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Development of indirect ELISA for the detection of canine adenovirus type 2 antibodies in dog sera

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ABSTRACT

Background: Canine adenovirus type 2 (CAV-2) induces infectious laryngotracheitis in members of the family Canidae, including dogs. To date, no ELISA kits specific for CAV-2 antibody have been commercialized for dogs in Korea.
Objectives: We aimed to develop new indirect enzyme-linked immunosorbent assay (I-ELISA) to perform rapid, accurate serological surveys of CAV-2 in dog serum samples.
Methods: In total, 165 serum samples were collected from dogs residing in Chungbuk and Gyeongbuk provinces between 2016 and 2018. The Korean CAV-2, named the APQA1701-40P strain, was propagated in Madin–Darby canine kidney cells and purified in an anion-exchange chromatography column for use as an antigen for I-ELISA. The virus-neutralizing antibody titers of CAV-2 in the dog sera were measured by virus neutralization (VN) test.
Results: We compared the results obtained between the VN and new I-ELISA tests. The sensitivity, specificity, and accuracy of new I-ELISA were 98.6%, 86.4% and 97.0% compared with VN test, respectively. New I-ELISA was significantly correlated with VN ($r = 0.91$).
Conclusions: These results indicate that new I-ELISA is useful for sero-surveillance of CAV-2 in dog serum.

Keywords: Canine adenovirus type 2; antibody; ELISA

INTRODUCTION

Canine adenoviruses infecting a wide range of species are divided into canine adenovirus type 1 (CAV-1) and 2 (CAV-2) based on the molecular biological characterizations. The CAV-1 and 2 belong to the same genus *Mastadenovirus* in the family *Adenoviridae*. Their genomes contain a double-stranded DNA that is characterized by an inverted terminal repeat (ITR) ranging from 35 to 200 bp in size. Since CAV-1 and 2 infections in dogs were first reported in 1954 and 1975, respectively [1], CAV-2 has been reported in many countries, including Turkey, Austria, Japan, and Korea [2-5]. CAV-2 has an affinity for the respiratory tract epithelium and causes infectious laryngotracheitis in dogs, raccoon dogs, foxes, and wolves. The most common symptoms in CAV-2-infected animals are coughing accompanied by a fever, runny nose, or red watery eyes. CAV-2 can even be life-threatening in vulnerable dogs; therefore, it is important to monitor the immune status of the dog population and implement appropriate measures, such as booster immunizations.

Author Contributions

Conceptualization: Yang DK; Funding acquisition: Hyun BH; Methodology: Lee S, Oh D, Yoo JY; Project administration: Yang DK, Hyun BH; Validation: Kim HH; Writing - original draft: Yang DK; Writing - review & editing: Yang DK, Kim HH.

There are several methods for detecting canine adenovirus (CAV) antigen, including virus isolation, polymerase chain reaction, histopathology, rapid immunochromatographic strip assay and immunohistochemistry [6-9]. CAV antibodies induced by inoculation of vaccine or natural infection can also be measured by several methods, such as hemagglutination inhibition (HI), virus neutralization (VN), enzyme-linked immunosorbent assay (ELISA), and the indirect immunofluorescence assay in animal serum samples [10,11]. HI is a serological method for detecting CAV-2 antibodies. However, the complicated test procedures and need to use human O type erythrocytes have limited the use of HI test in the laboratory. The VN test is used frequently to detect CAV-2 antibodies induced by vaccination in dog serum samples. Although VN test is accurate and useful for detecting CAV-2 antibodies, the method takes 4 to 5 days and is not suitable for large-scale sero-surveillance [11]. Previously, we reported a sero-survey of CAV-2 conducted with the VN test in various animal species [7]. However, ELISA is the preferred method for detecting CAV-2 antibodies in large-scale investigations because it does not require live CAV-2 and only a small amount of serum is needed; the procedure is also relatively simpler and safer than that of the VN test. A few ELISA kits specific for CAV-2 antibody have been commercialized for dogs, but these kits are expensive with small quantity production. The use of purified antigen when developing an ELISA is important. Viral purification has relied on traditional methods, such as sucrose gradient or cesium chloride (CsCl) centrifugation after concentration with polyethylene glycol (PEG) 8,000 or zinc acetate dihydrate (Zn $[CH_3CO_2]_2$). Recently, a chromatographic method for purifying adenovirus was reported [12]. Based on the report, we developed an indirect ELISA (I-ELISA) coated with CAV-1 antigen that was purified with chromatography column [13]. In this study, we focused on Korean CAV-2 strain and purified CAV-2 antigen using a currently commercialized Nuvia cPrime column. Using the purified antigen, we developed a new I-ELISA to detect CAV-2 antibodies in dog serum.

MATERIALS AND METHODS

Cells, virus and serum samples

Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, USA) containing 5% (v/v) fetal bovine serum (FBS; Gibco BRL, USA), penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) was used to maintain Madin–Darby canine kidney (MDCK) cells (CCL34; ATCC, USA) at 37°C in 5% (v/v) CO_2 incubator. The APQA1701-40P strain of CAV-2, which had undergone 40 serial passages in MDCK cell culture, was used as the antigen for I-ELISA and the VN test [7]. Bloods from 165 dogs residing in Chungbuk and Gyeongbuk provinces between 2016 and 2018 were collected and their sera were subjected to the VN and I-ELISA tests. It was not known whether the dogs had been immunized with dog vaccines, including for CAV. Dogs did not exhibit any symptoms related to CAV-2. Eight sera used for the optimization of I-ELIA were obtained from previous research project [5,7].

Growth kinetics

The growth kinetics of the APQA1701-40P strain were examined to determine the optimal harvest date. To assess the growth kinetics of the APQA1701-40P strain, MDCK cells grown in 25 cm^2 cell culture flasks were inoculated with virus containing 100 50% tissue culture infectious dose ($TCID_{50}$)/mL and harvested daily for 7 days. After performing three consecutive freeze–thaw cycles, 10-fold serial dilutions of each virus were titrated in 96-well microplates, and the cytopathic effects (CPEs) were observed under a microscope every day for 6 days post inoculation (DPI). The viral titers of the APQA1701-40P strain were

determined in accordance with the standard method of Reed and Muench [14], and expressed as the $TCID_{50}$/mL.

Virus neutralization test

VN test was performed in 96-well microplates using MDCK cells. Each serum sample, together with positive and negative controls, was evaluated in duplicate and serial two-fold dilutions. APQA1701-40P strain containing 100 $TCID_{50}$/50 µL was added to each well of the plate. After incubation at 37°C for 1 h, 0.1 mL MDCK cell suspension containing 2.0 × 10^5 cells/mL was added to each well. The 96-well microplates were put in a humidified incubator under 5% (v/v) CO_2 at 37°C, and each well was checked for CPEs for 4–5 DPI. The virus-neutralizing antibody (VNA) titer was the reciprocal of the highest serum dilution that completely inhibited the CPEs. All dog serum samples used in the VN test were diluted from 1:2 to 1:512. A VNA titer ≥ 1:2 was considered positive [7,15]. Eight sera collected from dogs were confirmed by VN test and used as positive and negative sera in optimization of I-ELISA.

Preparation of CAV-2 antigen

MDCK cells grown in roller bottles were inoculated with the APQA1701-40P strain without FBS, and were processed by three consecutive freeze–thaw cycles after checking the CPEs of the MDCK cells. The cell-associated and non-cell associated virus was harvested and filtered through a membrane filter (0.2 µm pore size). A peristaltic pump was used to drive the buffer and viral antigen. The clarified antigen was applied to a Nuvia cPrime column (Bio-Rad, USA), which is a hydrophobic cation exchange medium with an aromatic hydrophobic ring and a COO^- functional group. Before using the Nuvia cPrime column, we equilibrated it with 10 column volumes (CV) of 25 mM histidine (buffer A, pH 6.0). The mixture obtained by diluting the purified APQA1701-40P antigen with buffer A in a ratio of 1:4 was loaded onto the column, followed by 10 CV of buffer A for washing. The elution buffer (75 mM Tris, 525 mM NaCl, pH 8.5) was used for eluting APQA1701-40P antigen. The total virus protein concentration in elution fractions was measured by a NanoDrop 1,000 UV/Vis spectrophotometer (Thermo Fisher Scientific, USA). The elution fraction identified as the highest concentration in the spectrophotometer was subjected to electron microscope observation. A Formvar-coated grid was deposited on a drop of eluted antigen to allow absorption of viral particles for 2 min at room temperature. For negative staining, the grid was placed on top of a drop of 1% uranyl acetate for 1 min and dried for 1 h before examination. The viral particles of APQA1701-40P strain were observed using a Hitachi 7100 transmission electron microscope (Hitachi, Japan).

Establishment and application of I-ELISA

Optimal antigen coating concentrations and dilutions of serum were determined using a checkerboard titration test, as described previously [15]. The CAV-2 antigens ranging from 50 to 0.02 µg/mL, and eight serum panel samples at dilutions of 1:20 to 40,960 were used to determine the optimal concentrations for I-ELISA. The absorbance of the I-ELISA was measured at 405 nm in a spectrophotometer (Sunrise ELISA reader; Tecan, Switzerland). Under the newly established I-ELISA conditions, 100 µL of serum diluted 100-fold in dilution buffer (2% skim milk in phosphate-buffered saline [PBS]) was added to a 96-well microplate (MaxiSorp; Nunc, Denmark) coated with CAV-2 antigen. After incubation at 37°C for 1 h, the plate was washed with PBS containing 0.05% Tween 20 (PBST) 3 times and incubated with 100 µL anti-dog immunoglobulin G horse radish peroxidase conjugate (KPL, USA) for 1 h at 37°C. After washing, the 2′2-azino-bis-(3-ethylbenzothiazoline) substrate solution and 1.0% sodium dodecyl sulfate as a stop solution were added to all wells of the microplate according

to the manufacturer's indications. Serum samples were confirmed positive if the absorbance was higher than the cutoff value (0.4) based on the results of a test to determine the antigen and serum dilution factors. The specificity, sensitivity, and accuracy were calculated using previously reported formulas [16].

Statistical analysis

Linear regression analysis (least-squares method) was used to determine the correlation coefficient (*r* value) between the absorbance of the I-ELISA and the VNA titer. The *r* value was calculated using Microsoft Excel 2010 software (Microsoft Corp, USA).

RESULTS

Virus production

The optimal harvest time to obtain the highest CAV-2 titer is based on the growth kinetics. The highest mean viral titer ($10^{7.13}$ $TCID_{50}$/mL) was found in the virus harvested at 4 DPI (**Fig. 1A**). At 3 DPI, almost all MDCK cells infected with the APQA1701-40P strain revealed CPEs, such as rounding and detachment from the surface of the flask (**Fig. 1B**). Based on the APQA1701-40P growth kinetics, the Korean CAV-2 strain in roller bottle was harvested at 4 DPI and further purified.

Determination of the CAV-2 antibody titer

Two antibody detection assays were used to detect CAV-2 antibodies in 165 dog serum samples. **Table 1** shows that 143 were confirmed positive for VNA, with titers showing ≥ 1:2, while 144 serum samples were positive on new I-ELISA, with absorbances > 0.4. In both VN and new I-ELISA tests, there were 22 and 21 CAV-2-negative serum samples, respectively.

Purification of CAV-2 with Nuvia cPrime columns

CAV-2 antigen of APQA1701-40P strain for new I-ELISA was purified in a Nuvia cPrime column; the highest protein concentration (1.5 mg/mL) was found in the fifth elution fraction, as determined using a NanoDrop spectrometer (**Fig. 2A**). The fifth elution was subjected to electron microscopy, which showed intact icosahedral viral particles (**Fig. 2B**).
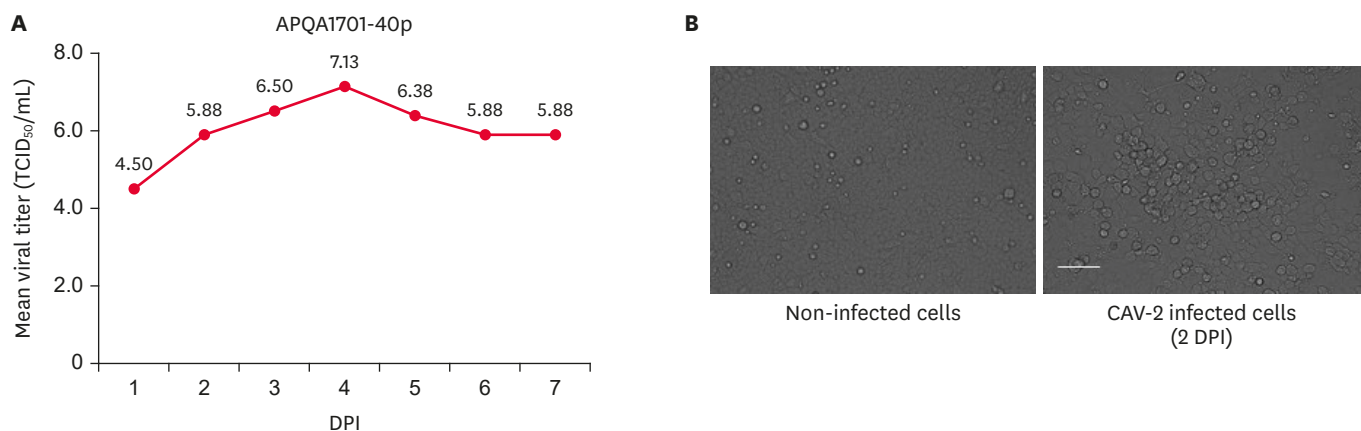

Fig. 1. The optimal harvest time was determined by measuring the APQA1701-40P growth kinetics (A) The titer of APQA1701-40P was measured two times and expressed as mean viral titer. The CPEs caused by CAV-2 were observed at 2 DPI (B). Scale bar indicates 100 μm.
CPE, cytopathic effect; CAV-2, canine adenovirus type 2; DPI, days post inoculation; $TCID_{50}$, 50% tissue culture infectious dose.

Table 1. The sensitivity, specificity, and accuracy of I-ELISA for the detection of CAV-2 antibodies in comparison with the VN test

Serological test	VN test		
	Positive	Negative	Sum
I-ELISA			
Positive	141	3	144
Negative	2	19	21
Sum	143	22	165
Sensitivity[a]		98.6%	
Specificity[b]		86.4%	
Accuracy[c]		97.0%	

I-ELISA, indirect enzyme-linked immunosorbent assay; CAV-2, canine adenovirus type 2; VN, virus neutralization.
[a]Sensitivity (%) = (number of positive results in both tests/total number of positive results in the reference test) × 100, [b]Specificity (%) = (number of negative results in both tests/total number of negative results in the reference test) × 100, [c]Accuracy (%) = (actual number of both positive and negative results/total number of samples) × 100.
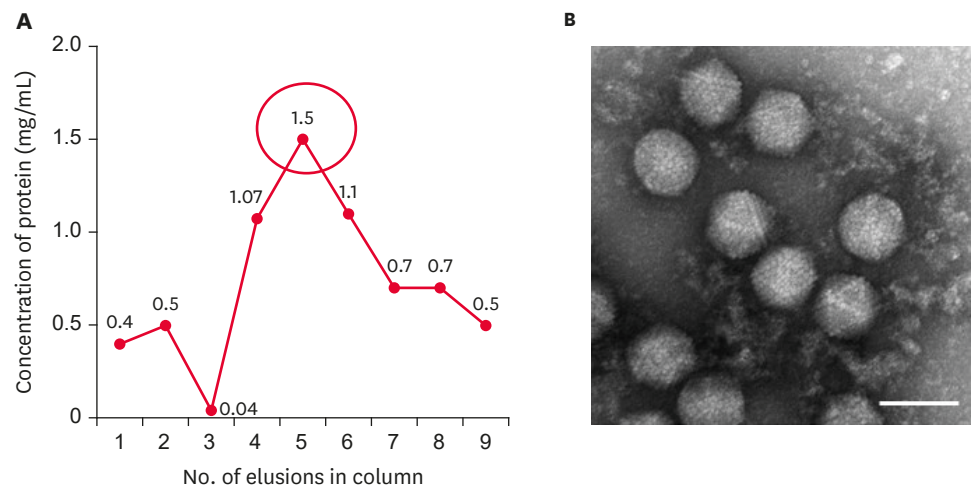
Fig. 2. Concentration of protein in the solutions eluted from the Nuvia cPrime column (A) and identification of canine adenovirus type 2 in the fifth elute by electron microscopy (B). Scale bar indicates 100 nm.

Optimization of I-ELISA

The CAV-2 antigens and dog serum samples were diluted to determine the optimal antigen concentration (10 μg/mL) and serum dilution (1:100) as shown in **Fig. 3A and B**. The optimal blocking buffer and conjugate concentration for I-ELISA were 5% skim milk in PBST and 10 ng/mL, respectively. We set an absorbance value of 0.4 as the cutoff value to distinguish positive from negative in serum samples. Therefore, serum samples showing an absorbance value > 0.4 on I-ELISA were considered positive.

Application of I-ELISA to dog sera

The absorbance value obtained from I-ELISA using the 165 dog serum samples was compared with the VNA titers determined in the VN test to evaluate the diagnostic reliability. The correlation between I-ELISA and VN is indicated by the regression lines and correlation coefficients (*r*) in **Fig. 4**. The *r* value for the VN test was 0.91. The diagnostic sensitivity, specificity, and accuracy of new I-ELISA were calculated from the CAV-2-positive/negative sera measured with the standard diagnostic assay, i.e., the VN test. As shown in **Table 1**, the sensitivity, specificity, and accuracy of I-ELISA turned out 98.6, 86.4, and 97.0%, respectively, compared with the results of the VN test.
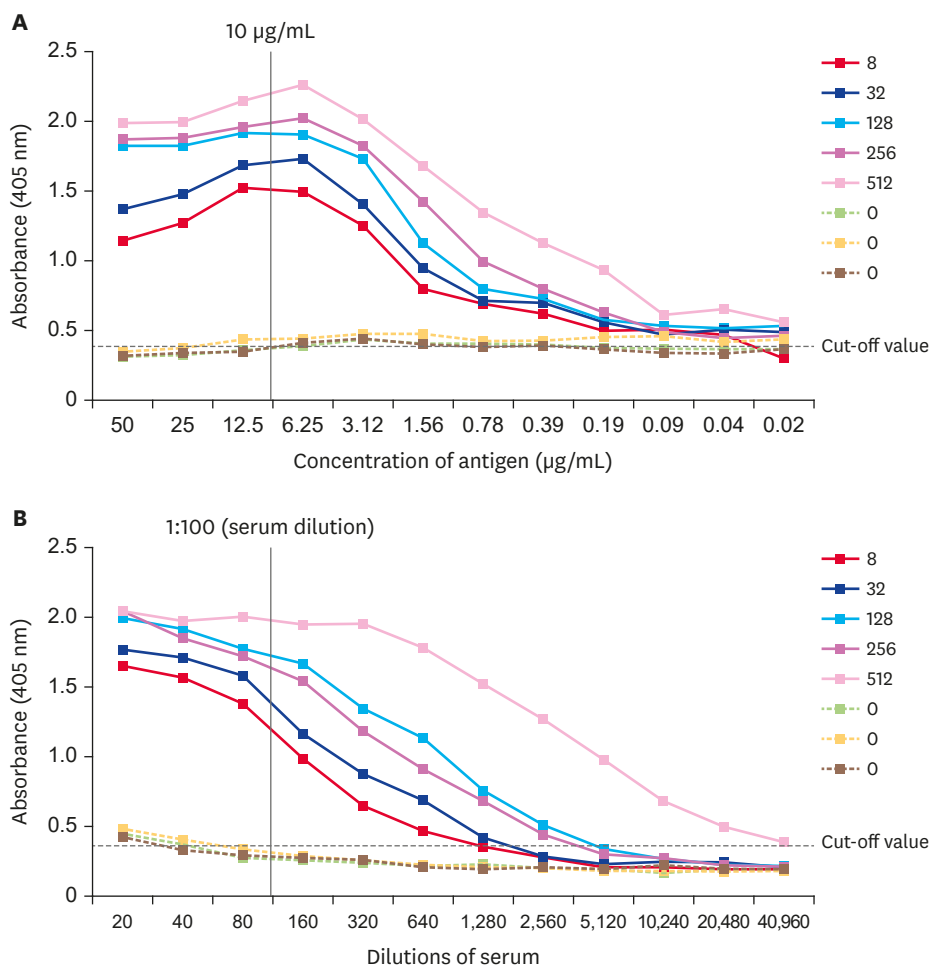
Fig. 3. Determination of the concentration of the purified CAV-2 antigen (A) and serum dilution (B) by I-ELISA. The concentrations of antigen and dilutions of serum were analyzed according to cut-off values. The numbers in the legend are the VNA titers (0–512) of CAV-2 in dog serum.
CAV-2, canine adenovirus type 2; I-ELISA, indirect enzyme-linked immunosorbent assay; VNA, virus-neutralizing antibody.
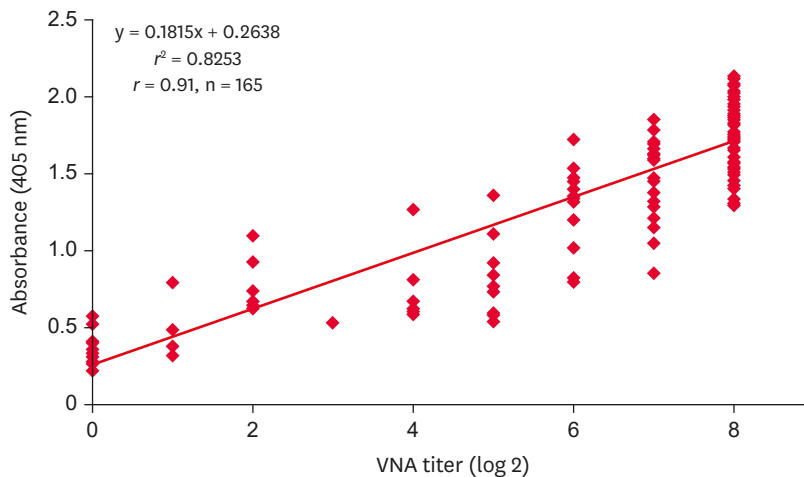
Fig. 4. Correlation between the VNA titer and absorbance of I-ELISA for detecting CAV-2 antibodies in 165 dog serum samples. The correlation is indicated by the linear regression line and r value (0.91).
VNA, virus-neutralizing antibody; I-ELISA, indirect enzyme-linked immunosorbent assay; CAV-2, canine adenovirus type 2.

DISCUSSION

Serological survey of CAV-2 has been used to evaluate the immune status of animal's post vaccination, and to investigate the exposure of naturally infected and recovered animals. Although the VN test has been used as a standard method, many researchers prefer a rather convenient method such as ELISA, because it is suitable for large numbers of serum samples and needs only small amounts of animal serum. In addition, the ELISA procedure is relatively simple and thus less time consuming. In previous study, we demonstrated that 88.5% of Korean dogs have CAV-2 antibodies in VN test [7]. These reasons encouraged us to develop new I-ELISA using CAV-2 antigen for sero-surveillance of CAV-2 in dogs.

Three reagents such as PEG 8,000, ammonium sulfate ($[NH_4]_2SO_4$), and zinc acetate dihydrate have been generally used for concentrating whole-virus antigen [17,18]. CsCl gradient centrifugation is the most commonly chosen method for purifying infectious adenovirus [19]. As the existing methods collect the antigen by centrifugation, they may be difficult to apply to concentrate or purify large volumes of bulk containing virus. The methods mentioned above are a bit complicated and require an ultracentrifuge. One of the most important factors for achieving high sensitivity and specificity of I-ELISA is highly purified viral antigen [15]. Therefore, we introduced a chromatographic method for purifying CAV-2 antigens for new I-ELISA. The most frequently used chromatographic methods for adenovirus are anion-exchange and size-exclusion chromatography, which take advantage of the strong negative charge and large size of adenovirus capsids [12,20-22]. We purified the entire CAV-2 antigen in the currently commercialized Nuvia cPrime column. This method results in high protein concentrations and allows for the identification of distinct viral particles using electron microscopy in the fifth solution eluted. Therefore, this simple process may be extended to purify other adenoviruses obtained from various animals such as chicken, horse and cattle. The disadvantage of the Nuvia cPrime column is that it combines with FBS protein present in the growth medium [21]. So, we propagated APQA1701-40P in DMEM without FBS.

The sensitivity, specificity, and accuracy of the I-ELISA, and their correlations with results obtained in the VN test, were determined using 165 dog serum samples. The overall accuracy of I-ELISA compared with VN test (97.0%) was similar to the 98% obtained by Noon et al. [10]. In our study, the sensitivity (98.6%) of new I-ELISA used to detect CAV-2 antibody was significantly higher than the specificity (86.4%). There were two and three differences, respectively, between the 143 positive and 22 negative samples in the two test methods. Three of 22 VN-negative samples were missed by I-ELISA (13.6% false-positive rate). In principle, two methods are different for antibody measurement. In other words, I-ELISA relies on antibody binding, while VN test evaluates antibodies to neutralize viral infection. These false positive reactions may be caused by several factors such as interactions between immunoglobulins in serum and coated antigen [23]. So, a new blocking buffer, highly purified antigen and new serum dilution buffer may contribute to reducing false positive reactions, which can be considered non-specific reactions. In addition, our result related to correlation showed that the absorbance values of the I-ELISA were significantly correlated with VNA titers of the VN test ($r = 0.91$), indicating a close relationship between I-ELISA and the VN test. As the newly developed I-ELISA is as reliable as the VN test, the related technologies can be transferred to companies that want industrialization of I-ELISA. Further studies on new type of I-ELISA using CAV-1 and 2 antigens are required to differentiate CAV-1 and CAV-2 antibodies in dog serum.

In conclusion, we demonstrated that the results of new I-ELISA using APQA1701-40P strain purified in Nuvia cPrime column correlate significantly with those of the VN test in terms of the rate of detection of CAV-2 antibodies in dog serum samples. Thus, this new I-ELISA assay can be used as a simple and efficient tool for examining many serum samples at once. Further study on assay of the intra-assay and inter assay coefficient of variation (CV) is required in order to be industrialized.

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