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Research Article

Development and Evaluation of an ELISA System for Detection of PEDV-Specific IgA Antibodies in Colostrum

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Abstract

Porcine epidemic diarrhea virus (PEDV) is a devastating swine infectious disease. Development of high-performance methods to diagnose and evaluate viral immune status remains very important to control PEDV. Here, a recombinant S1 protein-based indirect enzyme-linked immunosorbent assay (rpS1-iELISA) was developed to monitor IgA antibody in the colostrum. Optimized cut-off value of the rpS1-iELISA was determined as 0.448. Results yielded a sensitivity of 96.87% and a specificity of 100.0%. Repeatability tests indicated that the coefficients of variation of the colostrum samples within and between runs were both less than 8%. Test results of 523 field colostrum samples showed that the rpS1-iELISA had excellent agreement with immunofluorescence assay (*kappa* = 0.958) and better test performance than a commercial ELISA kit. This test will aid in future diagnostics and assessment of the protective levels of mucosal immune response against PEDV by measuring IgA levels in the colostrum.

Keywords: *ELISA, IgA antibody, Colostrum, Porcine epidemic diarrhea virus, Spike protein*

Kolostrumda Domuz Epidemik Diare Virus-Spesifik IgA Antikorlarının Belirlenmesinde ELISA Sisteminin Geliştirilmesi ve Değerlendirilmesi

Öz

Domuz Epidemik Diare Virus (DEDV) enfeksiyonu domuzların oldukça yıkıcı enfeksiyöz bir hastalığıdır. Virusa karşı bağışıklık durumunu değerlendirmek ve tanıyı koyabilmek için yüksek performanslı metotların gelişimi hastalığın kontrol altına alınabilmesi için elzemdir. Bu çalışmada, kolostrumdaki IgA antikorlarını taramak amacıyla rekombinant S1 protein temelli indirekt enzime bağlı immunosorbent metot (rpS1-iELISA) geliştirildi. rpS1-iELISA için optimize edilmiş eşik değeri 0.448 olarak belirlendi. Sonuçlar %96.87 duyarlılık ve %100 özgüllük gösterdi. Tekrarlanabilirlik testleri kolostrum örnekleri ve koşumlar arasında varyasyon katsayısının %8'den daha az olduğunu gösterdi. Toplam 523 adet saha kolostrum örneklerinin test sonuçları rpS1-iELISA'nın immunofloresans testi *(kappa* = 0.958) ile mükemmel uyumlulukta olduğunu ve ticari ELISA kitinden daha iyi performansa sahip olduğunu gösterdi. Mevcut test kolostrumda IgA seviyesini ölçerek DEDV'e karşı mukozal bağışıklık yanıtın koruyuculuk seviyesini tahmin etmede ve tanıyı koymada yardımcı olacaktır.

Anahtar sözcükler: *ELISA, IgA antikoru, Kolostrum, Domuz Epidemik Diare Virus, Spike proteini*

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the coronavirus family which causes vomiting, watery diarrhea, and dehydration in pigs and high mortality in neonatal piglets [1]. The first outbreak of PEDV was recognized in England in 1971 [2]. Since then, especially from 2010, PEDV outbreaks have been documented in many countries and severely affected the swine industry [3-5]. PEDV circulating in the world can be separated into three groups (G1, G2, G3), which have three subgroups (G1-1, G1-2, G1-3) [6]. Recent reports on PEDV recombinant strains in Italy

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and China have indicated the new evolution strategy of PEDV [7,8], which increases our insight into PEDV genetic variation and bring new challenge for PEDV control.

After an acute outbreak, PEDV can persist longer in affected herds and cause an enzootic infection [9]. Although several commercial vaccines have been used to control large outbreaks, PEDV remains one of the most important causes of economic loss in many pig farms in China [10]. In addition to effective vaccines, high-throughput and reliable methods to diagnose and monitor the immune levels of PEDV vaccine in herds are urgently needed for PEDV surveillance and control.

Porcine epidemic diarrhea virus is an enveloped coronavirus containing a positive-stranded RNA genome. This virus is approximately 28 kb in length and has a 5′ cap and a 3′ polyadenylated tail. Four structural proteins, including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins are encoded by the PEDV genome. The S protein, which is a type I membrane glycoprotein, can induce neutralizing antibodies and interacts with cell receptor in the host. The S protein can be divided into S1 (1-789 aa) and S2 (790-1.383 aa) domains. S1, as an immunodominant region, can induce an active immune response of host and has been an attractive target to develop PEDV vaccine and serologic test methods [9].

Given the special features of the porcine mucosal immune system, the presence of serum antibodies against PEDV is not always correlated with protection. Sow immunity plays an important role in preventing viral infection and disease in newborn piglets, and the IgA level in the colostrum is a better marker of protection from PEDV infection than serum neutralizing (SN) titer from serum samples [4,11]. The current vaccination evaluation systems for PEDV that focus mainly on the immune response at the systemic level and mucosal immunity associated with PEDV is relatively insufficient. Therefore, measuring the localized IgA immune responses is essential to evaluate the protection derived either by vaccines or by previous PEDV exposure [12]. In this study, an IgA ELISA method based on the recombinant partial S1 protein of PEDV isolate (CH/HNQX-3/14), which is a novel strain currently circulating in Henan, China, was developed to detect IgA antibody in the colostrum of PEDV-infected and vaccinated sows.

MATERIAL and METHODS

Ethics Approval

According to the Animals Use in Research Committee (AURC) of Henan Institute of Science and Technology, this study does not require any special approval.

Colostrum Samples

Thirty-six negative control colostrum specimens used in the test were derived from unvaccinated healthy sows, and 32 positive control colostrum specimens were supplied kindly by Prof. Yanyan Yang (Key Laboratory of Animal Immunology of Agriculture Ministry of China) and retested by Western blot and immunofluorescence assay (IFA). The positive control colostrum for transmissible gastro-enteritis virus (TGEV), classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), foot and mouth disease virus (FMDV), and porcine pseudorabies virus (PRV) were obtained from the Henan Center for Animal Disease Prevention and Control. These positive control colostrum were further validated with their corresponding ELISA kits and found to be strong positive and had no cross-reactivity with other swine pathogens.

Expression and Purification of Recombinant PEDV S1 Protein

The region encoding the partial S1 domain (aa 492-796) of PEDV variant CH/HNQX-3/14 (GenBank No. KR095279.1) was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using a forward primer (5′-GGGGATCC TCTTTTGTTACTTTGCC-3′) and a reverse primer (5′-GC GAATTCAGGCGTGTTGTAAAGC-3′). Reverse transcription was performed according to kit protocol (ThermoFisher Scientific, Shanghai, China). In brief, 1 μL of total RNA (1-2 μg) was added to reaction mixture containing 5×reaction buffer, 10 mM dNTP mix, 1 μL Oligo(dT)$_{18}$ Primer, 20 U RNAse inhibitor and 200 U M-MuLV Reverse Transcriptase to 20 μL of final volume. cDNA was synthesized at 42°C for 1 h. Remaining enzymes were heat-inactivated at 70°C for 5 min. PCR of S1 gene was performed as follows: initial denaturing at 95°C for 5 min; followed by 30 cycles at 95°C for 30 s, 57°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified products were purified by a QIAquick Gel Extraction kit (Qiagen China Co., Shanghai, China), and then cloned into pET-30a(+) (Invitrogen) between the *Bam*HI and *Eco*RI restriction sites to generate an expression construct designated as pET30a(+)-pS1.

The recombinant plasmid pET30a(+)-pS1 was transformed into *Escherichia coli* BL$_{21}$ (DE3) competent cells and grown at 37°C in Luria-Bertani broth supplemented with 100 μg/mL kanamycin to an optical density (OD) of 0.6 at 600 nm. The expression products were purified using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Shanghai, China) following the manufacturer's protocol. The final protein products were quantified by micro BCA protein assay kit (ThermoFisher Scientific) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot hybridization.

Development of Indirect ELISA Test with Recombinant S1 Protein

Colostrum samples were tested for IgA antibodies against PEDV by the recombinant partial S1 protein-based indirect enzyme-linked immunosorbent assay (rpS1-iELISA). Briefly, the optimal coating concentration of recombinant pS1

protein and optimum colostrum dilutions for ELISA were determined using a checkerboard titration. The antigen was diluted in coating buffer (50 mM carbonate buffer, pH 9.6) and incubated overnight at 4°C. The following day, plates were washed thrice with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and then blocked with casein/PBST blocking buffer (ThermoFisher Scientific) for 2 h at 37°C, followed by incubation with colostrum samples diluted in PBS for 30 min at 37°C. After a wash step, an optimal diluted horseradish peroxidase (HRP)-conjugated goat anti-porcine IgA secondary antibody (Bethyl Laboratories) was added and incubated at 37°C for 30 min. The reaction was developed using tetramethylbenzidine (TMB) peroxide (ThermoFisher Scientific) as the substrate and stopped by adding 50 µL of 2 M sulfuric acid. ODs were measured at 450 nm using an ELISA plate reader (BioTek). Positive, negative, and blank (sterile water) samples were tested in triplicate and recorded for statistical analysis.

ELISA Cut-Off Values and Assay Performance

The threshold cut-off value was evaluated by receiver operator characteristic (ROC) analysis (MedCalc software, Mariakerke, Belgium) using 36 PEDV negative colostrum samples and 32 PEDV positive colostrum samples, with IFA as the reference method to classify all samples. Positive colostrum samples from PEDV (n=10), TGEV (n=5), CSFV (n=9), PRRSV (n=7), FMDV (n=5), PRV (n=5), and 10 PEDV-negative colostrum samples were used to evaluate the diagnostic specificity. Each sample was tested in triplicate, and the mean $OD_{450\ nm}$ value and standard deviation (SD) were calculated.

The rpS1-iELISA reproducibility within and between runs was evaluated as previously proposed [13]. Six colostrum samples (3 IFA-positive and 3 IFA-negative samples) were selected for the reproducibility experiments. For intra-assay reproducibility, three replicates of each colostrum sample were assigned to the same plate. For inter-assay (between-run) reproducibility, three replicates of each sample were run on different plates. The mean $OD_{450\ nm}$ values, SD, and coefficient of variation (CV) were calculated.

Application of The rpS1-iELISA in The Field

The rpS1-iELISA was applied to test 523 field colostrum samples collected from 16 pig farms in Henan province. The colostrum samples were also evaluated for anti-PEDV IgA antibodies using IFA and a commercial ELISA kit (coELISA, BIONOTE) following the manufacturer's instruction. Cohen's kappa (κ) value was introduced as a measure of agreement between these assays. Additionally, the rpS1-iELISA was used to evaluate the immune efficacy of a commercial PEDV vaccine (WEIKE Biotech Company, Harbin, China) by measuring the IgA antibody in the colostrum from two sow herds with or without vaccination. The vaccinated sows were intramuscularly immunized with PEDV vaccine (2 mL per sow) at 6 and 2 (booster) weeks before parturition. The unvaccinated healthy pregnant sows were selected from a farm with no history of PEDV exposure.

IFA

IFA was performed as described previously [14]. Vero cells were grown in 24-well plates and inoculated with the cell culture-adapted PEDV strain (CH/HNQX-3/14) at a multiplicity of infection of 0.05 with MEM supplemented with 2.5 µg/mL trypsin (Sigma). PBS-treated cells were used as negative control. After incubation at 37°C for 72 h, the cells were washed thrice with PBST and fixed with 80% acetone for 20 min. Cells were then washed thrice with PBST and blocked with casein/PBST blocking buffer at 37°C for 2 h. After three washes with PBST, the colostrum samples were added and incubated at 37°C for 30 min. Next, fluorescein isothiocyanate-conjugated goat anti-porcine IgA (Thermo Scientific™ Pierce) was added at a dilution of 1:100 with PBST to each well. After 30 min of incubation at 37°C, the plates were rinsed thrice with PBST and examined using fluorescent microscopy (Zeiss, Germany).

Statistical Analysis

ELISA data were collected at $OD_{450\ nm}$ and expressed as the mean±SD. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison *post hoc* test (GraphPad Prism 6.0 software), and *P* value of <0.05 was considered significant.

RESULTS

The cloned pS1 gene of PEDV was expressed as a soluble protein with an N-terminal His-tag. SDS-PAGE results showed that, after the cells were induced with 0.8 mM IPTG (30°C for 8 h), the pS1 gene exhibited higher expression level at the position corresponding to the expected molecular mass of approximately 45 kDa *(Fig. 1)*. Expressed PEDV pS1 protein was purified by Ni-NTA, and the protein bound to Ni-NTA was eluted with 200 mM imidazole. SDS-PAGE and Western blot hybridization further demonstrated that purification removed, to a large extent, other proteins, leaving a predominant band at 45 kDa *(Fig. 1)* and achieving a protein yield of over 10 mg/L of culture, with purity greater than 95%.

The rpS1-iELISA was optimized in a checkerboard fashion to maximize signal-to-noise ratios. The optimal concentration of coating antigen was achieved at a concentration of 1.25 µg/mL recombinant pS1 protein. To further determine the optimum colostrum dilution for the testing platform, a well-characterized PEDV "high" positive control colostrum was serially diluted two-fold against antigen-coated ELISA wells at a fixed concentration. Colostrum dilution of 1/100 was found to provide the highest absorbance value and was selected for subsequent assays. The secondary antibody (HRP-conjugated goat anti-porcine IgA) dilutions of 1/10,000 was selected for this assay.

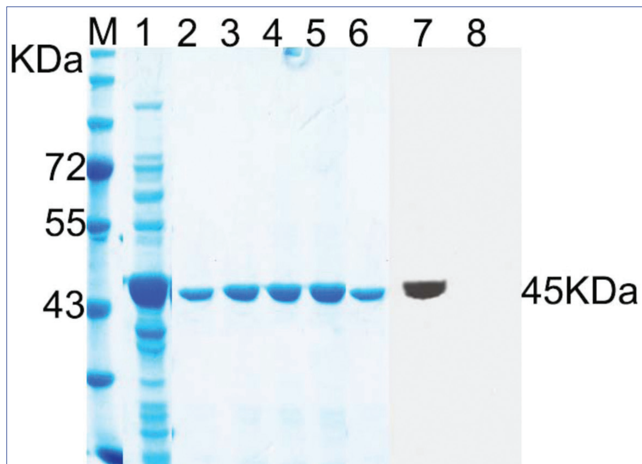

Fig 1. Expression and purification analysis of PEDV pS1 protein by SDS-PAGE and Western blot. Lane M: pre-stained protein molecular weight marker (10-180 KDa); Lane 1: SDS-PAGE of unpurified recombination pS1 protein, which was expressed in *Escherichia coli* BL_{21} (DE3) induced by 0.8 mM IPTG for 8 h at 30°C; Lanes 2-6: SDS-PAGE of purified recombination pS1 protein by Ni-NTA; Lane 7: Reaction of pS1 protein with PEDV positive swine sera by Western blot; 8: pET30a(+)/BL21 vector control

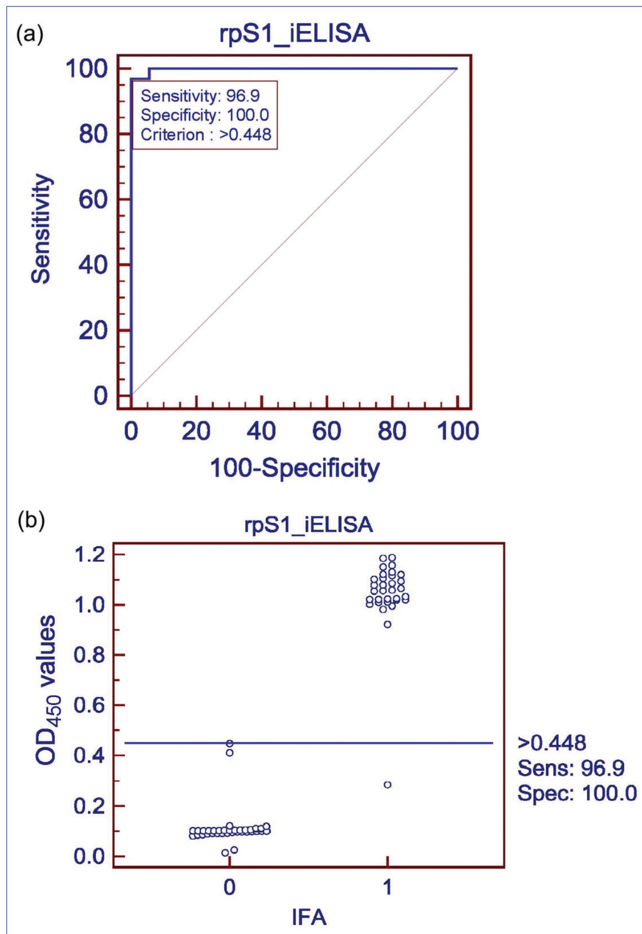

Fig 2. Determination of the cut-off value. (a) ROC analysis of the rpS1-iELISA. The area under the ROC curve was 0.998 when the optimal cut-off $OD_{450\ nm}$ value was 0.448. (b) Distribution of the rpS1-iELISA $OD_{450\ nm}$ values according to the classification of the colostrum by IFA results. The horizontal solid line represents the cut-off value

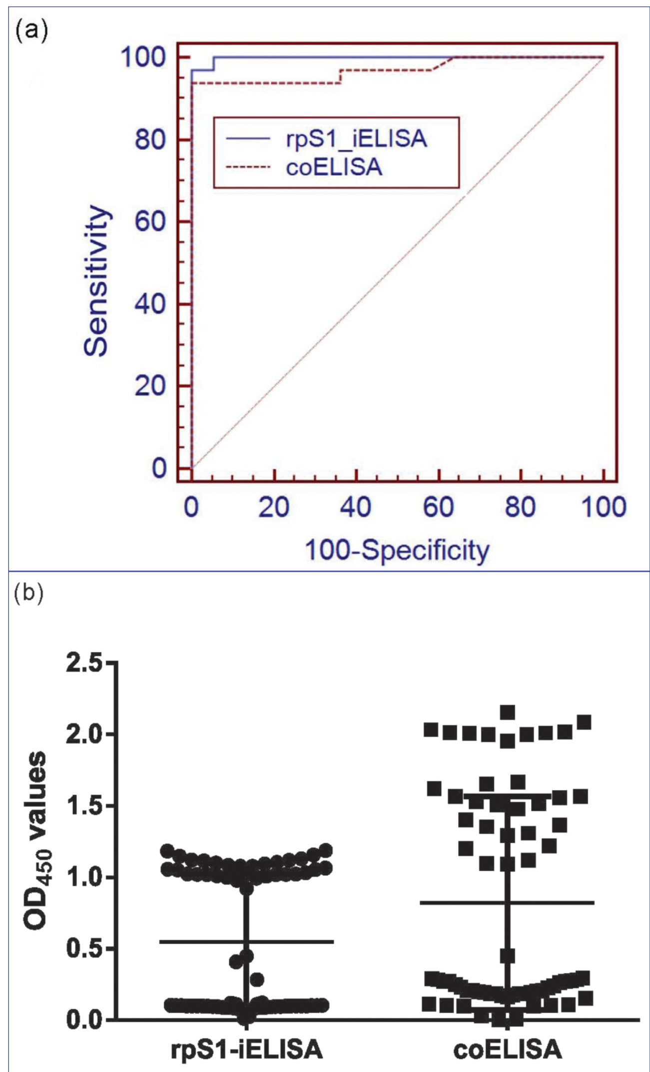

Fig 3. Comparison of test performance between rpS1-iELISA and coELISA by ROC analysis using known 32 positive and 36 negative colostrum samples. (a) The area under the ROC curve (AUC) for the rpS1-iELISA and coELISA are 0.998±0.00212 and 0.970±0.00223, respectively; (b) Distribution of the rpS1-iELISA and coELISA $OD_{450\ nm}$ values according to the classification of colostrum by IFA results. The horizontal solid line represents the cut-off value

Based on the ROC curve analysis of the rpS1-iELISA, the $OD_{450\ nm}$ values of the 36 negative colostrum samples varied from a minimum of 0.014 to a maximum of 0.448 and from 0.185 to 1.188 for the 32 positive colostrum samples. The $OD_{450\ nm}$ of 0.448 was selected as the optimal cut-off value, with an area under the curve (AUC) of 0.998±0.00212 and 95% confidence interval (CI) ranging from 0.944 to 1.000, giving sensitivity and specificity of 96.87% (95% CI: 83.8%, 99.9%) and 100.0% (95% CI: 90.3%, 100.0%), respectively *(Fig. 2)*. By contrast, the ROC analysis for the coELISA showed a lower AUC value (0.970±0.00223, 95% CI: 0.896-0.996) and lower sensitivity (93.75%, 95% CI: 79.2%-99.2%) compared with the rpS1-iELISA *(Fig. 3)*.

To evaluate the specificity of the developed rpS1-iELISA, positive colostrum samples for TGEV, CSFV, PRRSV, FMDV,

Table 1. *Assessment of intra-assay and inter-assay repeatability of the rpS1-iELISA using 3 positive- and 3 negative colostrum samples*

Colostrum Sample	Intra-assay Variability		Inter-assay Variability	
	X±SD	CV	X±SD	CV
1	1.043±0.045	4.30%	1.031±0.037	3.80%
2	1.036±0.033	3.20%	1.101±0.029	3.61%
3	1.048±0.036	4.40%	1.054±0.056	4.38%
4	0.087±0.014	3.46%	0.093±0.009	3.11%
5	0.081±0.005	4.72%	0.090±0.011	6.02%
6	0.091±0.008	7.94%	0.089±0.007	4.32%

Note: Data are the mean±standard deviation of three replications; CV, coefficient of variation; 1, 2 and 3 indicate positive samples; 4, 5 and 6 indicate negative samples

Table 2. *Evaluation of statistical agreement among three tests. Kappa values shown represent a statistical measure of test agreement and were calculated using MedCalc Software*

Reference test		rpS1-iELISA			coELISA		
		Positive	Negative	AP[a]	Positive	Negative	AP[a]
IFA	Positive	334	9	0.656	318	25	0.656
	Negative	1	179	0.344	2	178	0.344
AP[a]		0.641	0.359		0.612	0.388	
Kappa value [b]		0.958			0.888		

[a] *Apparent prevalence (AP) of the IFA, rpS1-iELISA or coELISA: positive ≡ positive number/total number; negative ≡ negative number/total number*
[b] *Observed proportional agreement between rpS1-iELISA and IFA: (334+179)/523=0.981; chance proportional agreement: (0.641×0.656)+(0.359×0.344)= 0.545; observed minus chance agreement: (0.981-0.545)=0.436; maximum possible agreement beyond chance level: (1-0.545)=0.455; agreement quotient (Kappa value) between rpS1-iELISA and IFA: (0.436/0.455)=0.958. The same method was used for calculating the agreement between the coELISA and IFA and obtained a Kappa value of 0.888*

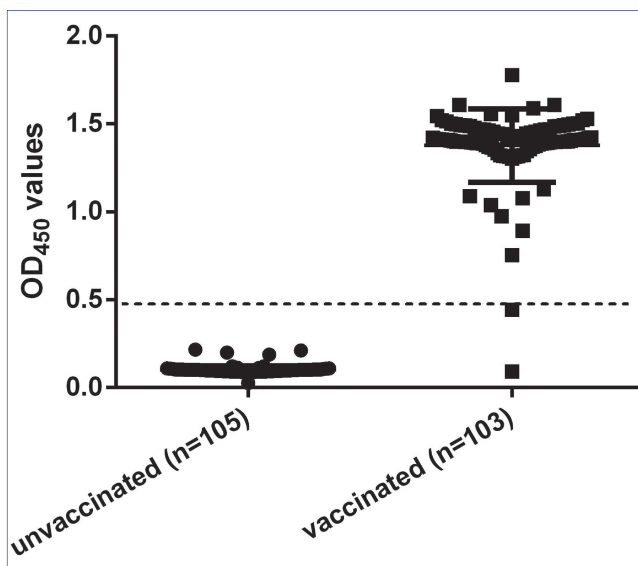

Fig 4. Distribution of anti-PEDV IgA antibodies in colostrum obtained from known PEDV vaccinated- and unvaccinated sows. Data presented as ELISA $OD_{450\ nm}$ values ± SD. The assay cut-off ($OD_{450\ nm}$ value of 0.448) is indicated by the dashed line

and PRV were used to analyze the cross-reaction with purified recombinant PEDV pS1 antigen. Three replicates of each sample were run on the same occasion. The OD values (mean±SD) were 0.083±0.008 for TGEV, 0.087±0.011 for CSFV, 0.056±0.019 for PRRSV, 0.091±0.014 for FMDV, and 0.073±0.010 for PRV. These results indicate that the PEDV pS1 protein-based ELISA had high specificity for detecting the anti-PEDV-specific IgA in the colostrum without antigenic cross-reactivity to the antibodies against other porcine viruses.

Reproducibility experiments within and between rpS1-iELISA plates were performed with three positive and three negative colostrum samples. The results showed that the CVs of intra-assay and inter-assay for three positive colostrum samples were both less than 5.0%. The intra-assay and inter-assay CVs of the three negative colostrum samples ranged from 3.46% to 7.94% and from 3.11% to 6.02%, respectively. These data indicated that the rpS1-iELISA was highly repeatable *(Table 1)*.

A total of 523 clinical colostrum samples were collected from 16 commercial pig farms in Henan province which had a history of diarrhea. From the 523 field colostrum samples, 65.58% (343/523) positive and 34.42% (180/523) negative samples were determined by IFA. Further analyses revealed that 334 of 343 positive colostrum samples determined by IFA were also positively analyzed by the rpS1-iELISA, while 179 of 180 negative colostrums were negatively confirmed by the rpS1-iELISA. The

ratios of positive and negative consistency for the two methods were 97.38 (334/334) and 99.44% (179/180), respectively.

To further assess the agreement between rpS1-iELISA and IFA, κ value was calculated using the formula described in *Table 2*. The *p* and *pe* values were calculated as 0.981 and 0.545, respectively. The agreement quotients (κ value) of the rpS1-iELISA was 0.958, which indicates that a satisfactory agreement between the rpS1-iELISA and IFA was obtained. Moreover, the rpS1-iELISA displayed significantly higher sensitivity and greater κ value than the coELISA (positive ratio of 334/523, 63.86% for rpS1-iELISA vs. 318/523, 60.8% for coELISA; κ value of 0.958 for rpS1-iELISA vs. 0.888 for coELISA, $P<0.05$) *(Table 2)*. This result suggested that rpS1-iELISA was preferable to the coELISA when applied to detect PEDV IgA antibody in the colostrum.

The IgA antibody in the colostrum was measured using the rpS1-iELISA to gain further insight into the efficacy of PEDV vaccine and impact of prefarrowing vaccination on maternal transfer of the IgA antibody. A total of 103 colostrum samples from healthy PEDV-vaccinated sows and 105 colostrum samples from unvaccinated healthy sows were examined. Comparing the average post-vaccination titers, the IgA levels of vaccinated sows were significantly higher than those of non-immunized ones ($P<0.05$). However, when the actual distribution values were evaluated within the groups, data from the two herds showed their respective homogeneous profiles, except for two cases in vaccinated sows with OD_{450} nm below the cut-off value of 0.448 *(Fig. 4)*. These data indicated that IgA levels in the two sow herds were reliable, and the vaccine actually increased IgA antibody responses in the colostrum.

DISCUSSION

Porcine epidemic diarrhea is a highly infectious disease that currently poses a serious threat to the swine industry. Precise diagnostics of PEDV and immune surveillance methods to monitor disease outbreaks and evaluate vaccine efficacy are required to implement control strategies. Previous studies confirmed that the colostral IgA level, but not the serum neutralization titer, induced by immunization against PEDV is correlated with protection of newborn piglets against virulent PEDV challenge [9]. Therefore, the evaluation of IgA level in the colostrum could be very useful to ensure that the piglets receive adequate passive immunity.

ELISA has been applied widely in diagnosis and antibody detection for animal infectious disease [15-21]. Several studies have used recombinant expressed and purified S protein of PEDV for ELISA serodiagnosis [12,22-24]. However, these studies focused mainly on the IgG test, and the numbers of known positive and negative samples used in these assays were relatively smaller than in the present study. Additionally, for mucosal infection, measurement of localized IgA level is critical to evaluate protection derived either by vaccines or by previous pathogen exposure [23]. In this study, a truncated spike gene pS1 domain from a novel PEDV variant (CH/HNQX-3/14) was expressed in *E. coli* and used as coating antigen to develop indirect ELISA to measure IgA antibody in the colostrum. Furthermore, a comparative analysis between rpS1-iELISA and IFA was performed, and the results showed an overall testing agreement of 95.8%. This phenomenon indicated that the rpS1-iELISA had approximately the same reliability as the PEDV whole virus-based IFA, whereas rpS1-iELISA avoided several disadvantages of IFA, such as high cost and relatively subjective interpretation. Moreover, rpS1-iELISA showed better test performance than a commercial IgA ELISA kit, and a higher specificity compared to another S1-protein based ELISA described by Gerber et al.[25]. No cross-reactivity with TGEV, CSFV, PRRSV, FMDV, or PRV was detected with the assay developed in the present study. Nevertheless, further studies are needed to assess whether this assay is suitable for the detection of IgA antibodies in fecal and oral fluid samples, and improve its test performance to distinguish IgA antibodies induced by vaccination from those resulting from PEDV infection.

In addition, the protection ability of IgA levels from immunized sows by artificial infection with PEDV was not evaluated because of the constraints of experimental conditions. However, our tracking survey confirmed that the proportion of piglets showing any abnormal intestinal symptoms at least once at any of the examination time points was lower in vaccinated sow herds than in unvaccinated ones (data not shown). This result suggested that the vaccine was apparently effective for protecting piglets from virulent PEDV challenge. An infectious challenge study is needed to determine whether such IgA levels can protect sows or piglets against PEDV infection, as well as clarifying IgA antibody kinetics in colostrum.

The current study reports the adaptation of a recombinant, highly purified partial S1 protein of PEDV to develop indirect ELISA platform for detecting PEDV IgA antibodies in the colostrum. This assay could be useful for PEDV diagnostics and evaluation of PEDV colostral immunity.

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