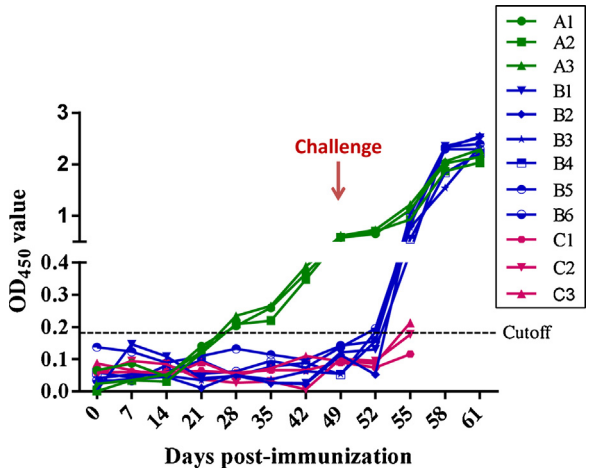

Fig. 4. Time course of the E^{rns} antibody development of pigs upon vaccination with C-strain or the marker vaccine rAdV-SFV-E2 and subsequent challenge with the CSFV virulent Shimen strain. A1–A3, C-strain-vaccinated pigs; B1–B6, rAdV-SFV-E2-vaccinated pigs; C1–C3, mock-immunized pigs.

(94.6%) and specificity (97.1%), and the consistence rates with VNT were 96.8% (61/63) for CSFV-challenged swine sera, 83.3% (15/18) for C-strain-vaccinated swine sera, and 95.0% (96/101) for the field swine sera (Table 1). For the sera from rAdV-SFV-E2-immunized pigs, no agreement between the yE^{rns}-ELISA and VNT was expected, and therefore these rates were not included for calculation of an agreement rate.

3.5. Agreement between yE^{rns}-iELISA and PrioCHECK CSFV E^{rns}

The yE^{rns}-iELISA was compared with the commercial PrioCHECK CSFV E^{rns} for detection of 66 clinical swine serum samples identified by VNT. The yE^{rns}-iELISA detected more positive samples (47/52 = 90.4%) compared with the PrioCHECK CSFV E^{rns} (31/52 = 59.6%) (Table 2).

4. Discussion

CSF remains a serious threat to the pig industry worldwide, including China (Edwards et al., 2000; Luo et al., 2014), and there is a need to develop novel DIVA vaccines and accompanying tests to facilitate the control and ultimate eradication of CSF. A marker vaccine, rAdV-SFV-E2, developed recently by our group, has been shown to be a promising DIVA vaccine with efficacy comparable with C-strain (Sun et al., 2011, 2013). Accordingly, we aimed at developing an accompanying DIVA test based on the detection of the CSFV E^{rns}-specific antibodies that are produced upon infection with wild-type CSFV, but not vaccination with rAdV-SFV-E2.

The methyltropic yeast *P. pastoris* has been widely used for recombinant protein production as a faster, easier and less expensive expression system than other eukaryotic systems (Cereghino and Cregg, 2000). Several factors such as codon usage of the expressed gene, promoter activity, and protein processing and folding may influence the expression levels of heterologous proteins (Outchkourov et al., 2002; Hohenblum et al., 2004). Improved production was obtained by codon optimization for some protein expression in *P. pastoris*, such as CSFV E2 (Cheng et al., 2014) and human glucocerebrosidase (Sinclair and Choy, 2002). Although E^{rns} of CSFV has previously been expressed in *P. pastoris*, the production level was relatively low, that is, 27 mg/L culture (Huang et al., 2006). To improve the yield of yE^{rns}, we optimized the codon usage of the E^{rns} gene according to the *P. pastoris* preferred codons.

Table 2
Comparison of the yE^{rns}-based iELISA and the PrioCHECK E^{rns} ELISA for the detection of swine serum samples identified by virus neutralization test (VNT).

Swine sera	yE^{rns}-iELISA		PrioCHECK E^{rns}	
	+	–	+	–
Negative by VNT	0	14	0	14
Positive by VNT	47	5	31	21
Total	47	19	31	35
Agreement (%)	90.4	100	59.6	100

Note: +, positive; –, negative.

As expected, the E^{rns} protein was successfully expressed in the yeast and the expression level (500 mg/L) was increased remarkably after the codon optimization. The E^{rns} protein was identified by Western blotting using anti-His tag MAb, with a molecular weight of approximately 37 kDa, which was consistent with the previously reported size of yeast-expressed E^{rns} in the presence of a reducing agent (β-mercaptoethanol) (Huang et al., 2006).

Different from the secreted expression of E^{rns} in *P. pastoris* by Huang et al. (2006) using the vector pGAPZαC with the α-factor signal sequence, the protein was expressed intracellularly in Baffled culture flasks, although we also used the expression vector pPIC9K containing the α-factor signal sequence. However, both secreted (1/3) and intracellular expression (2/3) of the protein can be achieved with a fermentor (data not shown). For the purification of proteins, especially the proteins with a low expression level, cells are easier to handle than supernatant when large amount of the purified protein is needed, since an additional procedure including concentration and/or dialysis is required for supernatant (medium).

Next, the iELISA based on the purified yE^{rns} was developed with the optimized conditions and validated with different swine serum samples. The assay was specific as no cross-reactions were observed with other swine viruses, except 1 out of 19 sera from pigs experimentally infected with BVDV, which showed weak cross-reaction with an $OD_{450\,nm}$ value very close to the threshold of the assay. Moreover, the assay was sensitive enough to detect the E^{rns}-specific antibodies during early infection. The iELISA clearly demonstrated the presence of anti-E^{rns} antibodies in swine immunized with conventional live vaccine (C-strain) or CSFV-infected pigs, whereas it is absent in pigs vaccinated with rAdV-SFV-E2, demonstrating the DIVA potential of the assay for discriminating CSFV-infected pigs from those vaccinated with marker vaccines.

Generally, VNT is performed to confirm the results of E2-ELISAs. However, it is illogical to perform VNT after vaccination with DIVA vaccines containing antigenic domains of CSFV E2, because false-positive results caused by cross-reactivity with the DIVA vaccine itself cannot be excluded. Due to the unavailability of the commercial Chekit E^{rns} ELISA kit (IDEXX) in China by far and the low sensitivity of the commercial PrioCHECK CSFV E^{rns} kit (Prionics) (Schroeder et al., 2012), we compared the yE^{rns}-iELISA with VNT for detection of diverse swine serum samples, despite that VNT is unsuitable as a confirmatory test when testing pigs vaccinated with marker vaccines expressing CSFV E2 protein. The iELISA showed high sensitivity and specificity for detecting diverse swine serum samples, except for the marker vaccine rAdV-SFV-E2-immunized swine sera (all negative in iELISA, some positive in VNT), emphasizing the infeasibility of VNT for detection of anti-E^{rns} antibodies in the pigs vaccinated with the E2-based marker vaccine.

The yE^{rns}-iELISA showed higher sensitivity (90.4%) compared with the PrioCHECK CSFV E^{rns} (59.6%) for the detection of positive samples identified by VNT, which is consistent with the previous report that the PrioCHECK CSFV E^{rns} was less sensitive (59%) than IDEXX Chekit E^{rns} (85%) (Schroeder et al., 2012), indicating that the yE^{rns}-iELISA is more reliable and applicable.

In summary, the enhanced expression of the E^{rns} protein of CSFV was achieved in yeast by codon-optimization, and an iELISA based on yE^{rns} was established with high specificity and sensitivity, which has potential to be an accompanying test for DIVA vaccines and applicable for routine serological diagnosis of CSF.

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References

Aebischer, A., Müller, M., Hofmann, M.A., 2013. Two newly developed E(rns)-based ELISAs allow the differentiation of classical swine fever virus-infected from marker-vaccinated animals and the discrimination of pestivirus antibodies. Vet. Microbiol. 161, 274–285.
Ahrens, U., Kaden, V., Drexler, C., Visser, N., 2000. Efficacy of the classical swine fever (CSF) marker vaccine Porcilis Pesti in pregnant sows. Vet. Microbiol. 77, 83–97.
Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45–66.
Cheng, C.Y., Wu, C.W., Lin, G.J., Lee, W.C., Chien, M.S., Huang, C., 2014. Enhancing expression of the classical swine fever virus glycoprotein E2 in yeast and its application to a blocking ELISA. J. Biotechnol. 174, 1–6.
Clavijo, A., Lin, M., Riva, J., Zhou, E.M., 2001. Application of competitive enzyme-linked immunosorbent assay for the serologic diagnosis of classical swine fever virus infection. J. Vet. Diagn. Invest. 13, 357–360.
Colijn, E.O., Bloemraad, M., Wensvoort, G., 1997. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. Vet. Microbiol. 59, 15–25.
Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., de Kruif, A., 2001. An E2 sub-unit marker vaccine does not prevent horizontal or vertical transmission of classical swine fever virus. Vaccine 20, 86–91.
Edwards, S., Fukusho, A., Lefèvre, P.C., Lipowski, A., Pejsak, Z., Roehe, P., Westergaard, J., 2000. Classical swine fever: the global situation. Vet. Microbiol. 73, 103–119.
de Smit, A.J.,2000. Evaluation of an ELISA for the detection of antibodies against Erns envelope protein of classical swine fever virus. In: Classical Swine Fever—Efficacy of Marker Vaccines and Laboratory Diagnosis. Faculteit Diergeneeskunde Thesis University, Utrecht, pp. 117–131.
Floegel-Niesmann, G., 2001. Classical Swine Fever (CSF) marker vaccine: trial III. Evaluation of discriminatory ELISAs. Vet. Microbiol. 83, 121–136.
Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D., 2004. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol. Bioeng. 85, 367–375.
Huang, C., Chien, M.S., Hu, C.M., Chen, C.W., Hsieh, P.C., 2006. Secreted expression of the classical swine fever virus glycoprotein E(rns) in yeast and application to a sandwich blocking ELISA. J. Virol. Methods 132, 40–47.
König, M., Lengsfeld, T., Pauly, T., Stark, R., Thiel, H.J., 1995. Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. J. Virol. 69, 6479–6486.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Li, M., Wang, Y.F., Wang, Y., Gao, H., Li, N., Sun, Y., Liang, B.B., Qiu, H.J., 2009. Immune responses induced by a BacMam virus expressing the E2 protein of classical swine fever virus in mice. Immunol. Lett. 125, 145–150.
Luo, Y., Li, S., Sun, Y., Qiu, H.J., 2014. Classical swine fever in China: a minireview. Vet. Microbiol. 172, 1–6.
Meyers, G., Thiel, H.J., 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47, 53–118.
Moormann, R.J.M., Bouma, A., Kramps, J.A., Terpstra, C., de Smit, H.J., 2000. Development of a classical swine fever subunit marker vaccine and companion diagnostic test. Vet. Microbiol. 73, 209–219.
Moser, C., Ruggli, N., Tratschin, J.D., Hofmann, M.A., 1996. Detection of antibodies against classical swine fever virus in swine sera by indirect ELISA using recombinant envelope glycoprotein E2. Vet. Microbiol. 51, 41–53.
Outchkourov, N.S., Stiekema, W.J., Jongsma, M.A., 2002. Optimization of the expression of equistatin in *Pichia pastoris*. Protein Expr. Purif. 24, 18–24.
Qiu, H.J., Shen, R.X., Tong, G.Z., 2006. The lapinized Chinese strain vaccine against classical swine fever virus: a retrospective review spanning half a century. Agric. Sci. China 5, 1–14.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.
Reimann, I., Depner, K., Trapp, S., Beer, M., 2004. An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. Virology 322, 143–157.
Rümenapf, T., Unger, G., Strauss, J.H., Thiel, H.J., 1993. Processing of the envelope glycoproteins of pestiviruses. J. Virol. 67, 3288–3294.
Schroeder, S., von Rosen, T., Blome, S., Loeffen, W., Haegeman, A., Koenen, F., Uttenthal, A., 2012. Evaluation of classical swine fever virus antibody detection assays with an emphasis on the differentiation of infected from vaccinated animals. Rev. Sci. Tech. 31, 997–1010.
Sinclair, G., Choy, F.Y., 2002. Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. Protein Expr. Purif. 26, 96–105.
Sun, Y., Li, H.Y., Tian, D.Y., Han, Q.Y., Zhang, X., Li, N., Qiu, H.J., 2011. A novel alphavirus replicon-vectored vaccine delivered by adenovirus induces sterile immunity against classical swine fever. Vaccine 29, 8364–8372.
Sun, Y., Tian, D.Y., Li, S., Meng, Q.L., Zhao, B.B., Li, Y., Li, D., Ling, L.J., Liao, Y.J., Qiu, H.J., 2013. Comprehensive evaluation of the adenovirus/alphavirus-replicon chimeric vector-based vaccine rAdV-SFV-E2 against classical swine fever. Vaccine 31, 538–544.
Tautz, N., Elbers, K., Stoll, D., Meyers, G., Thiel, H.J., 1997. Serine protease of pestiviruses: determination of cleavage sites. J. Virol. 71, 5415–5422.
van Gennip, H.G., van Rijn, P.A., Widjojoatmodjo, M.N., de Smit, A.J., Moormann, R.J., 2000. Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. Vaccine 19, 447–459.