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Development of a Blocking ELISA for Measuring Rabies Virus-specific Antibodies in Animals

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Rabies virus (RABV)-specific antibodies in animals and humans are measured using standard methods such as fluorescent antibody virus neutralization (FAVN) tests and rapid fluorescent focus inhibition tests, which are based on cell culture systems. An alternative assay that is safe and easy to perform is required for rapid sero-surveillance following mass vaccination of animals. Two purified monoclonal antibodies (4G36 and B2H17) against RABV were selected as capture and detection antibodies, respectively. A genetically modified RABV, the ERAGS strain, was propagated and concentrated by polyethylene glycol precipitation. Optimal conditions for the RABV antigen, antibodies, and serum dilution for a blocking enzyme-linked immune sorbent assay (B-ELISA) were established. We evaluated the sensitivity, specificity, and accuracy of the B-ELISA using serum samples from 138 dogs, 71 raccoon dogs, and 25 cats. The B-ELISA showed a diagnostic sensitivity of 95.8–96.3%, specificity of 91.3–100%, and accuracy of 96.0–97.2% compared to the FAVN test. These results suggest that the B-ELISA is useful for sero-surveillance of RABV in dogs, raccoon dogs, and cats.

Keywords: **Rabies, blocking ELISA, antibody detection, dogs**

Introduction

Among animals, carnivores infected with rabies are responsible for more than 99% of human rabies cases. Controlling human rabies caused by dog bites and animal rabies circulating in carnivores means combating rabies at the animal source. The best solution for controlling animal rabies is to implement mass vaccination policies because this is the only way to interrupt the infection cycle between carnivores and domestic animals, or among carnivores. Although the Korean government has implemented a mass vaccination policy, many animal rabies cases have been reported in the provinces of Gyeonggi and Gangwon since 1993 [1, 2]. The Korean government started sero-surveillance programs for rabies using the fluorescent antibody virus neutralization (FAVN) test with dog serum collected from the two provinces in 2002 because revaccination based on data obtained through serological monitoring is important to prevent disease recurrence.

There are several methods for measuring rabies virus (RABV)-specific antibodies in serum samples. The mouse neutralization test (MNT), FAVN test, and rapid fluorescent focus inhibition test (RFFIT) are standard methods for detecting rabies-specific antibodies in animals and humans designated by the World Organization for Animal Health (OIE) and World Health Organization (WHO), respectively [3–5]. Although the two virus neutralization methods are accurate and useful for measuring anti-RABV antibodies, both tests require skilled technicians to perform them and are not suitable for large-scale serum screening [6]. Therefore, it is neces-

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sary to develop a relatively simple assay such as an enzyme linked immuno-sorbent assay (ELISA) to measure RABV-specific antibodies. An ELISA is the preferred replacement for virus-neutralization assays because it does not require handling live RABV in a biosafety laboratory, and it is quick, simple to conduct, and requires only a small amount of serum [7]. An indirect ELISA (I-ELISA) has been developed using recombinant RABV nucleocapsid (N) protein expressed in *E. coli*, insect cells, and yeast [8–10]. A competitive ELISA (C-ELISA) using monoclonal antibodies (mAbs) against rabies has also been developed and evaluated for measuring rabies-neutralizing antibodies [7, 11].

Previously, we expressed recombinant RABV N protein using a baculovirus system and reported an I-ELISA with moderate sensitivity (88.1%) and high specificity (92.5%) compared with the FAVN test [12]. Therefore, we need an improved ELISA with high sensitivity and specificity that can be used to test serum collected from several animal species because raccoon dogs are also responsible for transmitting rabies in Korea. In this study, we developed and evaluated a blocking ELISA (B-ELISA) coated with the purified antibody and RABV for the detection of anti-RABV antibodies in animals.

Materials and Methods

Cells, viruses, and serum samples

Vero cells (African green monkey kidney cell line, ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with two antibiotics (100 IU/ml of penicillin and 10 μg/ml of streptomycin), one antifungal agent (0.25 μg/ml of amphotericin B), and 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, USA). The cells were used to propagate and titrate the RABV ERAGS strain, which has mutations at amino acids 194 and 333 in the glycoprotein of the ERA strain. BHK-21 cells (ATCC CCL-10) grown in DMEM and CVS11, a standard rabies strain, were used for the FAVN test. To optimize the B-ELISA, five positive and three negative RABV serum samples with virus neutralizing antibody (VNA) titers of 4.6–0.5 IU/ml and <0.06 IU/ml, respectively, were prepared from dogs. Serum samples were obtained from 138 dogs, 71 raccoon dogs, and 25 cats during development process of live and inactivated rabies vaccine and were subjected to the FAVN test and B-ELISA.

The FAVN test

The FAVN test was conducted in accordance with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE [13]. An OIE reference serum sample containing a VNA titer of 5.59 IU/ml was obtained from the OIE Reference Laboratory for Rabies (France), and serum diluted to 0.5 IU/ml was used as a positive control. The VNA titers against RABV were expressed in IU/ml with reference to those of the positive standard.

Propagation and purification of the ERAGS strain

Vero cells plated in 150-cm^2 tissue culture flasks were infected with the ERAGS strain at a multiplicity of infection of 0.1. After incubation at 37℃ for 96 h, the cells were frozen and thawed three times. The virus was harvested and titrated in 96-well microplates using 10-fold serial dilutions. After fixing and staining the cells with specific mAbs against RABV, the viral titers were calculated according to the method of Reed and Muench [14] and expressed as the 50% fluorescent assay infectious dose ($FAID_{50}$)/ml. The clarified antigen was first concentrated using Amicon® Ultra-15 centrifugal filter devices (Millipore, USA). Then, the concentrated RABV antigen was further concentrated by polyethylene glycol (PEG) precipitation, as described by Lewis and Metcalf [15]. Sodium chloride and PEG-8000 (Sigma, USA) were added to obtain final concentrations of 0.5 M and 0.8%, respectively. The mixture was placed at 4℃ for 10 h to precipitate RABV and then centrifuged at 10,000 ×*g* for 30 min. The supernatant was decanted and the centrifuged tubes left inverted until all of the supernatant was removed. The precipitate was resuspended in phosphate-buffered saline (PBS; pH 7.2) corresponding to 1% of the original volume. To eliminate any residual PEG, the second concentrated antigen was dialyzed with PBS (pH 7.2) overnight at 4℃. The RABV protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA).

Production and purification of mAbs against RABV

Four mAbs specific to RABV antibody were selected for use as capture or detection antibodies. Briefly, equal volumes of the purified RABV antigen and incomplete Freund's adjuvant were mixed and inoculated into

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BALB/c mice intraperitoneally three times. The spleens of the mice were collected aseptically 5 days after the final immunization, and the splenocytes were fused with SP2/0 mouse myeloma cells using PEG 1500 (Sigma). The resulting hybridoma cells were screened and cloned on hypoxanthine-aminopterin-thymidine (HAT) medium and confirmed with immunofluorescence assays. The four selected hybridoma cells were inoculated into mice intraperitoneally to generate ascites. The collected ascites fluid was subject to Protein G affinity chromatography (Bio-Rad, USA) and purified. The purified ascites fluid was quantified based on the absorbance at 280 nm. Four purified ascites samples were conjugated with horseradish peroxide (HRP), as described previously [16]. In brief, 16 mg of HRP were dissolved in 4 ml of distilled water and 0.8 ml of 0.1 M sodium periodate was added. Next, the mixture was incubated for 20 min. The activated HRP solution and ascites fluid were dialyzed with 1 mM sodium acetate (pH 4.0) and 20 mM carbonate, respectively. The HRP solution was blended with the ascites fluid at room temperature for 2 h. For return to a stable state, 4 mg/ml of sodium borohydride was added to the HRP conjugates that were dialyzed in PBS.

B-ELISA

First, 100 μl of purified 4G36 anti-rabies mAbs were diluted to 2 μg/ml in PBS (pH 9.6) and then coated onto a 96-well microplate (MaxiSorp; NUNC, Roskilde, Denmark) at room temperature for 16 h. Then, the solution was discarded and the microplate coated with mAbs was blocked with 5% skim milk. The same 96-well microplate was then coated with 100 μl of purified rabies antigen at a concentration of 0.92 μg/ml in carbonate buffer (pH 9.6) and incubated at 37℃ for 2 h. After incubation, the plate was washed with PBS containing 0.05% Tween 20 and dried. The plate was stored at 4℃ until use. Next, 100 μl of the serum samples (diluted to 1:5) were aliquoted into each well of the plate. The plate was covered with adhesive film and incubated at 37℃ for 1 h. After removing the adhesive film and rinsing the plate with PBS containing 0.05% Tween 20 three times, 100 μl of anti-rabies mAb-HRP conjugate were added to the plate and the plate was sealed with adhesive film and incubated for 1 h at 37℃. After washing the plate, 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to each well of the microplate, which was then incubated for 15 min at room temperature. Finally, 100 μl of 0.5 M sulfuric acid was added to stop the reaction. The absorbance at 450 nm was measured with an ELISA reader (Sunrise; Tecan, Basel, Switzerland). The percent inhibition (PI) was calculated as follows: PI = (1 – absorbance value of sample/absorbance value of negative sample) × 100. Serum samples were evaluated as positive if the PI was greater than 50. The specificity, sensitivity, and accuracy were calculated using the following formulas: sensitivity (%) = (number of positives in both tests/total number of positives in the reference test) × 100; specificity (%) = (number of negatives in both tests/total number of negatives in the reference test) × 100; and accuracy (%) = (number of both positives and negatives/total number of samples) × 100.

Results

Propagation and purification of the ERAGS strain

Vero cells infected with the ERAGS strain were frozen

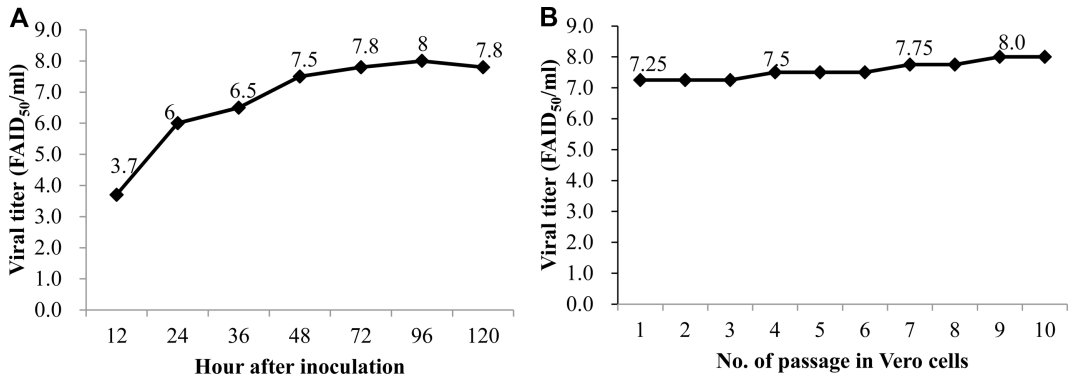

Fig. 1. Growth curve (A) for Vero cells infected with the ERAGS strain and viral titers according to the number of serial passages (B) in Vero cells. The ERAGS strain had the highest viral titer at 96 h post-inoculation and for over the 9th passage.

Table 1. Characterization of four mAbs against RABV.

Antibody designation	Protein specificity	Virus neutralizing antibody titer (IU/ml)*	Isotype	Chain
3E6	N	0.06	IgG2a	Kappa
4G36	G	41.6	IgG2a	Kappa
7G48	G	1.5	IgG2a	Kappa
B2H17	N	0.06	IgG1	Kappa

*The VNA titers in the hybridoma supernatants were measured with the FAVN test.

at 12, 24, 36, 48, 72, 96, and 120 h post-inoculation to determine the optimal harvest time and passage of the ERAGS strain. Each viral titer was determined in 96-well microplates. As shown in Fig. 1, the ERAGS strain propagated in Vero cells had the highest viral titer ($10^{8.0}$ $FAID_{50}$/ml) at 96 h post-inoculation and at the 9^{th} and 10^{th} passages. Therefore, the optimum harvest time of the ERAGS strain was 96 h post-inoculation, and the optimum passage number in Vero cells was ERAGS-10p.

Optimization of the B-ELISA

The ascites fluids produced in BALB/c mice inoculated with each of four hybridomas secreting specific RABV antibodies were characterized and then purified with a Protein A column. As shown in Table 1, two mAbs (3E6 and B2H17) that reacted with RABV N protein did not produce a VNA titer in the FAVN test, and the other two mAbs (4G36 and 7G48), which reacted with RABV G protein, had high and moderate VNA titers, respectively. The purified mAbs were applied to Vero cells infected with the ERAGS strain and their activities were identified using the FAVN test. As shown in Fig. 2, all of them showed specific fluorescence in Vero cells infected with the ERAGS strain. To select the optimal mAb for coating the 96-well microplates, microplates were coated with each of the four purified mAbs and their capture activity was evaluated. As shown in Fig. 3A, 4G36 had the highest negative to positive (N/P) ratio (N/P = 4.4) compared with the other three mAbs. Antigens were tested at three concentrations to establish the optimal coating concentration. As a result, antigen at 0.92 μg/ml had the highest N/P value (12.1; Fig. 3B). Serum samples diluted to 1:5, 1:10, and 1:20 were tested to find the optimal serum dilution rate. A serum dilution rate of 1:5 had the highest N/P value (28.4) and was selected as the serum dilution rate (Fig. 3C). The mAb to be used as the detector antibody was tested next; B2H17 had the highest N/P value (3.0) among the four mAbs.

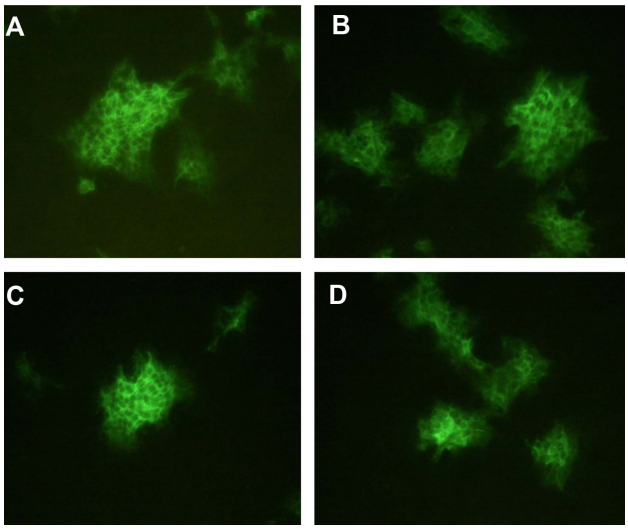

Fig. 2. Indirect fluorescent assay (IFA) to detect RABV antigens in cells. Vero cells infected with the ERAGS strain were stained with mAbs. 3E6 (A), 4G36 (B), 7G48 (C), and B2H17 (D) against RABV.

Application of the B-ELISA to standard reference and animal serum samples

The OIE reference dog serum was serially diluted to concentrations of 5.59 to 0.01 IU/ml. Three repeats of each dilution were tested, and high sensitivity was observed in the FAVN titer, ranging from 0.01 to 5.59 IU/ml (Fig. 4). An FAVN titer ≤ 0.69 had an absorbance value ≥ 0.40. The absorbance value with the B-ELISA was compared with the FAVN titers using 234 serum samples obtained from 138 dogs, 71 raccoon dogs, and 25 cats. The B-ELISA results were considered positive if the PI of the test serum was ≥ 50% and negative if it was < 50%. The sensitivity, specificity, and accuracy of the B-ELISA were calculated from the serum samples measured by the FAVN test. As shown in Fig. 5, serum samples showing < 0.5 IU/ml had a mean PI of 6.37 ± 19.41, and 172 (97.72%) out of 174 serum samples had a PI < 50. Serum samples with a VNA titer > 0.5 IU/ml had a mean PI of 82.29 ± 18.08, and 54 (93.1%) out of 58 serum samples had a PI > 50. Table 2 shows that the sensitivity, specificity, and accuracy of the B-ELISA

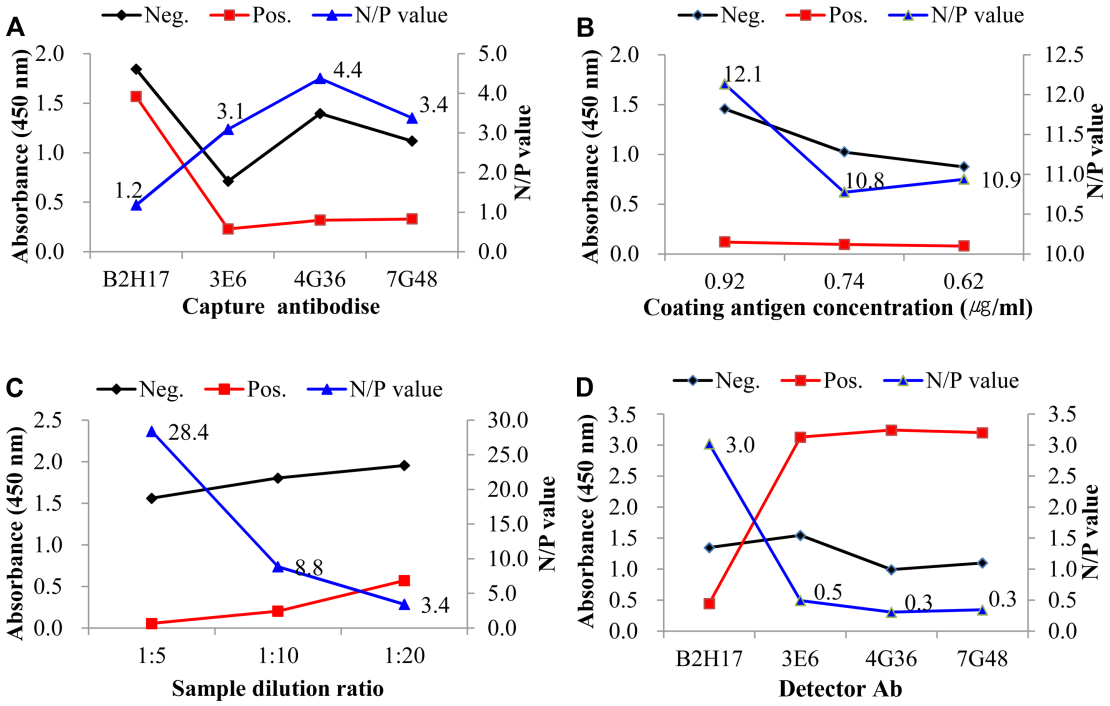

Fig. 3. Optimization of the B-ELISA for the detection of anti-RABV antibodies in serum. Based on the highest N/P value obtained in the B-ELISA, the optimal capture antibody (A), the concentration of coating antigen (B), sample dilution rate (C), and detector antibody (D) were selected.

with 138 dog serum samples were 95.8, 96.5, and 96.3%, respectively. The sensitivity, specificity, and accuracy of the B-ELISA using 71 raccoon dog serum samples were 91.3, 100, and 97.2%, respectively. The sensitivity, specificity and accuracy of the B-ELISA using 25 cat serum samples were 90.9, 100, and 96.0%, respectively.

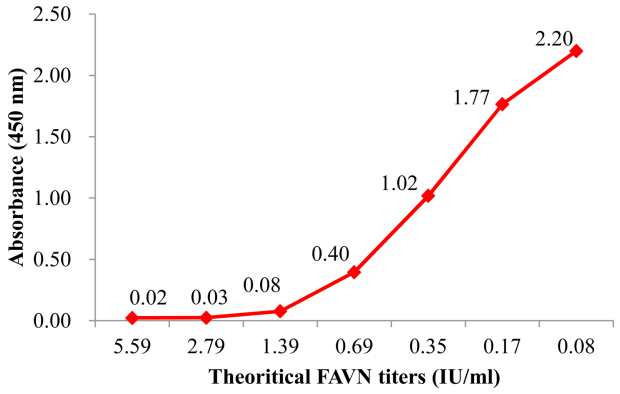

Fig. 4. Absorbances obtained with each OIE reference serum dilution in the B-ELISA. The theoretical FAVN titers are the expected titers for standard dog serum.

Discussion

The non-occurrence of animal rabies cases in Korea since 2014 supports the success of the national rabies control program, which was implemented with mass dog vaccinations, the distribution of a bait vaccine in high-risk regions, and sero-surveillance. However, the maintenance of a rabies non-occurrence status in Korea requires an adequate level of immunity within the carnivore and herbivore populations and a suitable surveillance system. Blocking the circulation of dog rabies requires more than 70% immunity within the dog population when the presence of neutralizing antibodies in serum indicates protective immunity [17]. At this time, the development of a B-ELISA for measuring RABV-specific antibodies in dogs, raccoon dogs, and cats is important.

Virus neutralization tests such as the MNT, FAVN test, and RFFIT for measuring anti-RABV antibodies following rabies vaccination or after ingesting a rabies bait vaccine require skilled technicians, rabies-specific antibodies, and specialized equipment such as fluores-

Table 2. The sensitivity, specificity, and accuracy of the B-ELISA for the detection of anti-RABV antibodies in comparison with the FAVN test.

	Species	No. of samples subjected to the FAVN test								
		Dogs			Raccoon dogs			Cats		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
B-ELISA	Positive	23	4	27	21	0	21	10	0	10
	Negative	1	110	111	2	48	50	1	14	15
	Sum	24	114	138	23	48	71	11	14	25
	Sensitivity*		95.8%			91.3%			90.9%	
	Specificity#		96.5%			100.0%			100.0%	
	Accuracy†		96.3%			97.2%			96.0%	

*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.

cence microscopes and polyclonal antibodies against RABV [3, 18]. Although the FAVN test is the reference test recommended by the OIE, many veterinary institutes prefer using ELISAs, which are easy to perform without specialized facilities. Therefore, we developed a B-ELISA with purified antigen and mAbs for measuring RABV-specific antibodies in immunized dogs, cats, and raccoon dogs that ingested a rabies bait vaccine.

Several ELISAs have been developed for measuring anti-RABV antibodies in animals. An I-ELISA developed using purified recombinant RABV N protein does not measure neutralizing antibodies and cannot be applied to raccoon dogs that have ingested a rabies bait vaccine containing recombinant vaccinia virus expressing RABV glycoprotein. This problem can be overcome using a B-ELISA, which allows serum samples to react with the captured virus coated on a solid phase. In the B-ELISA, B2H17 mAbs conjugated with peroxidase react with coated virus that is not blocked by RABV antibodies in serum. When the substrate TMB is added to the B-ELISA plate, a decrease in color intensity indicates the presence of anti-RABV antibodies in the test serum. Methodologically, a B-ELISA is similar to a C-ELISA. However, the two differ in that a B-ELISA allows serum samples to compete with a specific indicator antibody. Considering the situation in Korea, where

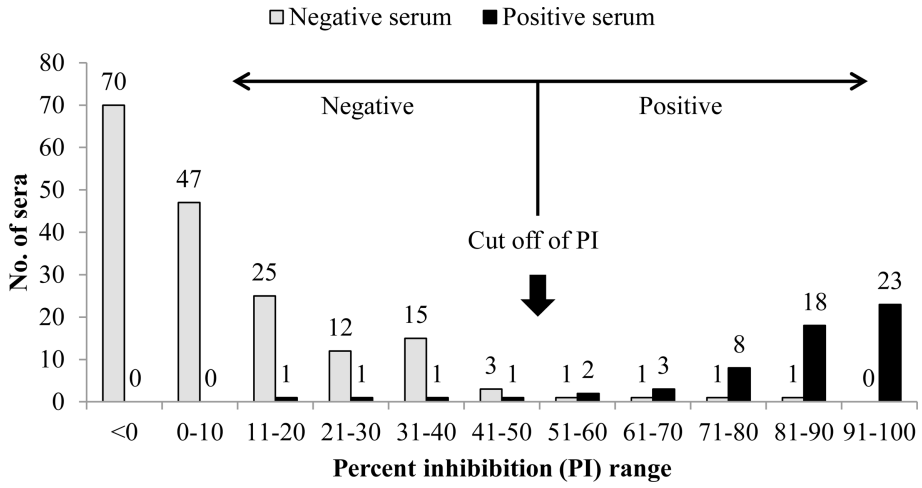

Fig. 5. Distribution of the PI values for 58 positive serum samples and 176 negative serum samples obtained by the B-ELISA. The arrow indicates the cutoff value of 50 in the B-ELISA. Of the serum samples, 54 of 58 (93.1%) positive serum samples had a PI > 50 and 172 of 176 (97.72%) negative serum samples had a PI < 50.

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a bait vaccine (V-RG) has been distributed since 2000 [1, 2], it is important to develop a tool for the sero-surveillance of raccoon dogs.

The sensitivity, specificity, and accuracy of the B-ELISA compared with the results of the FAVN test were determined using serum samples from 234 animals. The sensitivity (90.9–95.8%) of the B-ELISA for dog, raccoon dog, and cat serum compared with the FAVN test were significantly higher than those of the Platelia Rabies II Kit (80.4–88.9%) [19]. The B-ELISA was more sensitive than the I-ELISA (88.1%) based on recombinant RVN [12]. The specificity (96.5–100%) of the B-ELISA was similar to that of the Platelia Rabies II Kit (98.2–99.2%) using 0.5 IU/ml as a cutoff [19]. The specificity of an ELISA can be enhanced by the availability of capture antibody [20]. In our study, we used the mAb 4G36, which recognized the RABV glycoprotein as the capturing RABV antigen; this enhanced the sensitivity and specificity of the B-ELISA. Moreover, the B-ELISA showed high accuracy (96.0–97.2%) in three animal species, suggesting that it is suitable for the sero-surveillance of RABV in dogs, raccoon dogs, and cats. Applied studies of the B-ELISA in cattle are needed in the near future to assess sero-monitoring of cattle after rabies vaccination.

In conclusion, we found that the B-ELISA had high sensitivity, specificity, and accuracy compared with the FAVN test for the detection of anti-RABV antibodies in dog, cat, and raccoon dog serum samples, indicating that this new B-ELISA can be used to check the immune status of vaccinated animals and for serological surveys of the raccoon dog population in high-risk regions.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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