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Original Article

Indirect ELISA for the Detection of Rabies Virus Antibodies in Dog Sera

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Rabies is known as the most fatal disease in all warm-blooded animals, including dogs. Among animals that transmit rabies, dogs are mainly responsible for transmitting animal rabies in Asian countries. Detection of rabies virus (RABV) antibodies in dogs is performed by fluorescent antibody virus neutralization (FAVN) test or rapid fluorescent focus inhibition test. These standard assays are difficult to carry out in diagnostic laboratories without sufficient instruments, designated RABV, and cell culture systems. An alternative assay that is easy to conduct and time efficient is required for rapid sero-surveillance following vaccination. Recombinant baculovirus expressing RABV nucleoprotein (RVN) was constructed and the recombinant protein was purified using Ni-NTA and fast protein liquid column chromatography. We developed and evaluated an indirect enzyme-linked immunosorbent assay (I-ELISA) with recombinant RVN for the detection of RABV antibodies in 122 dog serum samples. The I-ELISA results obtained from these samples were compared with FAVN results. The sensitivity, specificity, and accuracy of I-ELISA were 88.1%, 92.5%, and 91.0%, respectively, compared with FAVN. Results of I-ELISA were significantly correlated with that of FAVN (r = 0.81). These results suggest that I-ELISA with recombinant RVN is useful for sero-surveillance of RABV in dog sera.

Key Words: Dog, Indirect ELISA, Rabies virus, Sero-surveillance, Rabies virus nucleoprotein, Recombinant baculovirus system

INTRODUCTION

Rabies is caused by rabies virus (RABV), a member of the genus *Lyssavirus* in the family Rabdoviridae. Its genome is a single-stranded negative sense RNA molecule and translates five viral proteins, namely the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L) (1). The N, P, and L proteins associated with the viral genomic RNA form the rabies viral Ribonucleoprotein (RNP) complex. The N protein takes part in encapsidation of the genomic RNA. The N protein in the genome sequence is the most abundant protein among the five structural proteins and RABV N (RVN) sequence is well conserved among Rabies lyssaviruses (2). Therefore, the N protein consisting of 450 amino acids has been used for the detection of RABV antibodies following administration of vaccination (1).

As the World Organization for Animal Health (OIE) has set its goal for elimination of dog-mediated rabies in the

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world by 2030, all OIE member countries including South Korea are strengthening efforts to eradicate rabies mediated by dogs through a national rabies control plan (3). Since the Livestock Disease Prevention Law in South Korea was enacted in 1961, rabies vaccination of dogs has been ongoing. Despite mass vaccination, animal rabies has been reported continuously in Gangwon and Gyeonggi provinces since 1993. The veterinary authority decided to conduct a sero-surveillance of rabies in dogs living in those two provinces in 2002 because it was important to monitor the immune status of the dog population and implement appropriate measures, such as booster immunization of dogs in rabies risk areas including demilitarized zone. The veterinary authorities in South Korea have conducted sero-surveillance of RABV in sera collected from dogs residing in 13 counties of the two provinces. An investigation of the prevalence of RABV antibodies in dogs is conducted using the fluorescent antibody virus neutralization (FAVN) test in approximately 250 dog sera samples collected from rabies risk regions each year (4).

FAVN and rapid fluorescent focus inhibition test (RFFIT) methods are used to detect RABV antibodies induced by vaccination in dog serum samples. These two methods have been designated as the standard method by the OIE and World Health Organization (WHO) to detect RABV antibodies in animals and humans. Although the FAVN and RFFIT can be accurate and useful for the detection of RABV antibodies, both test methods require 3 or 4 days to obtain results and are not suitable for large-scale sero-surveillance (5). Enzyme linked immunosorbent assay (ELISA) is the preferable method to detect RABV antibodies in a large-scale investigation because it does not require live RABV and only a small amount of serum is needed; additionally, the procedure is simple and safer than the FAVN test (6). A few ELISA kits specific for the RABV antibody have been commercialized for animals, but the kits are expensive and limited to use at the right time due to production limitations (7, 8). Therefore, an N protein-based ELISA was developed to test the quality of vaccines (9).

In the present study, we expressed recombinant RVN protein in Sf9 cells derived from *Spodoptera frugiperda* and developed and evaluated the indirect ELISA (I-ELISA) method using purified recombinant RVN protein for the detection of RABV antibodies in dogs.

MATERIALS AND METHODS

Cells, viruses, and serum samples

Sf9 cells (ATCC CRL-1711, Manassas, VA, USA) were grown in Grace's insect medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) for propagating recombinant baculovirus expressing RVN protein. BHK-21 cells (ATCC CCL-10) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with antibiotics (100 IU/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) and 5% FBS. The Rabies Challenge Virus Standard (CVS)-11 (ATCC VR-959), a standard rabies virus strain, was used for the FAVN test. Eight serum samples were prepared from dogs for optimization of I-ELISA. Animal experiment was reviewed and approved by institutional Animal Ethic Committee at Animal and Plant Quarantine Agency (No. 2016-453). In total, 122 dog serum samples were taken from part of the annual surveillance for the National Rabies Eradication Program and were subjected to FAVN test and I-ELISA.

Construction of plasmid-carrying N gene of RABV

Genomic RNA was extracted from the Evelyn-Rokitnicki-Abelseth (ERA) strain of RABV. Each forward and reverse primer for the amplification of RVN gene of RABV was designed to contain *Bam*HI and *Hind*III (Bioneer, Daejeon, Korea) restriction enzyme sites, respectively (Table 1). The RVN gene was amplified by reverse transcription and polymerase chain reaction (RT-PCR) using the primer set, and the product was separated on 1.8% agarose gel. After purification, the products were ligated into the cloning site of the pGEM-T vector system (Promega, Madison, WI, USA). The RVN gene fragment obtained by *Bam*HI/*Hind*III double digestion was ligated into the *BamH*I/*Hind*III site of baculovirus transfer vector, pBlueBac4.5/V5-His (Invitrogen), which contained a C-terminal peptide encoding a six-histidine tag for detection and purification. The plasmid containing the

Table 1. Oligonucleotide primers to amplify the RABV N gene and confirm the pure plaque of recombinant RVN baculovirus

Primer	Oligo nucleotide sequences (5' - 3')	Genomic region
NF	CCGGATCCGATGGATGCCGACAAGATTGTATTC	RABV N
NR	CCAAGCTTTGAGTCACTCGAATATGTCTTGTT	
Bac F	TTT ACT GTT TTC GTA ACA ACA GTT TTG	Baculovirus multi-cloning site
Bac R	CAA CAA CGC ACA GAA TCT AGC	

Underlined sequences indicate restriction enzyme sites (*BamH*I and *Hind*III) and start codon.
RABV N: rabies virus nucleoprotein.

RVN gene was transformed into DH5α competent cells (Invitrogen). The pBlueRVN plasmid was extracted and purified by a plasmid purification kit (Qiagen, Hilden, Germany).

Construction of recombinant RVN baculovirus and purification

For the construction of recombinant RVN baculovirus, 0.5 μg of Bac-N-Blue DNA (Invitrogen) and 10 μg of purified pBlueRVN plasmid DNA were mixed with Cellfectin reagent (Invitrogen) in Grace's insect medium without FBS and antibiotics. After incubation for 15 min at room temperature, the mixture was added into a 60-mm culture dish in which Sf9 cells had been cultivated at 27℃. After 3 days, supernatant was harvested and the cells were incubated continuously by adding fresh medium containing FBS. Plaque assay to purify recombinant baculovirus was performed in 1% agarose medium containing 150 μg/ml of X-gal. PCR assay against recombinant RVN baculovirus was carried out to confirm the isolation of a pure plaque using specific baculovirus primers (Table 1). Passage of the recombinant RVN baculovirus was conducted three times by introducing it to Sf9 cells at the multiplicity of infection (MOI) of 0.1. The third passage of the recombinant RVN baculovirus was used as viral stock and propagated for the production of recombinant RVN protein in Sf9 cells. The recombinant RVN protein was purified by Ni-NTA affinity column (Invitrogen) followed by fast protein liquid chromatography (FPLC) using a HisTrap^{TM} FF column (GE Healthcare Life Science, Pittsburgh, PA, USA) according to manufacturer's instructions. The purified recombinant RVN protein was used as antigen for I-ELISA.

Western blotting against recombinant RVN protein

Recombinant RVN protein expressed was identified by Western blotting. The recombinant RVN protein was separated on a 12.5% tris-glycine gel and transferred onto a nitrocellulose (NC) membrane. The NC membrane was reacted with anti-RVN monoclonal antibody (Median Diagnostics, Chuncheon, Korea) and anti-mouse IgG phosphate conjugate (KPL, Gaithersburg, MD, USA). After washing, the membrane was developed in TMB solution. The image of the developed NC membrane was captured by ChemiDoc XRS+ imaging system (BIO-RAD, Hercules, CA, USA).

Fluorescent Assay Virus Neutralization (FAVN) test

The neutralizing antibody titers in the serum samples were determined using a FAVN test in 96-well cell culture plates. The test was conducted in accordance with the OIE methodology that was described in the manual of diagnostic test and vaccines for terrestrial animals of OIE. In brief, all serum samples were heat-inactivated at 56℃ for 30 min prior to the test. Serial three-fold dilutions of serum samples with final volume of 100 μl were made in duplicate well. The CVS11 RABV strain at concentration of 100 $FAID_{50}$/50 μl was added to each well and the plates were placed for 1 h at 37℃. Next, 50 μl of a BHK-21 cell suspension (4×10^5 cells/ml) was added to each well, and the plates were incubated for 48 h at 37℃ in a humidified 0.5% CO_2 incubator. After incubation, the growth medium was discarded, the mono layers of BHK-21 cell was washed with phosphate-buffered saline (PBS) and was fixed in 80% cold acetone at -20℃ for 20 min. After dry for 1 h at room temperature,

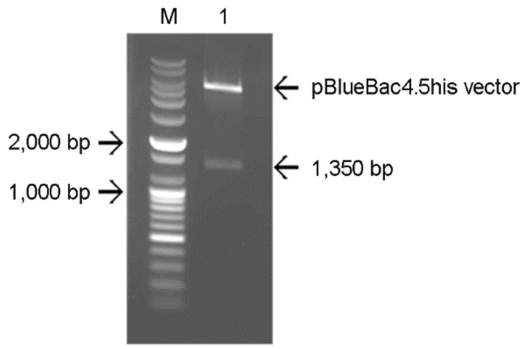

Figure 1. The confirmation of recombinant pBlueRVN plasmid construction. RABV nucleoprotein (RVN) gene was inserted into pBluebac4.5/V5-His vector to generate recombinant pBlueRVN plasmid. Insertion of RVN gene into pBluebac4.5/V5 His vector was confirmed by double-digestion with restriction enzyme *Bam*HI and *Hin*dIII followed by agarose electrophoresis. Lane M: 1 kb DNA ladder, lane 1: pBlueRVN.

the mono layers of BHK-21 was stained with a monoclonal antibody (Median Diagnostics) against the RABV N protein and fluorescein isothiocyanate (FITC)-conjugated goat/anti-mouse IgG+IgM (KPL) and read with fluorescence microscope (Nikon-TE2000U, Tokyo, Japan) at ×200 magnification. Serum VNA titers were compared to OIE standard dog serum (OIE Reference Laboratory for Rabies, Nancy, France) with a known antibody concentration to express the VNA titer as IU/ml.

Indirect ELISA

Optimal coating antigen concentrations and dilutions of serum and substrate were determined by a checkerboard titration test. The recombinant RVN antigen was diluted in coating buffer ranging from 5 to 0.002 μg/ml, and the serum samples were tested at dilutions of 1:10 to 20,480 in 5% skim milk. The 5% skim milk in PBS was used as serum dilution buffer and blocking buffer. Each 100 μl of serum in dilution buffer was placed in a 96-well microplate (Maxisorp; NUNC, Roskilde, Denmark) coated with the recombinant RVN antigen. After incubation at 37℃ for 1 h, the plate was washed with PBS containing 0.05% Tween 20 and incubated with 100 μl of HRP-conjugated anti-dog IgG (KPL) for 1 h at 37℃. After washing, 100 μl of ABTS substrate solution (KPL) was added to each well and then incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 μl of 0.5 M sulfuric acid. The absorbance at 405 nm was measured in a spectrophotometer (Sunrise ELISA reader; Tecan, Switzerland). Serum samples were evaluated as positive if the absorbance value was higher than the cutoff value of 0.4. The specificity, sensitivity, and accuracy were calculated using the following formula: sensitivity (%) = [(number of positives in both tests) / (number of positives in the FAVN test)] × 100; specificity (%) = [(number of negatives in both tests) / (number of negatives in the FAVN test)] × 100; and accuracy (%) = [(number of positives in both tests + number of negatives in both tests) / (total number of samples)] × 100.

Statistical analysis

Linear regression analysis (least-squares method) was used to determine the correlation between I-ELISA data and FAVN data. The correlation coefficient (*r* value) was computed using Sigma Plot version 10.0 for Windows (Jandel; San Rafael, CA, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

Expression of recombinant RVN protein in Sf9 insect cells

The RVN gene was cloned into pGEM-T and sub-cloned into pBluBac4.5/V5-His vector, which encodes RVN gene with a six-histidine tag in the C-terminal region (Fig. 1). After transfection into Sf9 cells, the production of recombinant RVN baculovirus was confirmed by plaque assay followed by PCR assay against recombinant RVN baculovirus and was identified by the presence of cytopathic effect and fluorescent antibody staining with anti-6-his monoclonal antibody or specific monoclonal antibody against RVN protein (Fig. 2).

Optimization of I-ELISA

Recombinant RVN protein was purified via two chromatographic steps and the purified protein was identified as a

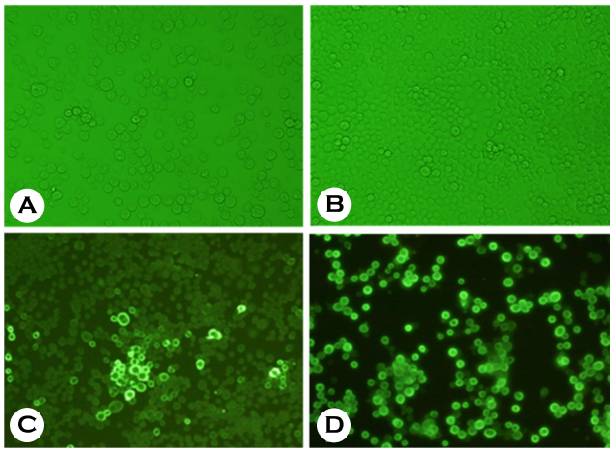

Figure 2. Identification of recombinant RVN baculovirus. Cytopathic effect in Sf9 insect cells infected with recombinant RVN baculovirus (A) and normal Sf9 insect cells (B). Immunofluorescence in Sf9 insect cells infected with recombinant baculovirus expressing RVN protein using anti-6 histidine antibody (C) and mouse monoclonal antibody against RVN protein (D).

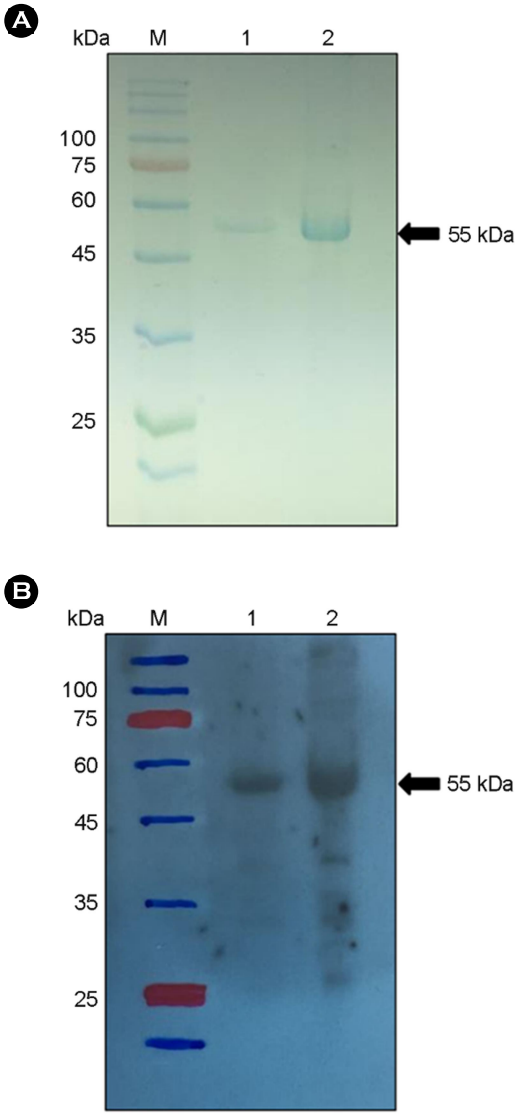

Figure 3. Identification of the recombinant RVN protein. Recombinant RVN protein expressed using recombinant baculovirus system was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue (A). RVN protein was identified using monoclonal antibody against RVN protein by Western blotting (B). The molecular weight of recombinant RVN protein was 55 kDa. M; protein ladder, lane 1 and 2; purified RVN protein.

55 kDa band by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using a monoclonal antibody to the RVN protein (Fig. 3). The optimal antigen concentration (1.0 μg/ml) and serum dilution (1:50) were determined by various positive/negative serum samples, which were measured by the FAVN test, as shown in Fig. 4. In addition, an absorbance value of 0.4 was set as the threshold (cutoff value) because the sensitivity and specificity were the highest value, compared with a cutoff value from 0.3, 0.4 and 0.5. Therefore, an absorbance value of >0.4 in I-ELISA was considered to be a positive result. Based on these results, the I-ELISA kit was prepared, and 122 dog serum samples were evaluated.

Determination of the RABV antibody titer

Two different antibody detection assays (FAVN and I-ELISA) were carried out on 122 dog serum samples to detect RABV antibodies. Table 2 shows that 42 serum samples were positive in VNA titers ranging from 0.29 to 4.6 IU/ml and 43 serum samples were positive in I-ELISA in absorbance values exceeding 0.4. The numbers of RABV-negative serum samples were 80 and 79, as evaluated by FAVN and I-ELISA, respectively.

Application of I-ELISA to dog sera

The absorbance value from I-ELISA for the 122 serum samples was compared with titers determined by FAVN to

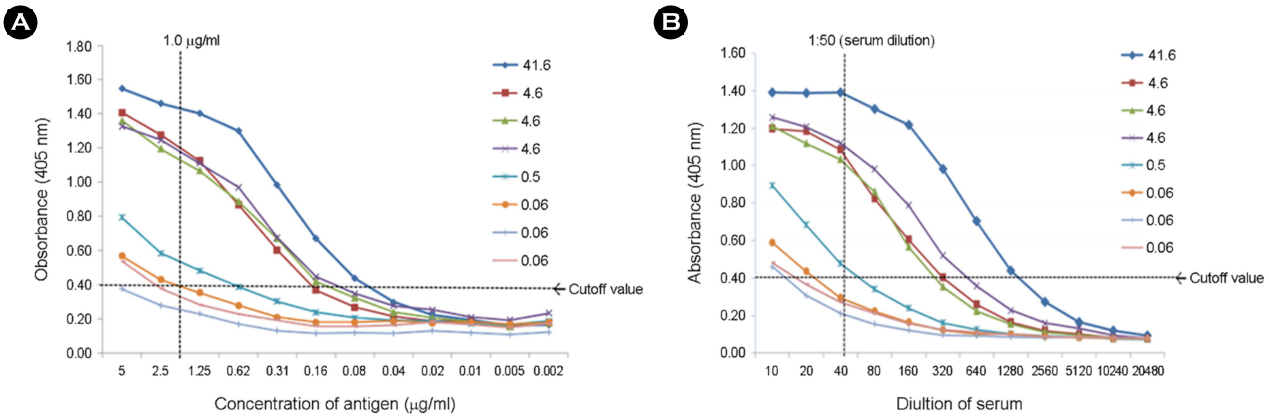

Figure 4. Optimization of Indirect-ELISA (I-ELISA). Concentration of the recombinant RVN antigen (A) and serum dilution factor (B) for indirect enzyme-linked immunosorbent assay (I-ELISA) were determined by a checkerboard titration test. The antigen was diluted to 1:300 (1.0 μg/ml) in carbonate buffer (pH 9.6) and coated wells in a 96-well microplate. The number of remarks indicates RABV antibody titer from fluorescent antibody virus neutralization (FAVN) test.

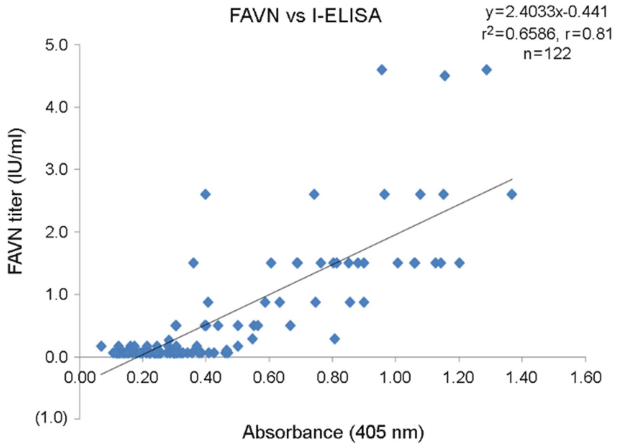

Figure 5. Correlation between RABV antibody titers obtained by FAVN test and I-ELISA in 122 dog serum samples.

Table 2. Determination of the sensitivity, specificity, and accuracy of I-ELISA for the detection of RABV antibodies in comparison with those of FAVN

		No. of samples with FAVN		
		Positive	Negative	Sum
I-ELISA	Positive	37	6	43
	Negative	5	74	79
	Sum	42	80	122
	Sensitivity*		88.1%	
	Specificity**		92.5%	
	Accuracy***		91.0%	

*Sensitivity (%) = [(number of positives in both tests) / (number of positives in the FAVN test)] × 100, **Specificity (%) = [(number of negatives in both tests) / (number of negatives in the FAVN test)] × 100, ***Accuracy (%) = [(number of positives in both tests + number of negatives in both tests) / (total number of samples)] × 100. I-ELISA: indirect enzyme-linked immunosorbent assay; FAVN: fluorescent antibody virus neutralization.

evaluate diagnostic reliability. The correlation between I-ELISA and FAVN was indicated by regression line and correlation coefficient (r), as shown in Fig. 5. The r-value for FAVN was 0.81 ($p < 0.05$). The diagnostic sensitivity, specificity, and accuracy of I-ELISA were calculated from the RABV positive/negative sera measured by standard diagnostic assay, FAVN test. The sensitivity, specificity, and accuracy of I-ELISA were 88.1%, 92.5%, and 91.0%, compared with FAVN test (Table 2).

DISCUSSION

Rabies infection in animals can be 100% prevented by vaccination and a reduction of transmitters (10). Therefore, all dogs living in South Korea should be immunized at least once per year with rabies vaccine in accordance with the National Rabies Eradication Program, which can lead to

elimination of dog-mediated rabies. Because vaccination failure or missed vaccination can result in an outbreak of rabies in dogs, preventive measures such as issuing warnings and performing revaccination have been implemented in association with sero-surveillance of the RABV antibody in South Korea (4). It is noted that successful prevention of dog rabies requires more than 70% immunity within the dog population (3, 10).

Serological surveys of RABV are useful to determine the vaccination rate in dogs. Although the FAVN test has been used as a standard method, many veterinary institutes prefer using ELISA, which is suitable for large numbers of serum samples and is simple and rapid. For this reason, we developed an I-ELISA method using recombinant RVN protein for sero-surveillance of rabies antibodies in vaccinated dogs and compared it with the FAVN test.

Several expression systems have been reported for the rabies viral proteins. The RVN gene is the most conserved of the five structural genes of RABV and has been selected to detect wild RABV by RT-PCR. Therefore, the recombinant RVN protein has been expressed in several hosts such as *Escherichia coli* (6, 11, 12), insect cells (13), yeast (14), and plant (15) for use as an antigen of serological assays. In this study, we established recombinant RVN protein expression system using recombinant baculovirus system. One of the most important factors affecting the sensitivity and specificity of ELISA is the use of highly purified antigen (16). Considering the above, we used the recombinant RVN protein purified with Ni-NTA affinity chromatography and FPLC column as an antigen to increase the sensitivity and specificity of I-ELISA.

The sensitivity, specificity, and accuracy of the I-ELISA as well as their correlations with results obtained using FAVN test were determined using 122 dog serum samples. The sensitivity of I-ELISA compared with FAVN (88.1%) was similar to that obtained from Inoue *et al*. (91.7%) who expressed it in *Escherichia coli*. However, the specificity (92.5%) was slightly lower than that (100%) of a previous study, indicating that improvement is needed (11). Our results also showed that the absorbance result of the I-ELISA was significantly correlated with that of the FAVN titer ($r = 0.81$). Moreover, the I-ELISA correlations are consistent with the result of other study that used rapid ELISA for detection of RABV antibodies in dogs (17), suggesting that the I-ELISA is suitable for sero-surveillance of RABV in dogs. Further studies using other types of ELISA, such as a blocking ELISA, are needed for sero-surveillance after rabies vaccination in several species, including cattle.

In conclusion, we showed that the I-ELISA results using recombinant RVN protein expressed in a recombinant baculovirus system have a significant correlation with FAVN test results in terms of the detection of RABV antibodies in dog serum samples, indicating that this new I-ELISA assay can be used as a simple and efficient tool for examining a large number of serum samples at once.

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