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Development and Validation of a Recombinant Nucleocapsid Protein-Based ELISA for Detection of the Antibody to Porcine Reproductive and Respiratory Syndrome Virus

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Three indirect enzyme-linked immunosorbent assays (iELISA) based on the North American like (NA-like), European like (EU-like) and co-expressed NA- and EU-like recombinant nucleocapsid proteins (N-protein) of porcine reproductive and respiratory syndrome virus (PRRSV) were validated for the detection of the antibodies in porcine sera. A total of 422 serum samples from unvaccinated pigs were tested. The cut-off value was optimized by a two-graph receiver operating characteristics analysis at a 95% confidence level. This assay was validated with Western blot analysis and IDEXX HerdChek™ ELISA. Cross-reactivity results showed that iELISA was PRRSV-specific. Repeatability tests revealed that the coefficients of variation of positive sera within and between runs were less than 10%. The results indicate that iELISA is simpler to produce and perform, time-saving and suitable for large scale surveys of PRRSV infection at low cost, and is potentially useful to evaluate the efficiency of various vaccines against PRRSV.

***Keywords*:** PRRSV, nucleocapsid protein, indirect ELISA

Porcine reproductive and respiratory syndrome virus (PRRSV) has been described as a small enveloped RNA virus classified within the family Arteriviridae, order Nidovirales (Cavanagh, 1997). PRRSV is the causative agent of the global disease that has an immense economic impact on the swine industry. The disease is characterized by respiratory disorders in young pigs and reproductive failure in sows (Collins *et al.*, 1992). It was first described as the Lelystad (Wensvoort *et al.*, 1991) virus in the Netherlands and VR-2332 (Collins *et al.*, 1992) in the U.S. The two prototype virus strains share approximately 60% nucleotide identity and have been divided into two main PRRSV genotypes: European (EU) type (genotype 1) and North American (NA) type (genotype 2) (Nelsen *et al.*, 1999; Oleksiewicz *et al.*, 2005).

PRRSV is an approximately 15 kb single-stranded polyadenylated RNA genome that encodes at least nine partially overlapping open reading frames (ORFs). ORFs 1a and 1b occupy more than two-thirds of the genome and encode the viral RNA polymerase (Dea *et al.*, 2000a). The major structural proteins of PRRSV are the envelope glycoprotein (GP) 5 (≈ 26 kDa), the membrane protein M (≈ 19 kDa), and the nucleocapsid protein (N-protein) (≈ 15 kDa), which are encoded by ORF5, ORF6, and ORF7, respectively (Dea *et al.*, 2000a). ORFs 2a, 2b, 3, and 4 encode minor structural proteins designated as GP2a, GP2b, GP3, and GP4, respectively (Meulenberg *et al.*, 1995b; Murtaugh *et al.*, 1995; Dea *et al.*, 2000a). Since the N-protein seems to be immunodominant, several groups explored the use of recombinant PRRSV N-proteins as an antigen for the detection of serum antibodies to the N-protein in indirect and competition ELISAs (Meulenberg *et al.*, 1995a; Cho *et al.*, 1996; Denac *et al.*, 1997; Dea *et al.*, 2000b; Seuberlich *et al.*, 2002; Ferrin *et al.*, 2004).

Various assays have been developed for detection of PRRSV-specific antibodies in swine sera. The immunoperoxidase monolayer assay (IPMA) (Wensvoort *et al.*, 1992) and the indirect immunofluorescence assay (IFA) (Yoon *et al.*, 1992) are based on the specific binding of antibodies to viral antigens presented in infected porcine alveolar macrophages or MA 104-derived cells. Indirect or blocking enzyme-linked immunosorbent assays (ELISAs) (Albina *et al.*, 1992; Cho *et al.*, 1996; Takikawa *et al.*, 1996) have been described by several groups of investigators. Antigens used in these assays were mostly derived from the infectious virus. The common disadvantage of these tests is the necessity to propagate PRRSV in cell culture, which is time-consuming and expensive. The IDEXX HerdChek PRRS ELISA (IDEXX Laboratories Inc., USA) is primarily used for the screening of PRRSV infections of pigs because of the high sensitivity, repeatability, and specificity (Ferrin *et al.*, 2004). It can detect the antibodies against both types of PRRSV, but this type of ELISA is expensive and can produce positive values that are not supported by any other evidence of pig infection (Plagemann, 2006).

In the present study, we investigated the presence of antibodies against PRRSV of 422 field serum samples by three individual indirect ELISA (iELISA), which were based on NA-like, EU-like, and the co-expression of NA- and EU-like

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Table 1. Primers used for the amplification and cloning of EU- or NA-type PRRSV ORF7 gene

Genotype	Oligo name	Sequence (5'-3')[a]	Restriction site
European (EU)	LENP7_F	CCCAAGCTTTCAAGTATGGCCGGTAA	*Hin*dIII
	LENP7_R	AAACTCGAGGTAACTTGCACCCTGACTG	*Xho*I
North American (NA)	VRNP7_F	CGCGGATCCTATGCCAAATAACAACGGCAA	*Bam*HI
	VRNP7_R	TTAGAGCTCGCTGAGGGTGATGCTGTGA	*Sac*I

[a] The restriction enzyme sites are underlined

recombinant N-protein expressed in *Escherichia coli*. Extensive validation of iELISA was undertaken, with special emphasis on repeatability, cross-reactivity, and diagnostic accuracy. This assay was validated by comparison with Western blot analysis and IDEXX HerdChek™ ELISA.

Materials and Methods

Serum stocks

Porcine serum containing NA-like (Strain DsSem4V, GenBank accession no. EU424163) or EU-like (Strain PicLun5L, GenBank accession no. EU424160) PRRSVs were selected from our serum stocks for cloning of complete ORF7 sequences (Chu *et al.*, 2008). A total of 422 field serum samples were collected from individual unvaccinated pigs with reproductive and respiratory syndromes. Porcine serum seropositive for *Mycoplasma hyopneumoniae* (MH), porcine circovirus type 2 (PCV2), swine influenza virus (SIV), *Pasteurella multocida* type A (PmA), and D (PmD), *Actinobacillus pleuropneumoniae* serotype 2 (App2) and 5 (App5) were taken from our serum collection. All of the serum samples were stored at -70°C.

RNA extraction, amplification, and cloning

Viral RNA was extracted with the Viral RNA Mini kit (QIAamp®, QIAGEN, Germany) from the porcine sera. Viral cDNAs were synthesized by RT-PCR using random primers and SuperScript™ III Reverse Transcriptase (Invitrogen, USA) following the supplier's instructions. The full length of PRRSV ORF7 coding sequence was amplified by polymerase chain reaction (PCR) using two pairs of primers (Table 1) designed according to the reference strains VR-2332 (GenBank accession no. U87392) and Lelystad (GenBank accession no. M96262). The PCR cycle conditions were as follows: 94°C for 5 min and 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final prolongation for 5 min at 72°C. The DNA fragments were then cleaved with appropriate restriction enzyme, and inserted into the pET-32b plasmid (Novagen, USA). The resulting plasmids were named as pET32b_VR7, pET32b_LE7 and pET32b_VR7_LE7 for the plasmids inserted with the ORF7 sequences of NA-like, EU-like and both NA- and EU-like PRRSVs, respectively. The recombinant plasmids were transfected into DH5α *E. coli* cells and confirmed by restriction analysis and PCR sequencing (Genotech Co., Korea).

Expression and purification of recombinant protein

For protein expression the plasmids pET32b_VR7, pET32b_LE7, pET32b_VR7_LE7, and pET32b were individually transfected into BL21 (DE3) *E. coli* cells. The overnight culture of bacteria was inoculated into Luria-Bertani medium containing 50 μg/ml ampicillin and incubated at 37°C until optical density (OD) at a wavelength of 600 nm reached 0.8. Then isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega, USA) was added for a final concentration of 1.0 mM to induce the expression of the recombinant protein. After an additional 4 h incubation, cells were harvested by centrifugation at 5,000×g for 20 min at 4°C. Protein expressed from empty pET32b vector and recombinant protein was purified using Chelating Excellose Spin kit (TaKaRa, Japan) according to the manufacturer's instructions. The purified fusion proteins were named as rNA-Np, rEU-Np, rNA-EU-Np, and pET 32b-Hisp, respectively.

SDS-PAGE and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12% polyacrylamide gels (Laemmli, 1970), and the fractionated proteins were visualized by Coomassie-brilliant blue staining. For immunoblotting, proteins were electrophoretically transferred to polyvinylidene difluoride membranes, and then incubated at room temperature for 1 h with Tris Buffered Saline-Tween 20 buffer (TBST, pH 8.0) containing 5% (w/v) skim milk. The membranes were washed with TBST for 3 times, and then incubated with mouse anti-His IgG (Santa Cruz Biotechnology, Inc., USA) (1:1,000 dilution) for 2 h. Unreacted antibodies were washed out with TBST, followed by incubating with horseradish peroxidase-conjugated goat anti-mouse IgG for 2 h at room temperature (1:1,000 dilution). The membranes were developed using Western Blot Detection System (WEST-ZOL® Plus, iNtRON Biotechnology, Inc., Korea).

Indirect ELISA (iELISA)

The iELISAs were performed using 96-well microtitre plates (EIA/RIA Plate, Costar®). Optimal antigen and serum concentrations were determined by standard checkerboard titration of fusion protein in twofold dilution steps from 1:200 to 1:25,600 and positive sera dilutions from 1:10 to 1:320. After diluting with coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6), 100 μl of recombinant protein was applied to columns 1, 3, 5, 7, 9, 11, and 100 μl of pET32b-Hisp was pipetted into columns 2, 4, 6, 8, 10, 12. The plates were incubated at 4°C overnight. The following day, 200 μl of PBS containing 2% (w/v) bovine serum albumin (BSA) was dispensed into all wells and the plates were incubated for 1 h at 37°C. Plates were washed with 300 μl of PBS containing 0.05% (v/v) Tween 20 (PBS-T_{20}). Subsequently, 100 μl of diluted sera was pipetted into appropriate wells of the plates and incubated for 1 h at

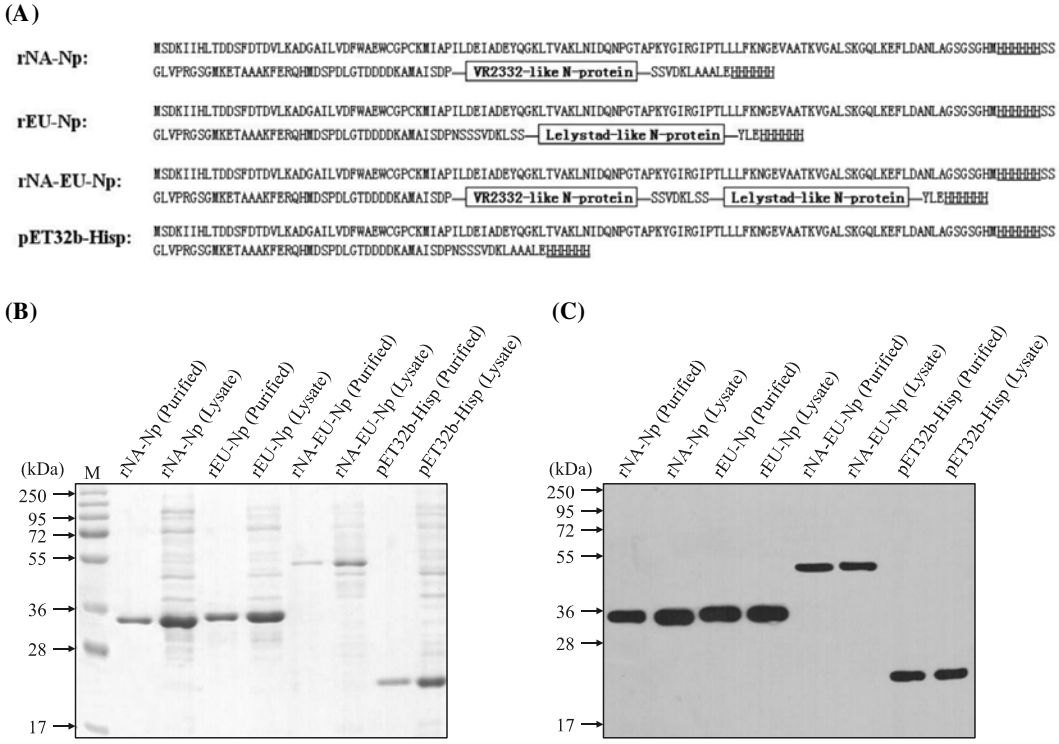

Fig. 1. Recombinant proteins used in the iELISA. (A) Comparison of the three recombinant proteins (rNA-Np, rEU-Np, and rNA-EU-Np) and the protein (pET32b-Hisp) expressed from empty pET32b vector plasmid. The hexahistidine tag used for Western blotting is underlined. (B) Purified recombinant N-protein and cell lysates were analyzed by SDS-PAGE and visualized by staining with coomassie blue. Lane M, Prestained protein ladder (Fermentas, USA). (C) Western blotting analysis for expressed recombinant N-protein using anti-His antibody.

37°C. Plates were washed with 300 μl of PBS-T_{20}, followed by the addition of 100 μl of peroxidase conjugated anti-pig IgG (Sigma, USA), then 1 h incubation at 37°C. Washing was performed as described above before the addition of 100 μl of substrate [24.3 mM citric acid, 51.4 mM Na_2HPO_4, 0.04% (w/v) o-Phenylenediamine dihydrochloride (Sigma), 0.01% (v/v) H_2O_2]. The color development was stopped after 15 min by addition of 50 μl of 3 M H_2SO_4 and the plates were read with a precision microplate reader (492 nm; Emax, Molecular devices).

After determining the optimal concentrations of antigen and serum, microtitre plates were coated with optimal dilutions of protein, and serum samples were tested with the same procedure as described above. The sample to positive (S/P) ratio was calculated using the following formula:

S/P = (OD_{492} of sample for well containing recombinant antigen - OD_{492} of sample for well containing pET32b-Hisp) / (OD_{492} of positive control for well containing recombinant antigen - OD_{492} of positive control for well containing pET 32b-Hisp).

For quality assurance, each plate contained four controls: a strong positive serum, a weak positive serum, a negative serum, and a reagent blank.

The ELISA tests performed with rNA-Np, rEU-Np, rNA-EU-Np were named as NA_iELISA, EU_iELISA, and NA/EU_iELISA, respectively.

Validation of iELISA

To determine an optimal cut-off value for this assay, 96 field serum samples were tested by iELISA, IDEXX ELISA, and Western blot. Western blots were used as the reference method to detect the presence of antibodies to PRRSV. A receiver-operating characteristic analysis (ROC) was performed using Two-graph ROC (A software template for MS-EXCEL) (Greiner, 1995; Greiner *et al.*, 1995), and a cut-off point was determined so that the diagnostic sensitivity (Se) and specificity (Sp) were maximized while the sum of false negative and false positive results were minimized. To compare with the IDEXX PRRSV ELISA, Se, Sp, and accuracy of iELISA were calculated according to the formula as previously described (Livingston *et al.*, 2002): Se = 100 × TP/(TP+FN); Sp = 100 × TN/(TN+FP); positive predictive value = 100 × TP/(TP+FP); negative predictive value = 100 × TN/(TN+FN); accuracy = 100 × (TP+TN)/(TP+FP+TN+FN), where TP, FP, TN, and FN indicated true-positive, false-positive, true-negative and false-negative, respectively.

To further validation of this assay, its precision within and between runs was tested as previously described (Jacobson, 1998). Twenty four field serum samples (14 IDEXX ELISA positive samples, 10 IDEXX ELISA negative samples) were selected for the repeatability experiments. For intra-assay (within-plate) precision, three replicates of each serum samples were assigned in the same plate. For inter-assay (be-

Table 2. Evaluation of IDEXX ELISA and iELISA

	Western blotting[a] (standard)	IDEXX ELISA	iELISA		
			NA	EU	NA/EU
True positive (TP)	59	54	56	56	57
True negative (TN)	37	37	37	36	37
False positive (FP)	-	0	0	1	0
False negative (FN)	-	5	3	3	2
Sensitivity (%)	-	91.5	94.9	94.9	96.6
Specificity (%)	-	100	100	97.3	100
Positive predictive value (%)	-	100	100	98.2	100
Negative predictive value (%)	-	88.1	92.5	92.3	94.9
Accuracy (%)	-	94.8	96.9	95.8	97.9

[a] A total of 96 field serum samples were tested in parallel by Western blotting, IDEXX ELISA and iELISA for detection of antibody against PRRSV. Sensitivity (%) = 100 × TP/(TP+FN); Specificity (%) = 100 × TN/(TN+FP); positive predictive value (%) = 100 × TP/(TP+FP); negative predictive value (%) = 100 × TN/(TN+FN); accuracy (%) = 100 × (TP+TN)/(TP+FP+TN+FN).

tween-run) precision, three replicates of each sample were run on different plates. Mean S/P ratio, standard deviation (SD) and coefficient of variation (CV) of each sample were calculated.

Cross-reactivity of antibodies against other important respiratory system diseases of pigs was tested using positive sera against MH, PCV2, SIV, PmA, PmD, App2, and App5.

Results

Expression and purification of recombinant N-protein

The ORF7 sequences were cloned into the expression vector (pET32b) and sequencing of the plasmids confirmed the sequences encoding the respective viral N-proteins. The predictive protein structures were shown in Fig. 1A. IPTG induced expression of recombinant N-protein in *E. coli* was evaluated at different expression times. The incubation time yielding the largest amount of recombinant protein as determined by SDS-PAGE was chosen (4 h) (data not shown). Bacterial cells were collected, disrupted by sonication, and its protein characteristics (soluble or insoluble proteins) analyzed. Purification was performed under denaturing conditions because the expressed protein was found in the insoluble form.

As shown in Fig. 1B, both purified proteins and cell lysates were analyzed by SDS-PAGE followed by Coomassie blue staining. The visualized bands were in agreement with the calculated molecular mass of approximately 34 kDa, 34.7

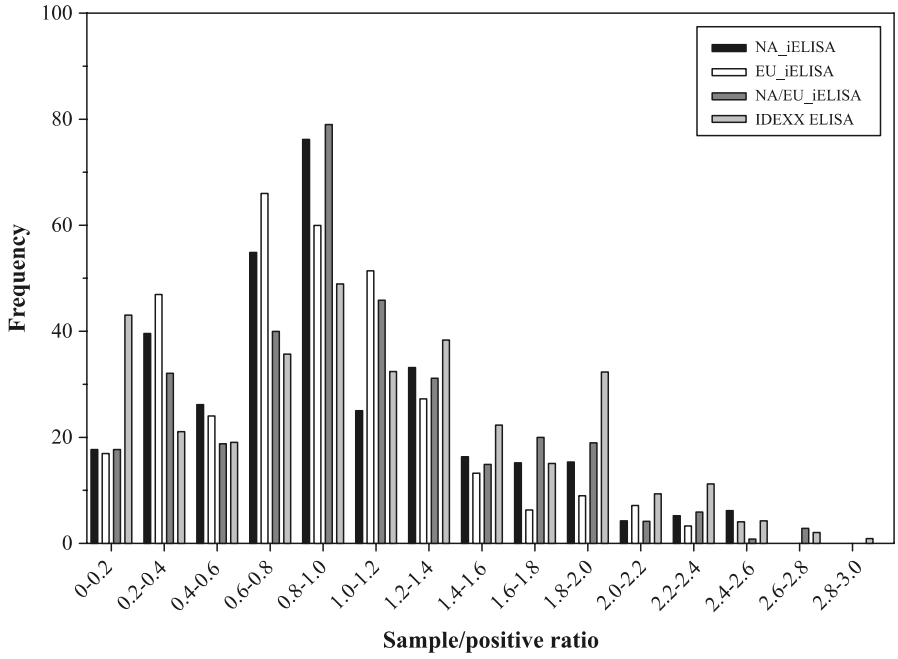

Fig. 2. Frequency distribution of the sample/positive (S/P) ratio determined via iELISA and IDEXX ELISA from 326 field serum samples from unvaccinated pigs with respiratory disorders.

kDa, 48 kDa, and 20.5 kDa for rNA-Np, rEU-Np, rNA-EU-Np, and pET32b-Hisp, respectively. The purified proteins and cell lysates were also analyzed by Western blotting using anti-His antibody to prove further the identity of the respective protein bands (Fig. 1C).

Performance and validation of iELISA

Based on the checkerboard titration method, the optimal antigen concentration and serum sample dilution were set at 2 μg/ml and 1:200. Thus, the P/N ratio between positive and negative control serum was the highest and background was relatively low. In order to determine a cut-off value, we performed a Two-graph ROC analysis with 96 pig sera that were randomly selected from the 422 field serum samples. The cut-off values were determined as 0.393, 0.342, and 0.308 for NA_iELISA, EU_iELISA, and NA/EU_iELISA, respectively. All of the 96 pig serum samples were detected in parallel by Western blotting, IDEXX ELISA, and iELISA. Overall, 59 samples were positive via western blot analysis and thus were assigned to the positive sera, whereas 37 samples were negative and represented the PRRS-negative sera.

The sensitivity, specificity and accuracy of the two ELISA methods were calculated and summarized in Table 2. For the IDEXX ELISA, 91.5% sensitivity and 100% specificity were obtained. In contrast, the iELISA revealed sensitivities of 94.9% (NA_ and EU_iELISA) and 96.6% (NA/EU_iELISA). The calculated specificities were 97.3% (EU_iELISA) and 100% (NA_ and NA/EU_iELISA). The results indicated that the iELISAs (96.9%, 95.8%, and 97.9% of accuracy in NA_, EU_ and NA/EU_iELISA, respectively) were more accurate than the IDEXX ELISA (94.8% of accuracy).

The assay was highly repeatable when tested with 24 serum samples (from serum samples for cut-off value determination) in intra-assay and inter-assay tests with iELISA. The intra-assay CV ranged from 3.6% to 8.4% and the inter-assay CV was between 3.2% and 9.3%.

Positive sera containing antibodies to other pathogenic microorganisms (MH, PCV2, SIV, PmA, PmD, App2, and App5) associated with porcine respiratory disease were included to determine the cross-reactivity of the iELISA. There were no cross-reactions and all the OD values were below the cut-off point.

To further demonstrate the usefulness and reliability of the iELISA in the diagnosis of PRRSV infection, a total of 326 serum samples from unvaccinated pigs with respiratory disorders were tested with IDEXX ELISA and iELISA (Fig. 2). Of these, 265 of 326 (81.3%) tested positive by IDEXX ELISA, and 268 (82.2%), 263 (80.7%), and 275 (84.4%) were positive with NA_iELISA, EU_iELISA, and NA/EU_iELISA, respectively.

Discussion

Monitoring the serostatus of swine herds for the prevalence of PRRSV is important in most swine producing countries. Compared to other serologic tests (including IFA, IPMA, and virus neutralization test), ELISA has been used most often to detect PRRSV because it is easy to perform and can be used as a large scale screening method, while other serologic tests have the expensive and time-consuming disadvantage of having to propagate PRRSV in cell culture (Mengeling *et al.*, 2000). The IDEXX HerdChek™ PRRS ELISA, a commercially available indirect ELISA, has become the industry standard for monitoring the serostatus of swine herds. Although IDEXX reports excellent sensitivity (97.4%) and specificity (99.6%) values, the test is expensive and the unexpected positive results occur in samples from negative-testing or low prevalence herds have caused great concern (Ferrin *et al.*, 2004). A blocking ELISA was developed as a follow-up test to the IDEXX ELISA to increase the method's effectiveness (Ferrin *et al.*, 2004). However, the blocking ELISA is based on the recombinant N-protein of NA-type PRRSV (ATCC VR-2332), which may not be appropriate to detect antibodies to EU-type PRRSV (Seuberlich *et al.*, 2002), which prompted the present study to develop and validate an indirect ELISA based on the N-proteins of VR2332-like, Lelystad-like and co-expression of VR2332- and Lelystad-like PRRSV for the detection of the antibody to the virus.

Although the N-protein is a relatively conserved protein of PRRSV, considerable amino acid differences exist between European and North American PRRSVs. For example, the N-proteins of Lelystad virus and VR-2332 exhibit only about 60% amino acid identity (Mardassi *et al.*, 1995; Meng *et al.*, 1995) as demonstrated in our previous study (Chu *et al.*, 2008) by comparing VR2332-like and Lelystad-like PRRSV from porcine herds in Korea. In addition, several linear epitopes have been identified in the N-proteins of Lelystad virus and North American PRRSV, but they differ for the two PRRSVs and little serologic cross-reaction occur between them (Dea *et al.*, 2000a). This may reduce the diagnostic accuracy of ELISA, which uses a single antigen of NA- or EU-type PRRSV N-protein.

The NA-like (DsSem4V) and EU-like (PicLun5L) strains of PRRSV were confirmed in our previous study, which shared 90.3% and 94.3% identity of nucleotide sequence of ORF7 as compared with the NA prototype (VR-2332) and EU prototype (Lelystad), respectively. However, there was only 64.6% identity between the two determined strains. Here, a recombinant antigen including single or both NA- and EU-like PRRSV N-proteins was expressed in *E. coli* and utilized in the iELISA system. Western blotting was set as the reference test and iELISA was optimized with the cut-off point determined by Two-graph ROC analysis.

It should be mentioned that an unexpected immune response to the vector expressed peptides could occur, causing false-positive results. To remove any aspecific reactions of the tested serums, the rows of the plates were alternately coated with diluted recombinant antigen or mock antigen (pET32b-Hisp). Finally, the OD value obtained from wells coated with recombinant antigen minus the OD value obtained from wells coated with mock antigen to increase the specificity of the tests.

To our knowledge, it is the first test (NA/EU_iELISA) for detection of antibodies against PRRSV based on the combined antigens derived from different types of PRRSV N-protein. Overall, the three subsets of iELISAs (NA_iELISA, EU_iELISA, and NA/EU_iELISA) showed a sensitivity and specificity greater than 94%, which are considered

to be excellent for a diagnositic test (Greiner *et al.*, 2000). When the diagnostic efficiencies of these iELISA sets were compared, NA/EU_iELISA presented the highest sensitivity, specificity and accuracy of the three. This suggests that the effectiveness of iELISA may be enhanced by the co-expressed NA- and EU-like PRRSV N-protein as the antigen. In contrast, the IDEXX ELISA showed a relative low sensitivity (91.5%). However, excellent specificity (100%) was seen in the IDEXX ELISA and iELISAs except for the EU_iELISA (97.3%) (Table 2).

Of the 326 field sera from unvaccinated pigs with respiratory disorders, 275 (84.4%) sera tested positive with NA/EU_iELISA, and 265 (81.3%) were positive with IDEXX ELISA (Fig. 2). This result was in agreement with the validation procedure, indicating the NA/EU_iELISA has a superior sensitivity compared with the IDEXX ELISA.

In conclusion, the iELISA established in the present study was sensitive and specific for PRRSV antibody detection, and was simple and quick to produce and perform in comparison to existing serological methods for the detection of PRRSV. The data in this study may facilitate the development of a reliable tool or kit for the low cost, large scale detection of PRRSV antibodies, and may have the potential to evaluate the efficiency of various vaccines against PRRSV.

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