

## Recharacterization of the Canine Adenovirus Type 1 Vaccine Strain based on the Biological and Molecular Properties

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Canine adenovirus type 1 (CAV-1) infection results in hepatitis in dogs. In this study, we investigated the biologic and genetic characteristics of the CAV-1 vaccine strain (CAV1V) to improve quality control about CAV vaccine. The identity of CAV1V as CAV-1 was confirmed based on its cytopathic effects and the results of hemagglutination (HA) and immunofluorescence assays, and electron microscopy. The CAV1V strain reached  $10^{7.5}$  TCID<sub>50</sub>/mL in MDCK cells at 4 days post-inoculation and exhibited hemmagglutination activity of 256 U using guinea pig erythrocytes. Intranuclear fluorescence in the infected cells was observed and typical adenoviruses were observed in electron microscope. CAV1V strain was identified as a CAV-1 strain by nucleotide sequence analysis. In a comparison of the nucleotide sequences of the fiber genes of several CAV strains, CAV1V showed the highest similarity (99.8%) with the GLAXO strain, which was isolated in Canada. Our biological characterization of CAV1V will facilitate quality control of the canine hepatitis vaccine.

**Key Words:** Canine adenovirus type 1, Genetic characterization, Vaccine

No potential conflict of interest relevant to this article was reported.

## INTRODUCTION

Canine adenoviruses (CAVs) belonging to the family Adenoviridae are classified into CAV type 1 (CAV-1) and type 2 (CAV-2) based on the results of hemagglutination (HA), virus neutralization (VN), and molecular biological assays (1, 2). CAVs can infect several animal species, including pet dogs, and wild animals such as wolves, coyotes, skunks, foxes, bears, and lions (3, 4). CAV infection of unvaccinated wild raccoon dogs was confirmed by serological testing (5, 6). CAV-1 and CAV-2 give rise to infectious canine hepatitis and infectious laryngotracheitis, respectively, due to their propensity to infect different cell types (7, 8). CAVs are transmitted by direct contact or by exposure to infectious saliva, feces, urine, or respiratory discharges (9).

CAV-1 can be characterized based on its cytopathic effects (CPEs) in cell culture, HA of erythrocytes, and regular icosahedral shape by electron microscopy. Diverse biological techniques, including immunofluorescence (IF) assays, are used to diagnose CAV infections in dogs (4, 10). VN, hemagglutination inhibition (HI), and indirect IF assays also provide information on the biological features of CAVs (6, 11).

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CAV has a non-enveloped, double-stranded DNA genome of approximately 32 kb with 30 open reading frames (12, 13). The capsid is composed of hexon, fiber, and penton base structural proteins. The fiber protein comprises tail, shaft, and knob domains and mediates CAV binding to the coxsackievirus and adenovirus receptor on host cells (14). The fiber protein protrudes at each of the 12 vertices of the surface of the CAV capsid. An analysis of the fiber gene has enabled assessments of the HA and molecular characteristics of CAV (15).

Although a live CAV vaccine has been manufactured for dogs in Republic of Korea since the late 1980s, its identity has not been verified by genetic characterization such as nucleotide sequencing. It is necessary for the manufacturer to improve quality control based on the biological and genetic characteristics of the vaccine strain before releasing vaccines. In this study, we investigated the biological and genetic features of the CAV-1 vaccine strain, CAV1V, which has been used to prevent CAV infection in dogs in Republic of Korea. This study will expand our understanding of CAV1V and will facilitate quality control during vaccine production.

## MATERIALS AND METHODS

### Virus and growth kinetics

CAV1V, provided by KBNP standing for Korea Biologicals eNvironmental products Pharmaceuticals (Yesan, Korea), was propagated in Madin-Darby Canine Kidney (MDCK) cells (CCL-34; American Type Culture Collection, Manassas, VA, USA). The MDCK cells were cultured to identify cytopathic effect (CPE) of the CAV1V strain in six-well plates at 37°C in a 5% CO<sub>2</sub> incubator. Confluent MDCK cells were washed twice with phosphate-buffered saline (PBS; pH 7.2), inoculated with 200 µL of 10-fold diluted CAV1V strain, and incubated for 1 h at 37°C. After adding 2 mL of Dulbecco's modified Eagle's medium (DMEM), the plate was incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 2 h. Next, 5 mL of fresh DMEM containing 5% fetal bovine serum (FBS) were added and the plate was incubated at 37°C in 5% CO<sub>2</sub> for 7 days post-inoculation (DPI). CPEs were photographed. To assess the growth kinetics of CAV1V, MDCK cells grown in 25 cm flasks were inoculated with the virus containing 100 TCID<sub>50</sub>/mL and harvested daily for 7 days. After freezing and thawing three times, 10-fold serial dilutions of the virus were titrated in 96-well microplates, and CPEs were observed under a microscope at 6 DPI. The CAV1V titer was determined according to the Reed and Muench method (16) and expressed as the 50% tissue culture infectious dose (TCID<sub>50</sub>/mL).

### Indirect immunofluorescence (IF)

MDCK cells were infected with CAV1V strain in 96-well plates and fixed in cold acetone (−20°C) for 20 min. After three successive washes with PBS (pH 7.2), the cells were reacted with a mouse monoclonal antibody against CAV-1 (VMRD, Pullman, WA, USA) for 1 h at 37°C and subsequently stained with a 100× dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM (KPL Laboratories, Gettysburg, PA, USA). After washing with PBS, the MDCK cells were examined under a fluorescence microscope at 200× magnification (TE2000-U; Nikon Instruments Inc., Tokyo, Japan). Cells showing nuclear fluorescence were considered positive for CAV-1.

### Electron microscopy

MDCK cells infected with CAV1V were harvested at 4 DPI to identify viral particles in the cells and the MDCK cells were collected from cell culture flask using a cell scraper. The infected MDCK cells were centrifuged at 1,500 × *g* for 5 min, and the pellet was fixed with 2.5% glutaraldehyde in PBS at 4°C for 2 h and subsequently with 1% osmium tetroxide in PBS for 2 h. After dehydration with ethanol, the fixed cells were embedded in resin, and ultrathin sections were cut and stained with uranyl acetate and lead citrate. The stained MDCK cells were subjected to a Hitachi 7100 electron microscope (Tokyo, Japan).

CAV1V-infected MDCK cells were harvested 72 h post-inoculation to identify purified virus particles and frozen and thawed three times. After centrifugation at  $4,000 \times g$  for 30 min to remove cell debris, the supernatant was treated with saturated ammonium sulfate (MW 8,000; Sigma-Aldrich, St. Louis, MO, USA) and stirred for 2 h at room temperature. After centrifugation at  $2,500 \times g$  for 30 min, the pellet was resuspended in TE buffer (5 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid [EDTA]; pH 7.8) at 5% of the original volume. The suspension was layered on top of cesium chloride (density 1.43) and centrifuged at  $100,000 \times g$  for 90 min using a SW-41 rotor (Beckman, Danvers, MA, USA). The band on top of the cesium chloride layer was collected and, to eliminate residual cesium chloride, dialyzed against PBS overnight at 4°C. One drop of purified virus was placed on a Formvar-coated grid and negatively stained with 1% uranyl acetate. The CAV1V particles were visualized under the same electron microscope.

## HA assay

HA assays were performed by incubating serial two-fold dilutions of CAV1V propagated in MDCK cells in 50  $\mu$ L of PBS (pH 7.2) at 4°C with 50  $\mu$ L of 0.6% pig, guinea pig, goose, fowl, mouse, or dog erythrocytes in 0.1% bovine serum albumin (BSA) fraction V. The HA titer was expressed as the reciprocal of the highest dilution of CAV1V that showed an HA reaction.

## Polymerase chain reaction (PCR)

DNA was extracted from CAV1V strain propagated in MDCK cells using a DNA Extraction Kit (Bioneer, Daejeon, Korea), according to the manufacturer's instructions. The extracted DNA was eluted in 50  $\mu$ L of RNase- and DNase-free DEPC-treated water. PCR amplification of the CAV fiber gene was carried out using three primers (Table 1). Each reaction in PCR premix (Bioneer) consisted of 10  $\mu$ L of denatured DNA, 1  $\mu$ L of each primer (10 pmol/ $\mu$ L), and 38  $\mu$ L of distilled water in a final volume of 50  $\mu$ L. The cycling profile was denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The products were visualized by 2.0% agarose gel electrophoresis and staining using Redsafe™ Nucleic Acid-Staining Solution (iNtRON, Seongnam, Korea).

**Table 1.** The primers used for the PCR-based analysis of canine adenovirus type 1

Name	Sequence (3'→5')	Expected size (bp)	Target gene
CAV1F1	ATGAAGCGGACACGAAGTGC	710	Fiber
CAV1R1	ACTAGGGCTCCATCCTGCAC		
CAV1F2	AACCTCCAGCAACAACCTAC	630	Fiber
CAV1R2	GGGCTCACTGATTGATAGCT		
CAV1F3	AGAGTATCCGGAGGTAGCCT	735	Fiber
CAV1R3	TCATTGATTTCCCCACATAGG		

## Sequencing of the fiber gene and phylogenetic analyses

The PCR products were purified using a gel extraction kit, ligated into pGEM-T easy vector (Promega, Madison, WI, USA), and the recombinant plasmids were identified by digestion with *Eco*RI (Bioneer). The sequences of the purified plasmids were determined using an MJ Research PTC-225 Peltier Thermal Cycler, ABI Prism BigDye™ Terminator Cycle Sequencing Kit, and AmpliTaq DNA Polymerase (FS enzyme; Applied Biosystems, Foster City, CA, USA). Both DNA strands were sequenced for verification. A phylogenetic analysis was performed based on the nucleotide sequences of the fiber genes of CAV-1/2, and 15 other CAV strains obtained from the GenBank database. Multiple DNA sequence alignments were performed using Clone Manager Basic ver. 9 software (Sci-Ed Software, Denver, CO, USA). A phylogenetic tree was constructed using the neighbor-joining method with MEGA ver. 7.0.20 software (<http://www.megasoftware.net/>). The bootstrap method with

1,000 replicates was applied to verify the reliability of the phylogenetic tree.

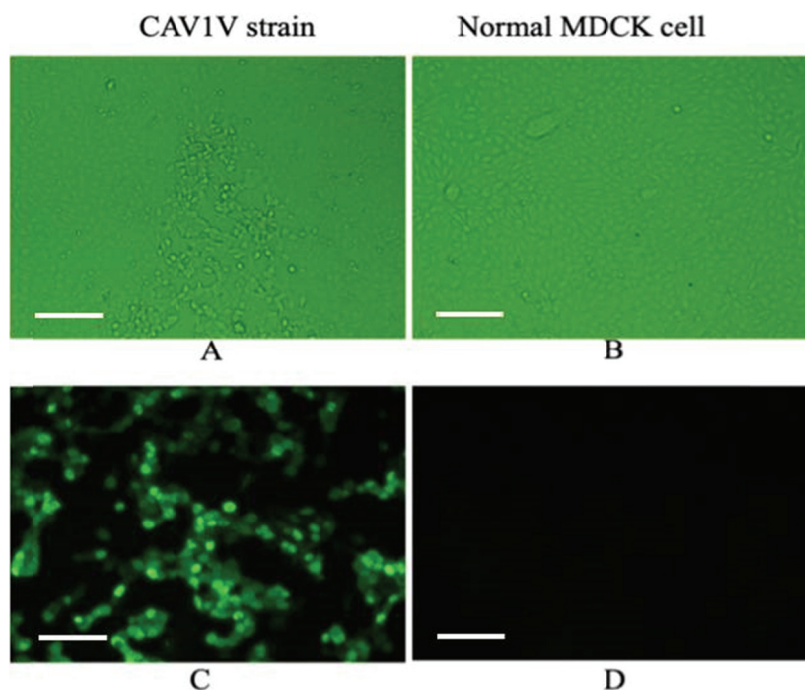
## RESULTS

### Biological characteristics of CAV1V

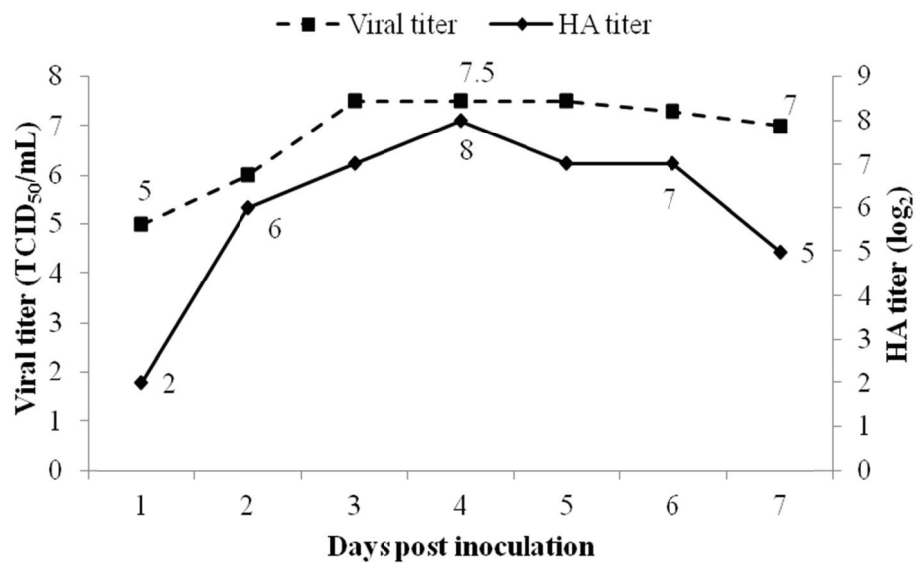
MDCK cells inoculated with CAV1V showed rounding and clustering (Fig. 1A). Also, specific fluorescence was observed in the nucleus of MDCK cells stained with the monoclonal antibody against CAV-1 (Fig. 1C). The CAV1V titer peaked at  $10^{7.5}$  TCID<sub>50</sub>/mL and 256 HA U at 4 DPI (Fig. 2). CAV1V showed the highest HA activity with guinea pig erythrocytes at 4°C (< 2–256 HA U) (Table 2). By electron microscopy, the CAV1V particles were 70–90 nm in diameter with penton capsomeres (Fig. 3), characteristics typical of Adenoviridae.

**Table 2.** Hemagglutination activity of CAV1V strain using erythrocytes from several animal species

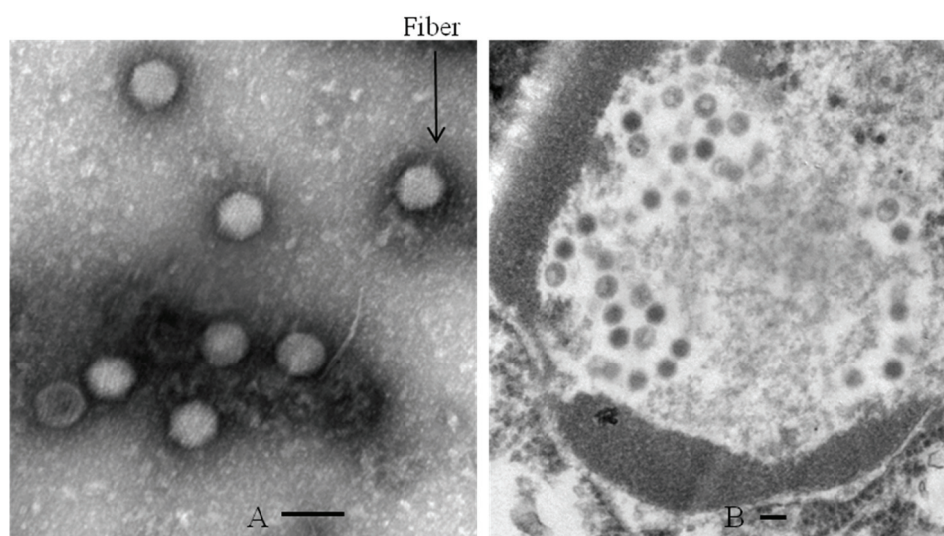
Species	Hemagglutination activity	
	CAV1V strain	Conditions
Guinea pig	256	4°C 0.6% erythrocytes containing 0.1 % BSA in PBS (pH 7.2)
Goose	32	
Fowl	64	
Mouse	< 2	
Pig	< 2	
Dog	4	



**Figure 1.** Cytopathic effects in MDCK cells infected with CAV1V (magnification, 200×) (A). Normal MDCK cells derived from kidney of an adult female cocker spaniel (B). Indirect IF using a monoclonal antibody against CAV-1 (C, D). Intranuclear fluorescence was observed in MDCK cells infected with CAV1V (C) but not in normal MDCK cells (D). Scale bars, 100 μm (A–D).



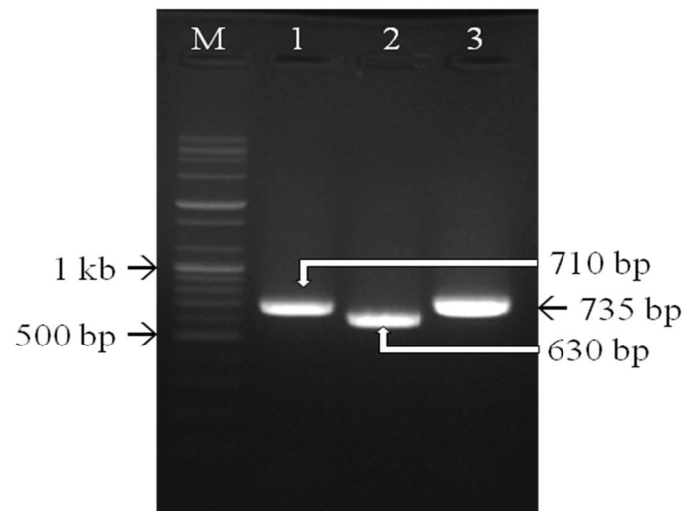
**Figure 2.** Growth kinetics of CAV1V in MDCK cells according to time of harvest. At 4 days post inoculation, CAV1V strain was present at  $10^{7.5}$  TCID<sub>50</sub>/mL and 256 HA U/50  $\mu$ L.



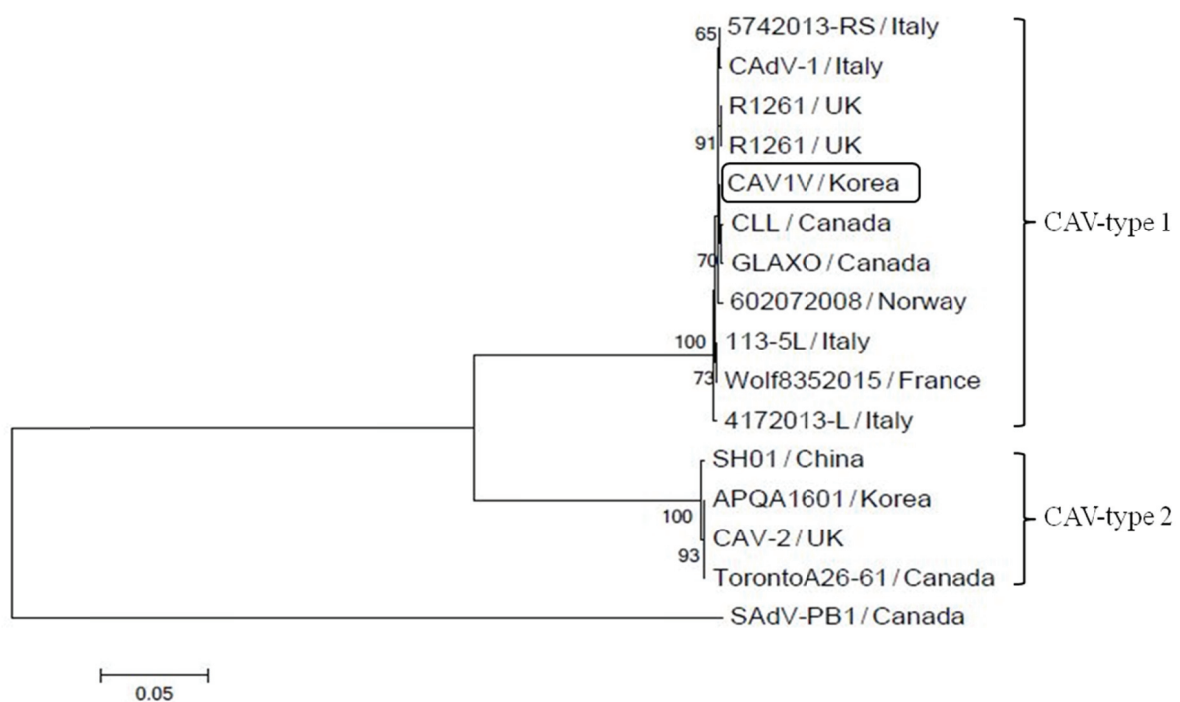
**Figure 3.** Electron microscopic images of virus particles of 70–90 nm diameter. Fiber proteins of CAV1V purified with cesium chloride gradient are protruding from the virion surface (A) (magnification, 100,000 $\times$ ). CAV particles are shown in the nucleus of MDCK cells (magnification, 50,000 $\times$ ) (B). Scale bars, 100 nm (A and B).

## Genetic characteristics of CAV1V

The PCR products of the three CAV1V fiber genes were 710, 630, and 735 bp in length (Fig. 4). The fiber gene of CAV1V was of 1,633 bp, encoding 544 amino acids. The fiber gene of CAV1V showed the highest nucleotide sequence homology (99.8%) with that of the GLAXO CAV-1 strain, which was isolated in Canada (Fig. 5). In a phylogenetic tree constructed based on the fiber genes of 15 CAV strains, CAV was divided into two clades, and CAV1V belonged to CAV-1 (Fig. 5). The fiber protein of CAV1V had high similarity to that of GLAXO (99.8%), and lower similarity to CAV-2 (79.7%). Compared with CAV-2, the fiber protein-coding gene of CAV1V had one deletion, one insertion, nine potential N-linked glycosylation sites, and the adenovirus conserved region (Fig. 6).

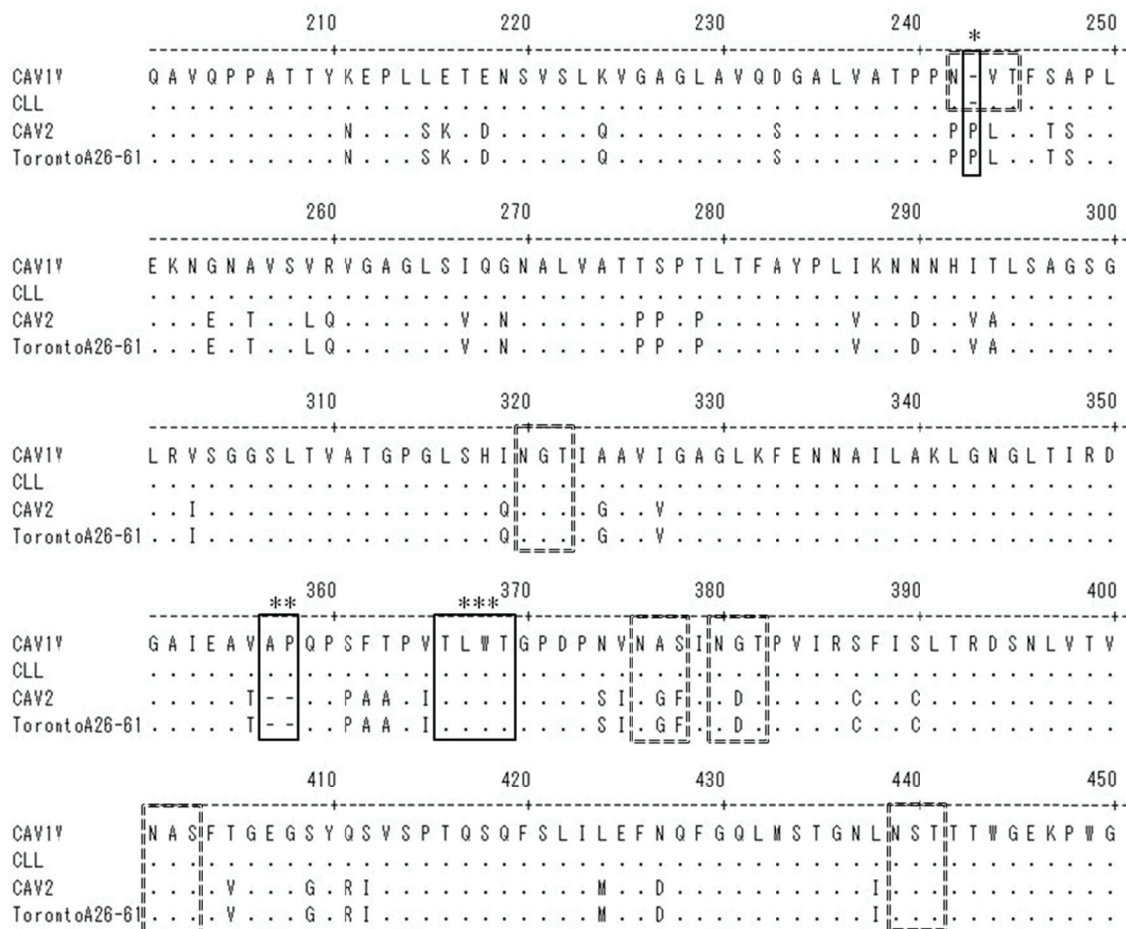


**Figure 4.** Three primer sets targeting the fiber gene of CAV-1 were used for PCR. PCR products of the expected sizes confirmed that CAV1V was a CAV-1 strain. Lane M, 1 kb ladder; lanes 1–3, DNA amplified from CAV1V.



**Figure 5.** Phylogenetic tree based on the nucleotide sequences of the fiber genes of 16 adenoviral strains. CAV1V belonged to the CAV-1 clade and had the highest homology with the GLAXO strain, which was isolated in Canada. The phylogenetic tree was constructed based on alignments of nucleotide sequences obtained using the neighbor-joining method.





**Figure 6.** Alignment of the predicted amino acids at positions 200–450 of the CAV1V fiber protein with the corresponding region of the fiber proteins of three other CAV strains. Dots indicate identical amino acids. Compared with CAV-2, there is one deletion (\*) and one insertion (\*\*) in the fiber protein of CAV1V. The region (\*\*\*) conserved in adenoviruses of all animal species was well preserved. Six potential N-linked glycosylation sites (N-X-S/T) are indicated by dotted-line boxes.

## DISCUSSION

CAV-1 infections of dogs and wild animals occur worldwide, including in Republic of Korea (17, 18). Although routine vaccination against CAV has significantly reduced the incidence of canine CAV infections worldwide, CAV infection of unvaccinated dogs can be fatal due to corneal edema and hepatitis. Therefore, CAV1V has since the 1980s been used in a pentavalent (distemper virus, hepatitis virus, parainfluenza virus, parvovirus, and *Leptospira*) vaccine against canine hepatitis. We propagated the CAV1V vaccine strain provided by KBNP in MDCK cells. The identity of CAV1V was confirmed as CAV-1 based on its CPEs and the results of IF, HA, electron microscopic, and PCR assays.

MDCK cells infected with CAV1V showed marked CPEs. The optimal harvest time was 4 DPI, when the CAV1V titer reached  $10^{7.5}$  TCID<sub>50</sub>/mL; this titer was similar to that of OD-N (19). These data suggest that CAV1V is suitable for vaccine production. CAV1V agglutinated erythrocytes of guinea pig, goose, fowl, and dog, but not those of pig or mouse. HA assays of CAV1V should use guinea pig erythrocytes, against which CAV1V showed the highest HA activity. The HA activity of CAV1V was similar to that of OD-N, which was isolated from a dog in Japan (19). Electron microscopic analyses of infected MDCK cells and purified virus revealed a morphology typical of Adenoviridae. The CAV1V particles were of a size and shape similar to those of CAV strains isolated from animals (17, 20).

PCR-based techniques can be used to diagnose CAV infections in animals (1, 21). PCR amplification of CAV1V yielded bands at 710, 630, and 735 bp, which will facilitate its identification in actual samples. The nucleotide sequence of the fiber gene of CAV1V showed a high level of similarity to those of other CAV-1 strains, likely because of the low mutation rate of double-stranded DNA viruses (22). A phylogenetic tree constructed based on the fiber genes of 15 CAV strains revealed CAV1V to be a CAV-1 strain and that it was most closely related to the GLAXO strain. Vaccination protects against CAV-1 and CAV-2 and should continue because CAV infections of non-immunized dogs have been reported (4). An alignment of amino acids 200–450 of the fiber protein with those of three other CAVs showed one deletion and one insertion at positions 241 and 357, respectively. These variations in the amino acid sequence of the fiber protein of CAV-1 and CAV-2 are reportedly responsible for differences in their HA activity and tropism (15). In addition, there is a need for further studies on the comparative analysis among vaccine strains.

In conclusion, our biological and genetic data confirm the identity of CAV1V as CAV-1. The methods and results of this study will be useful for manufacturer to carry out quality control and to prepare re-evaluation data about the CAV vaccine strain.

## Acknowledgments

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