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Original Article

Use of hydrophilic extra-viral domain of canine distemper virus H protein for enzyme-linked immunosorbent assay development

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Simple methods for measuring the levels of serum antibody against canine distemper virus (CDV) would assist in the effective vaccination of dogs. To develop an enzyme-linked immunosorbent assay (ELISA) specific for CDV, we expressed hydrophilic extra-viral domain (HEVD) protein of the A75/17-CDV H gene in a pET 28a plasmid-based *Escherichia* (*E.*) *coli* vector system. Expression was confirmed by dot and Western blotting. We proposed that detection of *E. coli*-expressed H protein might be conformation-dependent because intensities of the reactions observed with these two methods varied. The H gene HEVD protein was further purified and used as an antigen for an ELISA. Samples from dogs with undetectable to high anti-CDV antibody titers were analyzed using this HEVD-specific ELISA and a commercial CDV antibody detection kit (ImmunoComb). Levels of HEVD antigenicity measured with the assays and immunochromatography correlated. These data indicated that the HEDV protein may be used as antigen to develop techniques for detecting antibodies against CDV.

Keywords: canine distemper virus, enzyme-linked immunosorbent assay, H gene, hydrophilic extra-viral domain, immunochromatography

Introduction

Canine distemper virus (CDV) outbreaks in dogs have been dramatically reduced by vaccination using readily available modified live vaccines. CDV is a non-segmented single-stranded RNA paramyxovirus consisting of 15,690 nucleotides surrounded by an envelope [14]. The CDV envelope consists of a membrane-associated (M) protein incorporated with haemagglutinin (H) and fusion (F) proteins [9]. The H protein enables access of the viral ribonucleoprotein through the cellular membrane and is the major determinant of fusion efficiency, growth characteristics, and tropism of CDV [8,16]. An adequate host response against H protein is vital for protection against CDV infection via vaccination. The H gene sequence of the CDV-A75/17 strain, which is considered the prototype neurotrophic-virulent CDV [13], may have more than 90% sequence homology with the sequences of strains isolated in Asia.

A number of immunodiagnostic techniques have been developed for detecting CDV infection in dogs including enzyme-linked immunosorbent assays (ELISAs) specific for the nucleocapsid protein or complete viral antigen [15,17] and immunofluorescence assays using co-cultured CDV antigens [17]. Here, we describe the development of a novel ELISA using an *Escherichia* (*E.*) *coli*-expressed H gene antigen. This simple and effective ELISA may help facilitate the development of more efficient vaccine strategies such as ones designed to avoid maternal antibody interference in pups.

Materials and Methods

Cloning of the A75/17-CDV H gene and expression in *E. coli*

The H gene segment of the A75/17-CDV strain was isolated and inserted into the eukaryotic expression vector

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pCI (Promega, USA) as previously described [12], and provided to our laboratory by Dr. Andreas Zurbriggen (University of Bern, Switzerland). We designed three constructs containing the entire H protein (amino acids 1-607), extra-viral domain of the H protein (HEVD: amino acids 59-607), and an HΔ200 segment (amino acids 201-607). Primers for producing these three constructs are described in Table 1. Each insert of the H gene segment was amplified by PCR and cloned into Novagen pET 28a His-tag positive vector (Merck Millipore, USA) for expression in *E. coli* (Stratagene, USA). The cloned vectors were sequenced and used to transform BL21-CodonPlus (DE3)-Recompetent cells (Stratagene). Selected colonies were used to inoculate 10 mL Luria broth (Sigma, USA) with 500 μg kanamycin (final concentration 50 μg/mL; Sigma), expression of the exogenous proteins was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma) for 4 h, and the cells were harvested.

To determine whether the expressed proteins were insoluble or not, we performed a solubility test. Harvested BL21 cells were resuspended in 500 μL Tris-EDTA buffer, lysed by sonication, and centrifuged. SDS-PAGE was performed to separate proteins from both the pellet and supernatant.

Harvested BL21 cells were centrifuged at 6,000 × g for 30 min at 4°C, and the pellets were resuspended in suspension buffer (50 mM Tris-HCl and 25% sucrose, pH 7.8). Lysozyme (Sigma) was added at a final concentration of 0.2 mg/mL, and the suspension was stirred for 30 min at 4°C. $MgCl_2$ (final concentration of 5 mM; Sigma) and DNase I (final concentration of 50 μg/mL; Sigma) were then added. After stirring for an additional 60 min at 4°C, the solution was centrifuged at 13,000 × g for 15 min at 4°C. The pellet was resuspended in a wash solution containing 2% deoxycholic acid and 1 mM EDTA at a concentration of 20 mL/g of inclusion body and centrifuged twice at 13,000 × mL/g for 15 min at 4°C. This pellet was resuspended in a new wash solution composed of 1% Triton X-100 in PBS and centrifuged twice at 13,000 × g for 15 min at 4°C. The resulting pellet was resuspended in a new wash solution of 20 nM Tris-HCl (pH 7.8) and centrifuged three times at 13,000 × g for 15 min at 4°C. The precipitate was incubated at 4°C overnight with an unfolding reagent containing 6 M guanidinium-HCl (Sigma), 0.5 M NaCl, and 20 mM sodium phosphate (pH 7.8).

A commercial Ni-NTA column designed to remove His-tagged proteins (Qiagen, The Netherlands) was equilibrated with a denaturing binding buffer containing 8 M urea, 20 mM sodium phosphate, and 0.5 M NaCl (pH 7.8). The unfolded inclusion body material was loaded onto the column according to the manufacturer's instructions. The column was washed with a denaturing washing buffer composed of 8 M urea, 20 mM sodium phosphate, and 0.5 M NaCl (pH 6.0), and subsequently washed with a native wash buffer consisting of 50 mM sodium phosphate, 0.5 M NaCl, and 50 mM imidazole (pH 8.0). Finally, the extracted CDV proteins were eluted with an elution buffer containing 50 mM sodium phosphate, 0.5 M NaCl, and 250 mM imidazole (pH 8.0).

Table 1. Oligonucleotide primers used to amplify three H gene segments of CDV

Primer	H gene sequence
Entire H1	5′-GGGTCGACATATGCTCTCCTACCAA-3′
Entire H2	5′-GGGCGGCCGCAGGTTTTGAACGGTT-3′
HEVD1	5′-GGGTCGACATCGATTTCACCAAGTA-3′
HEVD2	5′-GGGCGGCCGCAGGTTTTGAACGGTT-3′
HΔ200 1	5′-GGGTCGACATCTATCAGTATCATTG-3′
HΔ200 2	5′-GGGCGGCCGCAGGTTTTGAACGGTT-3′

Western blot and dot blot analyses

The eluted HEVD and HΔ200 proteins were separated on sodium dodecylsulfate (SDS)-8% polyacrylamide gels under denaturing conditions. The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, USA) by electroblotting, and the membranes were incubated in TBS-Tween consisting of 25 mM Tris-HCl, 137 mM NaCl, 3 mM KCl, and 0.1% Tween 20 (pH 7.5) with 5% (v/v) skim milk (Sigma). After one 20-min wash and two additional 5-min washes, the membranes were incubated for 60 min at 20°C with a monoclonal anti-His6 antibody (Roche, USA) diluted 1 : 2,000 in 1% bovine serum albumin (BSA; Sigma) and PBS. A similar Western blotting assay was also performed using goat anti-CDV polyclonal antibody (VMRD, USA) diluted 1 : 1,000 with 1% BSA in PBS. Antibody binding was detected by incubation with rabbit anti-goat-peroxidase conjugate (1 : 2,000; Pierce Biotechnology, USA) and peroxidase substrate (Pierce Biotechnology).

Independent samples of HEVD and HΔ200 (0.5 μg each) were also dot blotted onto single nylon membrane segments (Roche) using routine methods. The anti-CDV polyclonal antibody and anti-goat-horseradish peroxidase (HRP) antibody were used as the primary and secondary antibodies, respectively. Independent dot blots were also incubated with anti-His6-peroxidase antibody.

Preparation of serum samples and dot-ELISA with ImmunoComb

Sera samples for ELISA were obtained from 12 dogs participating in a CDV challenge experiment as described elsewhere [7]. In addition, 32 other sera samples were obtained from animal clinics in Seoul (Korea) to evaluate the clinical use of a one-step immunochromatography technique. Anti-CDV antibody titers of all sera were

determined by dot-ELISA with a canine distemper ImmunoComb IgG antibody test kit (Biogal, Galed Laboratories, Israel) according to the manufacturer's instructions. This commercial kit should be stored with normal refrigeration (2～8°C). Before conducting the test, all kit elements and sera were brought to room temperature. This is a semi-quantitative procedure based on color comparison between a standard and test sample with the result usually expressed on a scale of 0 (S0) to 6 (S6). After the ImmunoComb test, 12 sera samples were stored at −20°C; 32 were inactivated by incubation at 56°C for 30 min and then stored at −20°C until the virus neutralization test was performed.

Serum neutralization (SN) test

SN test was conducted according to the method described by previous study [1]. Onderstepoort-CDV was propagated in monolayer cultures of Vero cells (African green monkey kidney cells). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 7% fetal calf serum (FCS; Sigma) and antibiotics at 37°C in an atmosphere of 5% CO_2. To screen for anti-CDV antibodies, two-fold dilutions of serum were added to the working virus solution containing 100 units of 50% tissue culture infectious dose ($TCID_{50}$) of CDV and incubated at 37°C for 1 h. Thereafter, an aliquot of each solution was added to duplicate microtiter wells containing subconfluented monolayers of Vero cells. Each plate was incubated at 37°C for 1 h and washed twice with FCS-free DMEM before DMEM containing 7% FCS was added. The plates were subsequently incubated for up to 4 days. After examining the cytopathogenic effect in each serum dilution, serum dilutions at which at least 50% of 100 $TCID_{50}$ virus infectivity was determined.

ELISA

The HEVD and HΔ200 antigens were each diluted 1 : 100 in carbonate buffer (pH 9.6) and used to coat the solid-phase of ELISA plates (0.5 μg/well) overnight. The coated wells were washed three times with wash buffer (0.05% Tween 20 in PBS). Next, 1% BSA in PBS was used to block non-specific binding and the plates were then washed three times with wash buffer. Sera samples were obtained from 12 dogs participating in a CDV challenge experiment as described elsewhere [7], which enabled the standardized sera to be grouped into samples likely to have high titers (S0), medium titers (S3), and low titers (S6) according to ImmunoComb data. All serum samples were incubated for 60 min at 20°C in each of the HEVD and HΔ200 antigen-based ELISA plates. Additionally, the samples were analysed with a commercial dot-ELISA using co-cultured CDV antigens [17] according to the manufacturer's instructions. After three washes, rabbit anti-dog IgG-peroxidase conjugate (Jackson ImmunoResearch Laboratories, USA) was diluted 1 : 5,000 in BSA-PBS and incubated with the plates for 60 min at 20°C. Incubation with secondary antibody alone was used as a control. After additional washes and incubation with peroxidase substrate (Pierce Biotechnology), the plates were read with an ELISA plate reader at 450 nm. Data for the sera samples were compared to the corresponding dot-ELISA results.

Colloidal gold-antibody coupling

Goat anti-canine IgG (GGHL-40A; Immunology Consultants Laboratory, USA) was conjugated to 40-nm colloidal gold beads as previously described [15]. Briefly, the goat anti-canine IgG was dialyzed against 2 mM borate buffer at pH 9.0 for 1 h at 4°C and centrifuged. The colloidal gold was adjusted to pH 9.0 with 0.1 M K_2CO_3 and incubated for 8 min at room temperature after reacted with the goat anti-canine IgG for 2 min. The test line was formed with gold conjugate stabilized with 1% BSA. Before use, the detector was washed three times with PBS containing 1% BSA. Mouse anti-biotin antibody (Jackson ImmunoResearch Laboratories) was conjugated to the colloidal gold beads according to the same methods.

Development of one-step immunochromatography rapid test

One-stop immunochromatography rapid test kit was prepared as previously described [10] The test kit was composed of a test strip in a plastic cassette. The capture antibodies were bound to three different lines on nitrocellulose membrane: control line 1 (C1 line; 5 g/mL biotin-BSA), the test line (1.5 mg/mL HEVD), and control line 2 (C2 line; 50 μg/mL biotin-BSA). Concentrations of the reagents were adjusted such that the band intensity of the C1 line corresponded to 1 : 16 SN titer and that of the C2 line corresponded to 1 : 128 SN titer. Gold-conjugated goat anti-canine IgG and gold-conjugated monoclonal mouse anti-biotin antibody were dried on glass fiber. The test strip was assembled in the order shown in Fig. 1: sample pad, gold pad, nitrocellulose paper, and adsorbent pad (cellulose paper). All pad overlapped made the IgG-antibody complexes migrate along the test strip. This one-step immunochromatography kit could be stored at room temperature (2～30°C) for 24 months.

One-step immunochromatography rapid test procedure

Thirty-two canine sera samples were collected at animal clinics in Seoul to evaluate the possible clinical use of this test. The sera samples were diluted 1 : 25 with test buffer (50 mM Tris, 0.5% gelatin, and 1% Tween 20 at pH 8.0), and 150 μL of the diluted sera were added to the sample hole of the kit. The following test procedure was carried out as previously described [10]. Anti-CDV antibody in each sample bound to purified HEVD and was then assembled into complex with the colloidal gold-conjugated

anti-canine IgG (GGHL-40A; Immunology Consultants Laboratory). These complex migrated to the T line and formed goat anti-canine IgG-canine serum antibody-purified HEVD mAb conjugated-colloidal gold, which was shown as a purple band. Color intensity was proportional to the quantity of anti-CDV antibody in each serum. The gold-conjugated monoclonal mouse anti-biotin antibody complexed with monoclonal mouse anti-biotin antibody in C1 and C2 lines was formed independently of the IgG antibody.

Results

Expression of the three CDV constructs and solubility test

Each expressed CDV protein was attached to 5 kDa of the tag protein. Thus, the entire H protein, HEVD, and HD200 had molecular weights of 72, 65, and 50 kDa, respectively. The entire H protein was not over-expressed in the BL21 cells compared to the other proteins. The HEVD and HΔ200 proteins were highly expressed and found to be hydrophobic.

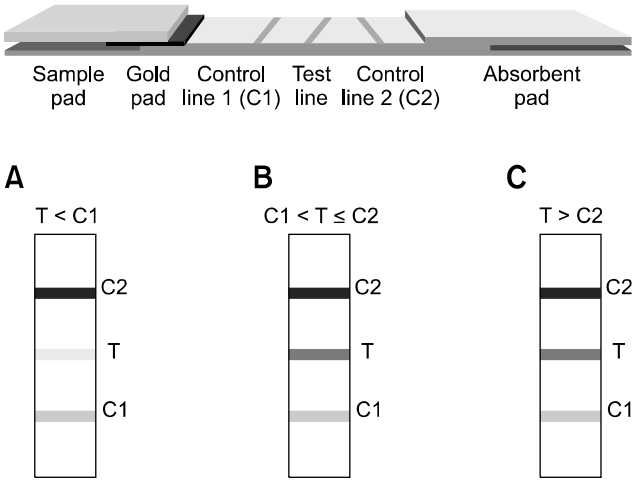

Fig. 1. Diagram of the test strip for detecting anti-CDV antibody and interpretation of the test results. Band shown in (A), (B), and (C) correspond to as SN titers below 1 : 16, from 1 : 16 to 1 : 64, and above 1 : 128, respectively.

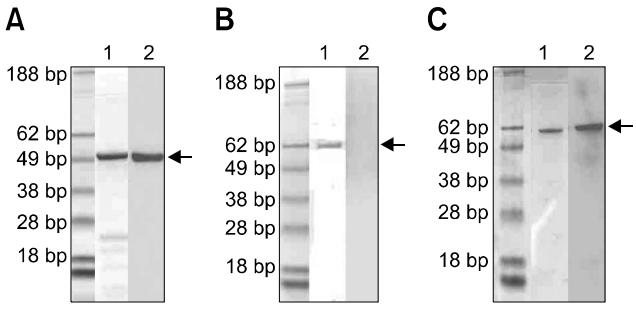

Fig. 2. (A) HΔ200 (lanes 1 and 2, SDS-PAGE and Western blotting analyses using anti-His6 antibody, respectively). (B) HEVD (lanes 1 and 2, SDS-PAGE and Western blotting analyses using anti-His6 antibody, respectively). HEVD antigenicity was observed with Western blotting (C) using anti-CDV polyclonal antibody (1 : 1,000) and anti-goat HRP-conjugated antibody (1 : 2,000).

Western blotting analyses

HΔ200 eluents were detected using SDS-PAGE and Western blotting with anti-His6 antibody. HEVD was detected by SDS-PAGE but not by Western blotting using the anti-His6 antibody (1 : 2,000; Roche) as shown in panel B in Fig. 2. HEVD had good antigenicity according to the Western blotting analyses using an anti-CDV polyclonal antibody (1 : 1,000; VMRD) as presented in panel C in Fig. 2. HEVD was also effective for detecting anti-CDV antibody in samples based on reactivity with the anti-polyclonal antibody.

Dot blotting and ELISA

HΔ200 was more clearly detected compared to HEVD when performing dot blotting. The anti-His6 antibody easily detected both HEVD and HΔ200. However, differences in detection were observed when using the anti-CDV polyclonal

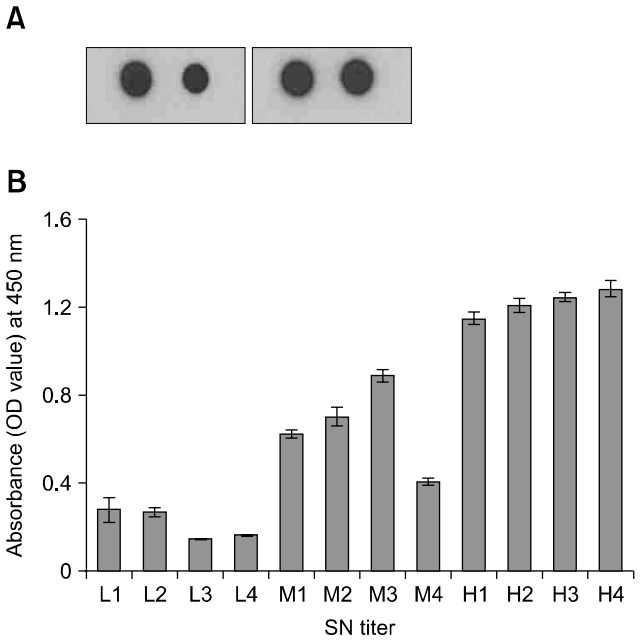

Fig. 3. (A) Dot blotting for HEVD and HΔ200 using anti-CDV polyclonal antibody (left) or anti-His6-peroxidase antibody (right) and ELISA. (B) performed for 12 sera samples divided into three categories: ones with low titers (S0, L1 ~ L4), medium titers (S3, M1 ~ M4), and high titers (S7, H1 ~ H4). S units (S0 to S6) were measured by ImmunoComb. For dot blotting, each serum sample was diluted 1 : 3,000. The anti-CDV polyclonal antibody detected HEVD more easily than HΔ200, while anti-His6-peroxidase antibody was sensitive to both proteins.

antibody. The anti-CDV polyclonal antibody was more sensitive to HEVD compared to HΔ200. Considering that the presence of HEVD was not confirmed using SDS-PAGE and Western blotting with the anti-His6 antibody, detection of HEVD may be dependent on the protein confirmation. ELISA results shown in panel B in Fig. 3 indicated that the sera samples from each group could be clearly arranged according to titer based on the ELISA performed at 450 nm. The control had an optical density value approximately 0.01 (data not shown).

One-step immunochromatography rapid test assessment

We collected 32 canine sera from animal clinics in Seoul in order to evaluate the applicability of this test in clinical practice. Bands on the control lines confirmed that the test was performed correctly. Additionally they were equivalent to high and low titers of antibody, as measured by SN titers. Therefore, the band density of the T line could be compared to known SN titers of T1 and T2 lines (Table 2).

Discussion

Current modified live CDV vaccines were developed before the 1960s. Differences between the vaccine strains and new isolates were found with virus-neutralizing test, indicating that these variations may be related to inadequate protection conferred by vaccine strains against new isolates. In a previous study [5], new isolates induced high SN and low ELISA titers against field strains but induced low SN and high ELISA titers against vaccine strains. The vaccine strains could not fully protect susceptible dogs against infection by new strains due to strain-specific virus neutralization caused by antigenic changes in the CDV H protein [6]. Phylogenetic analysis of the H protein suggested that the new isolates formed distinct clans according to geographical factors. Thus, there may be slight differences in protection against field isolates in the same area, and a new modified vaccine using recent isolates is needed for more effective protection.

A Swiss isolate, CDV-A75/17, is considered the prototypic virulent CDV [3]. The H gene of this strain has more than a 90% sequence homology with that of other isolates from Asia. The glycosylation sites in HEVD were not analysed in our study. Despite sequence differences and the existence of glycosylation sites, ELISAs using the HEVD of A75/17-CDV as an antigen can be effective for detecting CDVs from Asia as well as other continents. The F protein has an extra-viral domain, and a relatively high level of homology exists between A75/17 and other isolates from Asia. This protein could be suitable for the immuno-detection of CDV. However, an attempt to express F protein in *E. coli* was not successful (data not shown). Expression of the entire or parts of the extra-viral protein of any virus in *E. coli* may allow easier application of the protein as a diagnostic tool compared to other vector systems.

There are two major trans-membrane CDV glycoproteins: the F protein and H protein. Both proteins have the major epitopes for serum neutralization. This means that it is possible to measure the SN titer more accurately compared to other diagnostic methods using structural CDV proteins like the nucleocapsid protein. Among the different parts of the H protein, HEVD was over-expressed in *E. coli* and found to have excellent antigenicity that made this factor suitable for use in immuno-detection methods such as ELISAs and dot blotting. Although HΔ200 was expressed as well as HEVD, HΔ200 did not have adequate antigenicity

Table 2. Specificity and sensitivity of the immunochromatography rapid test for detecting anti-CDV antibodies in 32 serum samples compared to SN titers and ImmunoComb S units

ImmunoComb titer	SN titer	Specimen number	One-step immunochromatography rapid test results			
			T = ND	T < C1	C1 < T ≤ C2	T > C2
S0	< 1 : 2		2			
	1 : 2	1		1		
	1 : 4	1		1		
S1	1 : 8	2		2		
S2	1 : 16	8			8	
S3	1 : 32	10			10	
S4	1 : 64	3			3	
S5	1 : 128	3			1	2
S6	1 : 256	1				1
	1 : 512	1				1
Sum		32	2	4	22	4

ND: not detected.

for detecting the anti-CDV antibody. HEVD expression was most likely confirmation-dependent, and glycosylation of the H protein slightly affects antigenicity. HEVD was not detected by Western blotting using the anti-His6 antibody. It seemed that the amount of eluent was too small for detection. The H protein was not very well expressed as an entire protein, indicating that the 5' terminal sequences may affect translation of the entire protein. Additionally, the entire F protein was not successfully expressed in *E. coli* (data not shown). For both of these glycoproteins, the 5' terminal sequence may influence protein expression in *E. coli.* It has been suggested that the AUA codon of the F gene in A75/17-CDV plays a role in F protein translation compared to the Onderstepoort strain that has an AUG codon [3]. More likely, different start signals in the F gene determine whether the protein may be expressed in *E. coli*.

The *E.coli*-expressed-H protein was purified and used for an ELISA. We evaluated 12 sera samples from dogs that were grouped according to anti-CDV antibody titers determined with conventional anti-CDV antibody detection kit. A reasonable correlation between data from the two different assays was observed. However, the sample size used in this study was quite small and further testing is necessary. Although we were not able to perform a virus-neutralization test, it appears that the SN antibody production increased proportionally with generation of anti-CDV polyclonal antibody.

The specificity and sensitivity of the ELISA correlated with those of the SN titers and conventional anti-CDV antibody detection kit. ImmunoComb, regarded as the gold standard for quantifying anti-CDV antibody titers, is a dot-ELISA designed to measure distemper IgG antibody titers on scale of 0 (S0) to 6 (S6). According to the manufacturer's instruction, score of 3 (S3) for ImmunoComb is considered a positive response equal to 1 : 32 of the SN titer or 1 : 5 of the indirect immunofluorescent antibody titer. However, the SN titer ranging between 1 : 16 and 1 : 32 could inhibit the CDV infection [4,11]. The conventional test kit provides results within 30 min and requires prudent handling. On the other hand, our one-step immunochromatography could not only be performed rapidly without special equipment, but the test components are more easily stored. We tested the one-step immunochromatography rapid test with 32 canine sera samples collected from animal clinics in Seoul with the purpose of evaluating clinical use of the kit. Although anti-CDV antibody-negative sera or anti-sera against other viruses could make it difficult to assess the specificity of this ELISA more accurately, we focused on potential clinical use with field samples. Band intensity of the T line corresponded to titer more than 1 : 2 as measured by the conventional kit. We estimated that the specificity was close to 95.5% while the sensitivity was a little less than 93.1% to the detection kit.

In general, the half-life of maternal-derived antibodies is approximately 8 days. It is recommended that conventional vaccination should be performed for pups at the age of 6 to 8 weeks and boosted at an interval of 3 to 4 weeks at least two or three times [18]. Puppies without adequate maternal antibody titers are more likely to succumb to CDV. The prevention and diagnosis of CDV is based on conventional CDV antigen detection kits and anti-CDV antibody detection assays. In particular, it is essential to determine the time when maternal antibodies inactivate CDV-attenuated vaccines. In addition to the conventional anti-CDV antibody detection kit, our ELISA using HEVD could be helpful for the early serological evaluation of CDV infection. In addition, our test represents a valuable new strategy that could be performed before and during 6 to 8 weeks of age, which is the critical period when pups are most susceptible to CDV infection [2].

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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