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# Isolation and molecular characterizations of canine distemper virus from a naturally infected Korean dog using Vero cells expressing dog signaling lymphocyte activation molecule

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## ABSTRACT

**Background:** Canine distemper virus (CDV) infection results in high morbidity and mortality in dogs. There has been no report about Isolation of Korean CDV since 1980 in Korea.

**Objectives:** To investigate the biological properties and the genetic characterization of Korean CDV.

**Methods:** Vero cells expressing dog signaling lymphocyte activation molecule (dSLAM) gene named as Vero/dSLAM were used to isolate CDV using 17 samples. Diagnostic methods such as cytopathic effects, immunofluorescence assay, peroxidase linked assay, electron microscopy, rapid immunodiagnostic assay, and reverse transcription polymerase chain reaction were used to confirm the Korean CDV isolate as a CDV. The genetic analysis was performed through cloning and sequencing of hemagglutinin gene of CDV isolate.


**Results:** A virus propagated in Vero/dSLAM cell was confirmed as CDV (CD1901 strain) based on the above methods. The CD1901 strain showed the highest viral titer ( $10^{5.5}$  50% tissue culture infectious dose [TCID<sub>50</sub>]/mL) in the Vero/dSLAM cells at 4 days post inoculation, but did not form a fork on chorioallantoic membrane of 7-day-old egg. Ribavirin, a nucleotide analogue anti-viral agent, inhibits moderately the Korean CDV propagation in the Vero/dSLAM cells. The nucleotide and amino acid sequences of the H gene of CD1901 strain were compared with those of other CDV strains. The CD1901 strain belonged to Asia 1 group and had the highest similarity (99.9%) with the BA134 strain, which was isolated in China in 2008.

**Conclusions:** We constructed successfully Vero/dSLAM and isolated one Korean CDV isolate (CD1901 strain) from a naturally infected dog. The CD1901 strain belonged to Asia 1 genotype.

**Keywords:** Canine distemper; virus isolation; SLAM

## INTRODUCTION


Canine distemper is highly contagious and fatal in dogs and other animal species of *Canidae*, *Mustelidae*, and *Procyonidae* [1]. The etiological agent of canine distemper is canine distemper

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#### Conflict of Interest

The authors declare no conflicts of interest.

#### Author Contributions

Conceptualization: Yang DK; Data curation: Yang DK, Kim HH; Funding acquisition: Hyun BH; Methodology: Lee S, Yoon SS, Park J, Oh D, Yoo JY, Ji M, Han B, Oh S; Project administration: Yang DK; Resources: Yoon SS; Supervision: Yang DK; Validation: Kim HH; Writing - original draft: Yang DK; Writing - review & editing: Yang DK, Kim HH.

virus (CDV) belonging to the genus *Morbilivirus* in the family *Paramyxoviridae*. The CDV genome has 15,690 nucleotides encoding 8 proteins such as nucleocapsid (N), phosphoprotein (P), non-structural protein (C and V), matrix (M), fusion (F), hemagglutinin (H), and polymerase (L). Among 8 proteins, the H and F proteins taking a responsibility for the viral attachment, entry and membrane fusion have been used to determine the host range and tropism and to prove primary resistance mechanism [2-4]. The high genetic variability of H gene has enabled many researchers to carry out molecular epidemiological study through genetic analysis of field CDV isolates [5,6]. Clinical symptoms of canine distemper are divided into acute and subacute and the main symptoms are fever, nasal and ocular discharge, lethargy, anorexia, coughing, vomiting and diarrhea [7]. When a CDV infected dog develops symptoms, dog do not respond to treatment and die almost 100%.

A number of attempts were made to isolate wild CDV in primary cell culture system in the past and a limited number of CDV strains such as Onderstepoort and Lederle strains have been cultivated in chick embryo cells [8,9]. Recently, as CDV receptors have been identified, the isolation of wild CDV using cells expressing receptors has been reported in many countries [10,11]. To date, 3 cellular receptors related to CDV have been identified. First, signaling lymphocyte activation molecule (SLAM) known as CD150, membrane glycoprotein expressed on cells of T- and B- lymphocytes, macrophages, and dendritic cells is one of receptors for *Morbilivirus* [12]. Second, an inhibitor of complement activation known as CD46 acts as another receptor for attenuated measles virus [13]. Recently, nectin-4 consisting of 510 amino acids is also identified as an epithelial and neurotropic receptor for CDV and involved in neurovirulence [14]. Identification of CDV isolates have been made based on the biological features of CDV such as specific cytopathic effects (CPE) in cell culture system, a typical shape in electron microscopy (EM) and formation of forks on chorioallantoic membrane (CAM) of 7-day-old embryonated chicken eggs. Two diagnostic methods such as immunofluorescence assay and rapid diagnostic kit are designed to detect the antigen of CDV in infected tissue or in canine ocular/nasal discharge [15]. In spite of the fact that reports about CDV isolation are being made in many countries, results regarding the diagnosis of CDV in dogs have been reported in Korea. A few reports are available regarding successful isolation of Korean CDV isolates [5].

In this study, we isolated a field strain of CDV from a naturally infected dog using Vero/dog SLAM (dSLAM) cells that were expressing dog stimulating lymphocyte activation molecule gene. A Korean CDV isolate designated as CD1901 strain was propagated successfully, and the passage of CD1901 strain was conducted 10 times in the Vero/dSLAM cells. We investigated the biological properties of the CD1901-10P strain and the nucleotide sequence of H gene of the CD1901-10P strain was determined to evaluate genetic relationships. This study will expand information about the Korean CDV isolate.

## MATERIALS AND METHODS

### Samples

Seventeen homogenates including lung, spleen, brain, and liver from 7 dogs diagnosed with canine distemper in Animal and Plant Quarantine Agency from 2015 to 2019 were prepared for virus isolation. The organ tissues were used to generate a 10% homogenate in Dulbecco's modified Eagle's medium (DMEM). The 10% homogenates were centrifuged at  $2,500 \times g$  for 15 min to get rid of tissue debris and the supernatants were filtrated through a  $0.45 \mu m$  membrane filter. The filtered samples were applied for the virus isolation.

### Construction of dSLAM Vero cell

To construct stable cell expressing dSLAM gene using Vero cells (ATCC CCL-81), dSLAM gene was cloned into the lentiviral expression vector, pLV-CMV-dSLAM-eGFP-puro, which contains both green fluorescent protein (GFP) and puromycin resistance genes. Lentivirus was produced by co-transfection with the expression vector mentioned above and lentiviral packaging plasmid. The lentivirus was inoculated into Vero cells exhibiting 30% confluence in 6-well plate. The polycation named as Polybrene (Sigma-Aldrich, TR-1003G, USA) to increase binding between the lentivirus and Vero cell membrane was added to the 6-well plate at a concentration of 8 µg/mL. The next day DMEM medium and virus mixture was replaced with normal growth medium containing 10% fetal bovine serum (FBS; Gibco BRL, USA). At 3 days post lentivirus transduction, the cells were sub-cultured in the presence of 100 µg/mL puromycin for 5 days. The 2nd passaged cells were designated as Vero/dSLAM cell after confirming the expression of dSLAM gene by both fluorescence microscopy and polymerase chain reaction (PCR). DNA of Vero/dSLAM cells was extracted with a DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. The DNA attached to the column was eluted with 50 µL of the elution buffer provided in the kit. PCR was carried out using 2 kinds of primer sets to amplify the partial dSLAM gene (Table 1). For the PCR, 5 µL denatured DNA, 1 µL each primer (10 pmol) and 43 µL distilled water were added to AccuPure® PCR premix kit (Bioneer). The cycling profile of PCR consisted of 35 cycles for denaturation-annealing-extension (95°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min) and 1 cycle for a final extension at 72°C for 5 min.

### Virus isolation, growth kinetics and titration

The Vero/dSLAM cells used for virus isolation were cultured in DMEM supplemented with 2 kinds of antibiotics (penicillin and streptomycin) and one antimycotic and 10% FBS. The confluent Vero/dSLAM cells in 24-well plate were washed twice with phosphate buffered saline (PBS, pH 7.2) and inoculated with 200 µL of filtered samples. After incubation at 37°C for 1 h, the samples in 24-well plate were removed and 1 mL of fresh DMEM containing 3% FBS was added to each well. The plate was placed in a 5% CO<sub>2</sub> incubator at 37°C for 7 days. The supernatant in the well showing CPE was harvested and inoculated into newly prepared Vero/dSLAM cells for the second passage. If no CPEs were observed in wells of the 24-well plate for 7 days in the second passage, the samples were considered negative for the virus isolation.

Growth kinetics of the CD1901 strain was carried out to know the highest growth time. To carry out the growth kinetics of CD1901 strain, Vero/dSLAM cells grown in 25 cm<sup>2</sup> flasks were inoculated with the virus containing 100 50% tissue culture infectious dose (TCID<sub>50</sub>)/mL and harvested daily for 7 days. After conducting 3 consecutive freeze-thaw cycles, viral titer

**Table 1.** List of primers used for PCR to amplify dog SLAM gene and for reverse transcriptase PCR to amplify partial H gene of canine distemper virus

Name of primers	Oligonucleotide (5'-3')	Size (bp)	Target gene
digSLAMF1	GGGGCTTCTCCTCCCTGCGC	320	SLAM
dogSLAMR1	GCTTTCAGGATCCTCAGGG		
dogSLAMF2	GGCATTGACCACTGATCCC	380	SLAM
dogSLAMR3	GTGCAGGGGTCCTCAGCTGG		
CDVHF1	ATGCTCTCCRACCAQAGACAAGG	840	H
CDVHR1	GGCTTTGGAAGTTTCCGGGAGG		
CDVHF2	GGGGAGTTGACTCGAAAAG	642	H
CDVHR2	GGGAGGAATGGTAAGCCATCCGG		
CDVHF3	CGGTCCGGTGATACTGAATGGAG	544	H
CDVHR3	TCAAGTTCTGAACGATTACATGAG		

PCR, polymerase chain reaction; SLAM, signaling lymphocyte activation molecule; H, hemagglutinin.

was checked as follows: 10-fold serial dilutions of each virus were distributed into 96-well micro plates, and the Vero/dSLAM cells containing  $4 \times 10^4$  cells and 10% FBS were added into the same micro plate. CPE were observed under a microscope every day for 7 days post inoculation (DPI). The titer of CD1901 strain was determined according to the Reed and Muench [16] method and expressed as TCID<sub>50</sub>/mL. The CD1901 strain was also inoculated into 4 kinds of cells such as Vero/dSLAM cells, Vero/human SLAM (hSLAM; ECACC General collection, England), MDCK (ATCC CRL34, USA) and Vero cells (ATCC CCL81) grown in 25 cm<sup>2</sup> flask to know cell tropism. After incubation for 7 days, each flask was frozen and thawed 3 times. The clarified solutions were subjected to the viral titration mentioned above.

### **Immunofluorescent assay (IFA) and peroxidase linked assay (PLA)**

The Vero/dSLAM cells infected with the CD1901 strain in 96-well micro plate were fixed with cold acetone in  $-20^{\circ}\text{C}$  freezer for 15 min. After 3 consecutive washes with PBS, the cells were reacted with a mouse monoclonal antibody against CDV (VMRD, USA) at  $37^{\circ}\text{C}$  for 1 h and then stained with fluorescent isothiocyanate-conjugated goat-anti-mouse immunoglobulin (Ig) G + IgM antibodies (KPL Laboratories, USA) diluted 200-fold. After washing the plates with PBS, the Vero/dSLAM cells were air-dried and examined under a  $200\times$  fluorescence microscope (TE2000-U; Nikon Instruments Inc., Japan). Cells showing specific fluorescence in cytoplasm of the Vero/dSLAM cells were considered to be infected with CDV.

For the PLA, the Vero/dSLAM cells infected with the CD1901 strain were fixed with the same acetone. After reaction with CDV monoclonal antibodies, the Vero/dSLAM cells were stained with biotinylated anti-mouse IgG (Vector laboratories, USA) and applied with avidin-biotin complex solution for 1 h. Substrate (3,3'-diaminobenzidine) including peroxide was added to all well to develop infected cells. After washing with distilled water, the cells were observed under a microscope.

### **EM**

To take pictures of intact viral particles, the Vero/dSLAM cells infected with the CD1901 strain were harvested at 4 DPI and were frozen and thawed 3 times. After centrifugation at  $3,000 \times g$  for 30 min to remove cell debris, the viral suspension was subjected to precipitation using 10% (w/v) Polyethylene glycol 8000 (PEG 8000; Sigma-Aldrich) in the presence of 0.5 M (w/v) NaCl. The mixture was centrifuged at  $3,000 \times g$  for 10 min following overnight incubation at  $4^{\circ}\text{C}$ . The PEG-precipitated pellets were dissolved in TNE buffer (100 mM Tris-Cl, 100 mM NaCl and 1 mM EDTA, pH 7.6) at 5% of the original volume. The concentrated sample was subjected to discontinuous sucrose gradient centrifugation in a SW-41 rotor (Beckman, USA). For this procedure, 30%–50% (wt/wt) sucrose solution were prepared using TNE buffer. The resuspended sample was layered on the top of the 30% sucrose solution and centrifuged at  $100,000 \times g$  for 90 min. The clear band between 30% and 40% sucrose was collected carefully, and dialyzed against PBS to remove residual sucrose solution overnight. The dialyzed sample was centrifuged at the same condition again and then pellet was resuspended with TE buffer (5 mM Tris-Cl, and 1 mM EDTA, pH 7.8). One drop of the purified virus was placed on Formvar-coated grids and stained negatively with 1% uranyl acetate. The viral particles of CD1901 isolate were examined under the Hitachi 7100 electron microscope (Japan).

### **Rapid immunodiagnostic assay (RIDA)**

The use of commercial RIDA kit was used according to the manufacturer's indication (Bionote, Korea). Briefly, vial suspension of CD1901-10P strain was mixed with the dilution

buffer at a ratio of 1:1. A total of 50  $\mu\text{L}$  of the mixture was applied to the sample well of the kit. It took 5 min to determine result after application of the mixture and the appearance of 2 purple bands, which are the test and control band, was considered positive.

### Formation of fork in CAM of egg

The CD1901 strain and Lederle strain (CDV vaccine strain) showing viral titer of  $10^{5.0}$  TCID<sub>50</sub>/mL were inoculated onto the CAM of four 7-day-old embryonated chicken eggs, respectively. The inoculated eggs and 2 normal eggs were incubated for 6 days after inoculation at 37°C. The egg shells were opened on the 7th day after inoculation and the formation of fork on CAM was examined on a magnifying glass.

### Anti-viral activity of ribavirin against CD1901 strain

Ribavirin (1- $\beta$ -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a nucleotide analogue anti-viral agent, has been noted for its actions against a broad range of RNA viruses. The ribavirin stock solution (5 mg/mL) was diluted to 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0  $\mu\text{g}/\text{mL}$  in DMEM, respectively. The CD1901 strain showing viral titer of  $10^{5.0}$  TCID<sub>50</sub>/mL was added to Vero/dSLAM cells grown in 25 cm<sup>2</sup> flasks with 10 mL of DMEM including each concentration of ribavirin and the flasks were incubated for 4 days. After 3 freezing and thaw cycles, each viral titer was measured by the method described above.

### Reverse transcription PCR (RT-PCR), sequencing and phylogenetic analysis

Viral RNA of the CD1901 strain was extracted using a RNA extraction kit (Bioneer) according to the manufacturer's instructions. The RNA attached to the column was eluted with 50  $\mu\text{L}$  of the elution buffer provided in the kit. QIAGEN OneStep RT-PCR kit (Germany) was used to amplify 3 partial H genes of CD1901 strain with 3 kinds of primer sets (Table 1). A reaction mixture is as follows: 5  $\mu\text{L}$  denatured RNA, 1  $\mu\text{L}$  each primer (10 pmol), 10  $\mu\text{L}$  5 $\times$  buffer (12.5 mM MgCl<sub>2</sub>), 2  $\mu\text{L}$  dNTP mix, 2  $\mu\text{L}$  enzyme mix (reverse transcriptase and *Taq* polymerase), and 29  $\mu\text{L}$  distilled water. The cycling profile of RT-PCR composed of complementary DNA synthesis at 50°C for 20 min, 40 cycles for denaturation-annealing-extension (95°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec) and 1 cycle for a final extension at 72°C for 10 min. Three RT-PCR products were visualized by electrophoresis on 2.0% agarose gel containing the Redsafe Nucleic acid staining solution (iNtRON, Korea).

Three RT-PCR products purified by the gel extraction kit were ligated into the pGEM<sup>®</sup>-T Easy vector system (Promega, USA) according to the manufacturer's instruction. Plasmid DNAs were extracted from 6 white *Escherichia coli* (DH5 $\alpha$ ) organisms, and inserted DNA in the plasmids were identified by *Eco*RI enzyme digestion (Bioneer). Two DNA plasmids with each gene were submitted to an external agency (Macrogen Inc, Korea) to determine the nucleotide sequences. The sequences of the plasmids were determined with the MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM BigDye Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems, USA). Single-pass sequencing was performed for each template using 2 universal primer sets of SP6 and T7. Each DNA sequence of both strands were crosschecked to verify the sequences.

A phylogenetic analysis was performed based on the H gene of the CD1901 strain and 30 other CDV strains obtained from the GenBank database. A phylogenetic tree was constructed by applying the neighbor-joining method using MEGA version 7.0.20 software (<http://www.megasoftware.net>). The bootstrap method with 1,000 replicates was constructed to verify the reliability of the phylogenetic tree. Multiple amino acid sequence alignments between

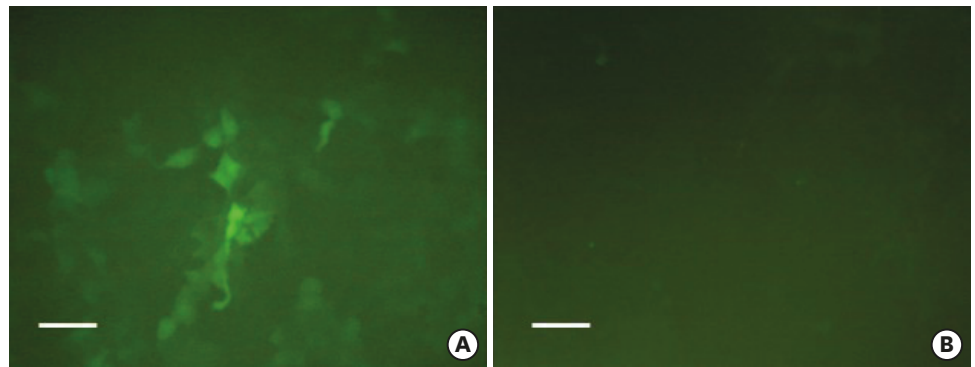


CD1901 and Lederle strains were examined using Clone Manager Basic version 9 software (Sci-Ed Software, USA).

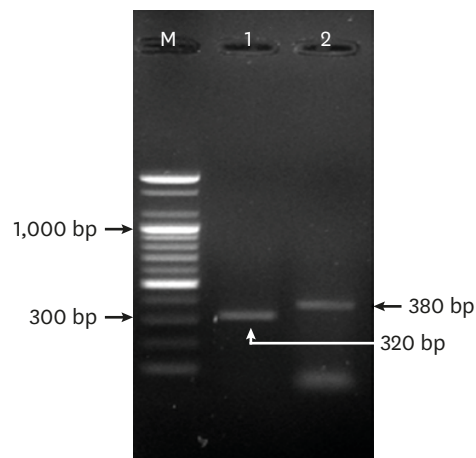
## RESULTS

### Construction of Vero cells expressing dSLAM gene

The dSLAM gene was cloned into the lentiviral expression vector containing GFP gene and the lentivirus containing vector was infected into Vero cells. There were 2 ways to determine whether the Vero cell infected with lentivirus expressed dSLAM gene or not. The first way was to check fluorescent when the cells were observed under a fluorescence microscopy. As shown in **Fig. 1**, fluorescence on the Vero cells infected with lentivirus was observed by fluorescence microscopy without any treatment. The second way was to identify the dSLAM gene in the cell's DNA. Therefore, the dSLAM gene was identified in the DNA of the Vero/dSLAM cells by PCR using 2 kinds of primer sets (**Fig. 2**).



**Fig. 1.** Identification of expression of green fluorescence protein in Vero/dSLAM cells (A) and normal Vero cells (B) under a fluorescence microscope. Fluorescences are observed in Vero/dSLAM cells (A), but not in normal Vero cells (B). Two pictures are magnified 200 times. Scale bars indicate 100  $\mu\text{m}$  (A and B). dSLAM, dog signaling lymphocyte activation molecule.

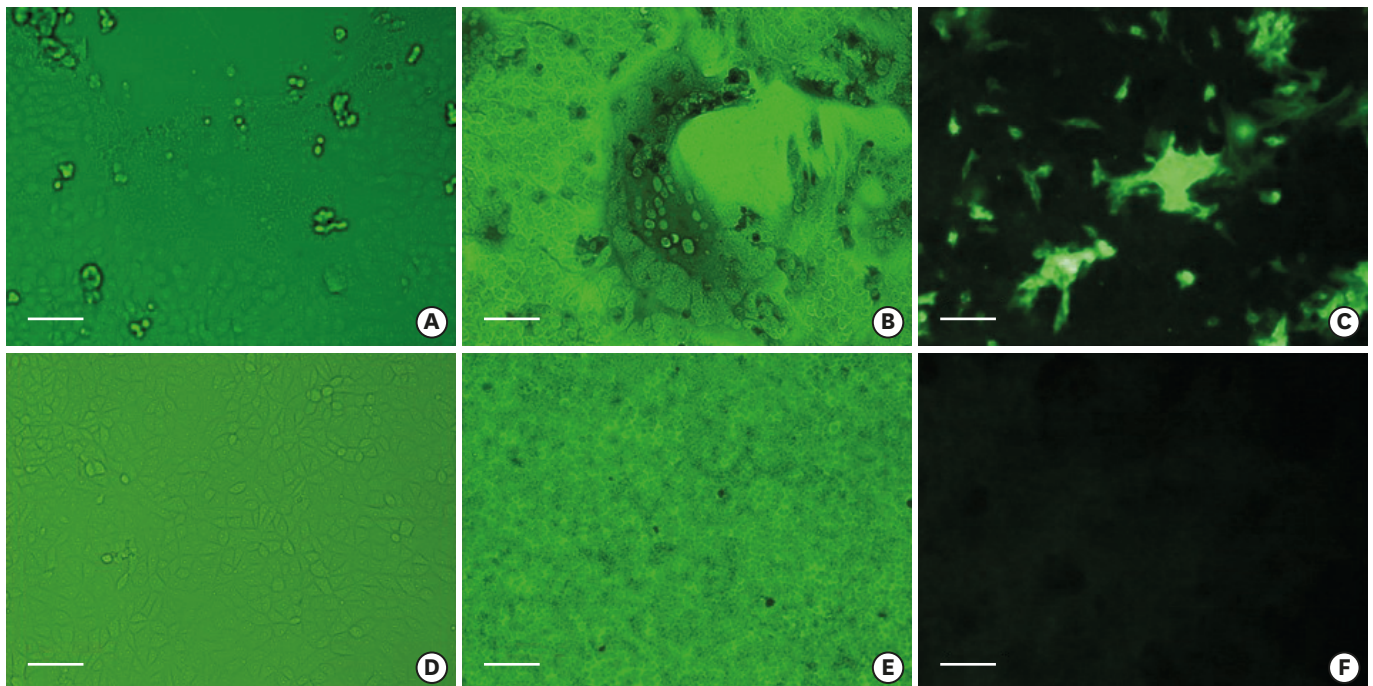


**Fig. 2.** Amplification of partial dSLAM gene in Vero/dSLAM cells by polymerase chain reaction. M: 1kb ladder, lane 1 and 2: Vero/dSLAM cells. The DNAs extracted from Vero/dSLAM cells are amplified with 2 specific primer sets for dSLAM gene. dSLAM, dog signaling lymphocyte activation molecule.

### Isolation and biological characterization of CD1901 strain

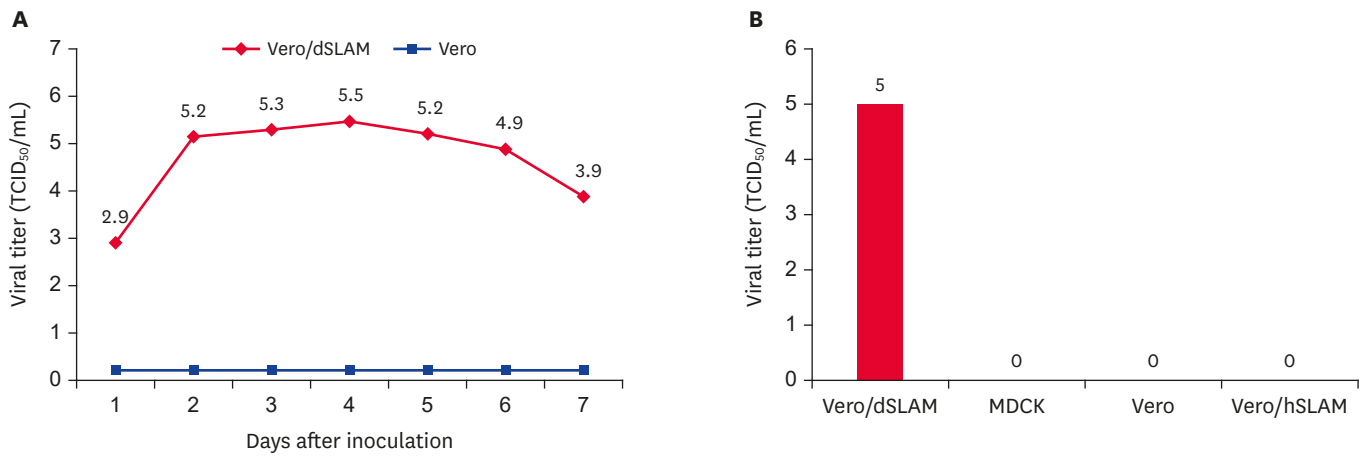
Of the 17 inoculated Vero/dSLAM cells, one dog spleen sample in 2016 showed specific CPE, which was characterized by cell fusion with large round syncytium and being similar to giant cells (**Fig. 3A**). A virus showing distinct CPEs in the Vero/dSLAM cells was named as CD1901 strain and the Vero/dSLAM cells infected with the CD1901 strain were fixed in cold acetone and stained with monoclonal antibodies against CDV. As shown in the **Fig. 3B and C**, brown color and specific fluorescence were observed in the cytoplasm of Vero/dSLAM cells stained with the CDV monoclonal antibody. These results confirmed that the CD1901 strain was CDV. Growth kinetics was performed to determine the biological features of CD1901 strain. As shown in **Fig. 4A**, the highest viral titer ( $10^{5.5}$  TCID<sub>50</sub>/mL) was founded in the virus harvested at 4 DPI. At 3 DPI, 70% Vero/dSLAM cells infected with CD1901 strain showed specific CPEs mentioned above. Based on the CD1901 growth kinetics, virus antigen for electron microscope was harvested around 4 DPI. The CD1901 strain was inoculated into 4 kinds of cells to identify cell tropism. The CD1901 strain had a titer of  $10^{5.0}$  TCID<sub>50</sub>/mL in the Vero/dSLAM cell among the 4 cells and did not proliferate in Vero/hSLAM, Vero, and MDCK cells (**Fig. 4B**).

CDV particles of the CD1901 strain purified by sucrose density gradient ultra-centrifugation were examined by EM. The viral particles that exhibit pleomorphic forms were 120–140 nm in diameter with crown-shaped projections, indicating a morphological appearance typical of *Paramyxoviridae* (**Fig. 5A**). The CD1901 strain propagated in Vero/dSLAM cells was applied to commercial RIDA kit. The 2 bands in the kit once again confirmed that the CD1901 strain was CDV (**Fig. 5B**). To determine the proliferative capacity of CD1901 strain in CAM, the virus was inoculated into 7-day-old eggs. The CD1901 strain did not form a fork in CAM (**Fig. 6A**), but the Lederle strain formed (**Fig. 6B**). The viral titer of CD1901 strain grown in culture media

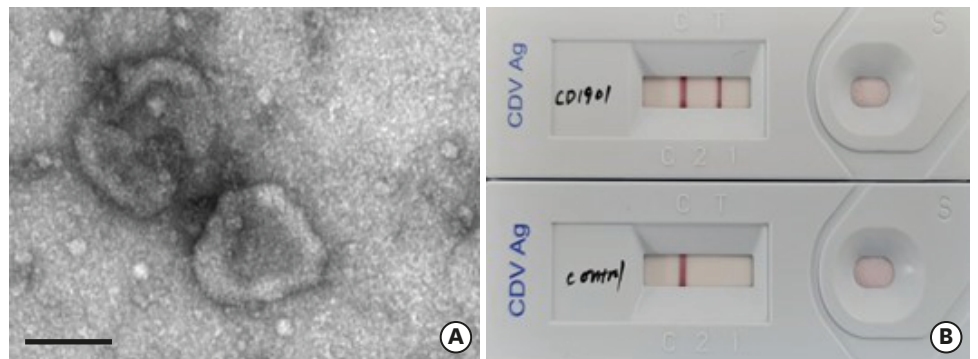


**Fig. 3.** Identification of canine distemper virus based on the CPE, fluorescence assay, peroxidase linked assay. CPEs of Vero/dSLAM cells infected with CD1901-10P isolate (A), stained with abidin-biotinlated conjugate (B), specific fluorescences observed in cytoplasm of Vero/dSLAM cells infected with CD1901 isolate (C), and normal Vero/dSLAM cells (D, E, and F). Six pictures are magnified 200 times (A-F). Scale bars indicate 100  $\mu$ m (A-F). CPE, cytopathic effect; dSLAM, dog signaling lymphocyte activation molecule.

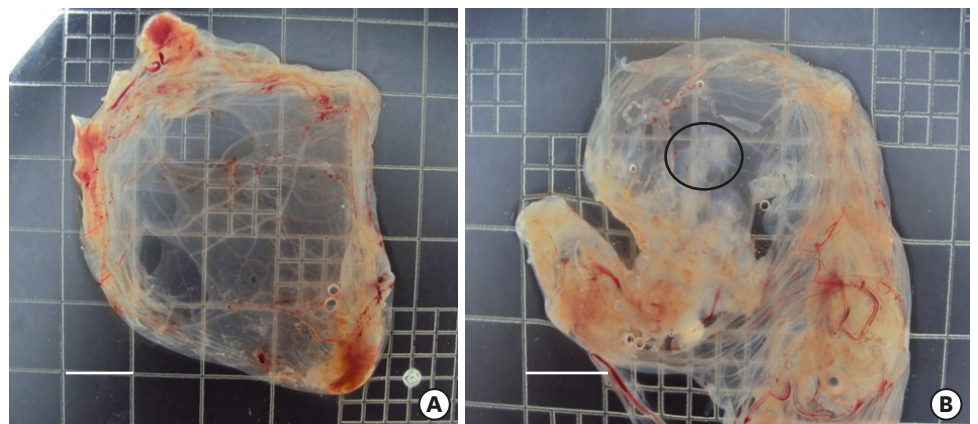
Isolation and identification of CDV in Korea



**Fig. 4.** Growth kinetics of the CD1901-10P strain according to the harvesting day in Vero/dSLAM cells (A) and viral titer of CD1901-10P propagated in 4 kinds of cells to check cell tropism (B). The CD1901 isolate showed the highest titer of  $10^{5.5}$  TCID<sub>50</sub>/mL at 4 days post inoculation (A), but did not proliferate in MDCK, Vero and Vero/hSLAM cells (B). TCID<sub>50</sub>, 50% tissue culture infectious dose; dSLAM, dog signaling lymphocyte activation molecule; hSLAM, human signaling lymphocyte activation molecule.

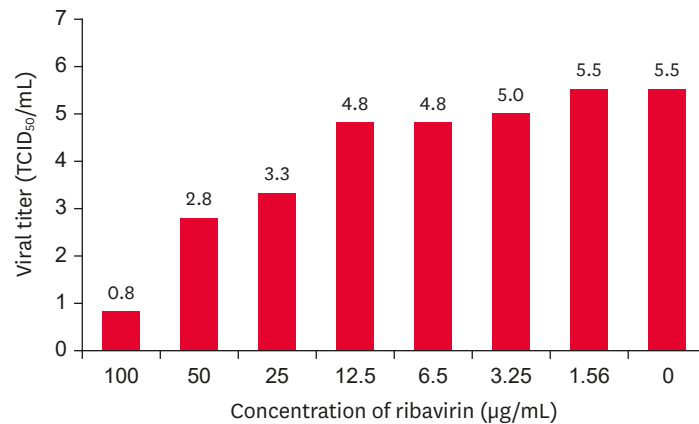


**Fig. 5.** Identification of CDV particles demonstrated by transmission electron microscopy (A), Magnification is 10,000 and scale bar indicates 100 nm. CD1901-10P antigens are detected in RIDA kit (B). The RIDA kit showing 2 lines considers a positive result. CDV, canine distemper virus; RIDA, rapid immunodiagnostic assay.



**Fig. 6.** Identification of the proliferative capacity of CD1901-10P strain in CAM of 7-day-old egg. The CD1901-10P strain did not form a fork in CAM (A), but the Lederle strain formed as indicated in dark circle (B). Scale bars represent 1 cm. CAM, chorioallantoic membrane.





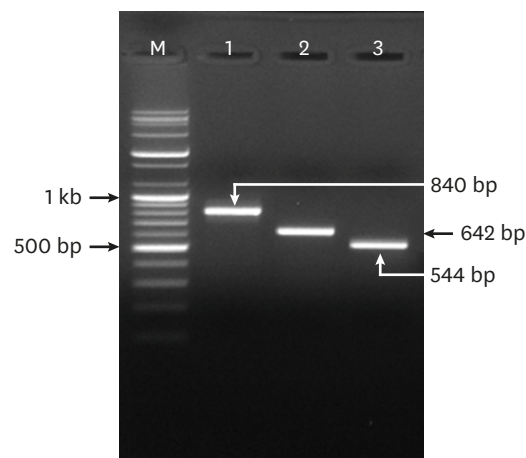
**Fig. 7.** Inhibition of CD1901-10P strain according to concentration of ribavirin. At the highest treated dose, 100 µg/mL, viral titer of CD1901-10P strain is 85.5% lower than that of non-treated control group (0 µg/mL). TCID<sub>50</sub>, 50% tissue culture infectious dose.

containing ribavirin at the concentration of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0 µg/mL was  $10^{0.8}$ ,  $10^{2.8}$ ,  $10^{3.3}$ ,  $10^{4.8}$ ,  $10^{4.8}$ ,  $10^{5.5}$ , and  $10^{5.5}$  TCID<sub>50</sub>/mL, respectively (**Fig. 7**). At the highest treated dose, 100 µg/mL, viral titer of CD1901 strain was 85.5% lower than that of non-treated control group (0 µg/mL), indicating that ribavirin inhibits moderately the Korean CDV propagation.

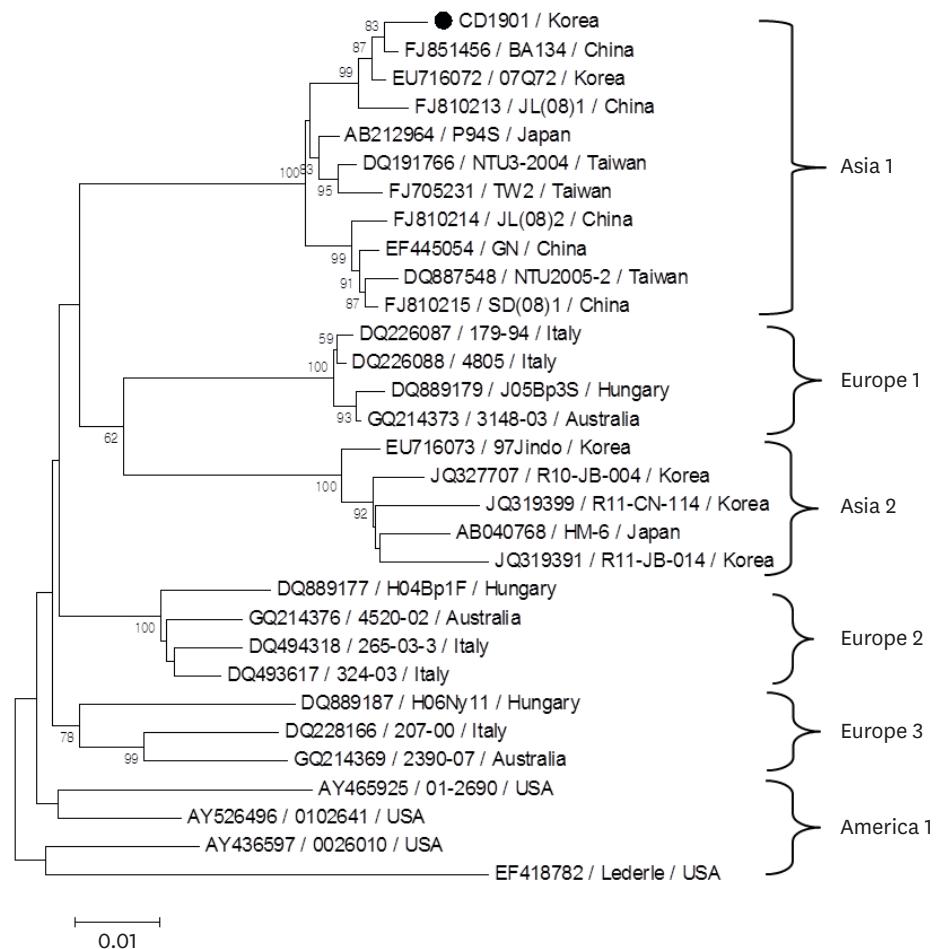
#### Molecular characterization of CD1901 strain

Molecular methods such as RT-PCR, cloning and sequencing were also applied to identify the genetic features of CD1901 isolate. Three partial H genes of the CD1901 isolate were amplified by RT-PCR using 3 specific primer sets for CDV. As shown in **Fig. 8**, 3 PCR products from the CD1901 strain were detected at sizes of 840, 642, and 544 bp after 2.0% agarose gel electrophoresis and confirmed that the CD1901 strain was CDV based on diagnosis of RT-PCR.

The entire H gene sequence of CD1901 strain was composed of 1,824 nucleotides encoding 607 amino acids and was compared with those of 30 CDVs available in GenBank to determine how its genetic relationship with other CDV strains. The CD1901 isolate showed the highest



**Fig. 8.** Three primer sets targeting the H gene of the CD1901 isolate were used for RT-PCR. RT-PCR products of the expected sizes confirmed the CD1901 isolate as canine distemper virus. M: 1 kb ladder, lane 1-3: RT-PCR products of partial H gene of CD1901 strain. H, hemagglutinin; RT-PCR, reverse transcriptase polymerase chain reaction.

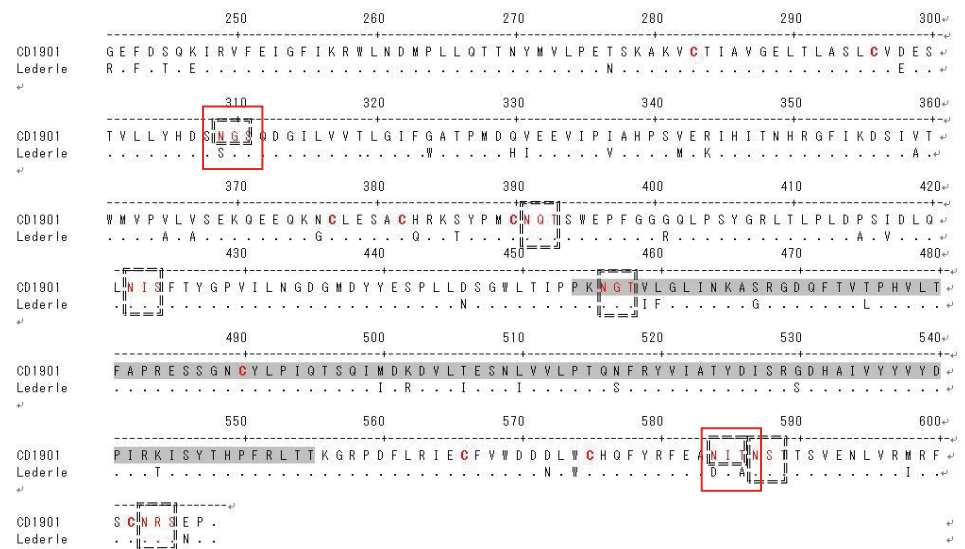


**Fig. 9.** A phylogenetic analysis based on the nucleotide sequences of the hemagglutinin genes from 31 CDV strains. The CD1901 isolate is classified into Asia 1 group. The phylogenetic tree was constructed based on alignments of nucleotide sequences obtained using the neighbor-joining method.

nucleotide homology (99.9%) with the BA134 strain reported in China in 2008 (**Fig. 9**), but had relatively low homology (90.8%) with Lederle strain, which has used as a vaccine strain in Korea since 1980's. A phylogenetic tree was constructed based on the H genes of 31 CDV strains to better understand the genetic relationships of the CD1901 strain. As shown in **Fig. 9**, CDVs were divided into 6 groups such as Asia 1, 2, Europe 1, 2, 3 and America, and the CD1901 strain belonged to Asia 1 group. The partial amino acid sequences (240–609) of the H gene of CD1901 strain and Lederle strain were aligned to find genetic features of CD1901 isolate. There were 12 cysteins in H genes of 2 CDV strains. However, a total of nine and 7 potential N-linked glycosylation sites (N-X-S/T) were identified in the CD1901 strain and Lederle strain, respectively (**Fig. 10**).

## DISCUSSION

Many countries including Republic of Korea have reported CDV infection in domestic dogs and wild animals such as raccoon dogs [5,17,18]. CDV vaccine strains such as Lederle, Onderssetpport, Rockborn and Synder Hill strains have been used to prevent CDV infections



**Fig. 10.** Alignment of the predicted 240–609 amino acid sequences of the CDV H gene between CD1901 and Lederle strain. The same amino acids are indicated by the dotted rectangles. There are 12 cysteins in H proteins of 2 CDV strains. However, there are 9 and 7 N-glycosylation sites in CD1901 and Lederle strain and differences are shown in red squares. The potential dog signaling lymphocyte activation molecule binding region is shaded. CDV, canine distemper virus; H, hemagglutinin.

since 1980s. Although routine vaccination has somewhat reduced CDV infection in dog population, there are questions about the suitability of CDV vaccine strains against current CDV outbreaks because CDV lineages circulating in Korea, China differ from current CDV vaccine strains [5,17,19].

SLAM has known as one of receptor against morbilliviruses and is used as a gate for viral entry because it is expressed on the surface of lymphocytes [12]. In this study, new Vero cells expressing dSLAM gene were constructed to isolate field CDV circulating in Korean dog population as other researchers constructed cells expressing dSLAM gene [10,20]. We successfully isolated only one virus in Vero/dSLAM cells from 17 suspected samples. This is presumed to be improper storage of some samples. The isolate was confirmed it CDV based on results of CPEs, IFA, PLA, EM, RIDA kit, RT-PCR and nucleotide sequence analysis of H gene. It was found that remarkable CPEs were observed in the infected Vero/dSLAM cells and the optimal harvest time and viral titer were 4 DPI and reached  $10^{5.5}$  TCID<sub>50</sub>/mL after the CD1901-10P strain was inoculated into Vero/dSLAM cells. The maximum viral titer was similar to that of 007Lm strain, which was isolated from dogs in Japan [21], indicating that isolation of CDV can be suitable in Vero cells expressing SLAM gene and the CD1901 strain have a similar growth kinetics. Further passages or adaptation may be required to increase viral titer for the preparation of inactivated vaccine. In our results, MDCK, Vero and Vero/hSLAM cells did not allow the growth of the CD1901 strain, indicating that dSLAM receptor be highly correlated with virus isolation. The transmission EM of the purified CD1901 strain revealed a typical paramyxovirus morphology. The size and shape of CD1901 viral particles were similar to that of CDV isolated from animals [22]. Formation of forks on CAM of 7-day-old embryonated chicken eggs inoculated with CDV isolate has been reported as a marker for virulence [18]. The Lederle strain for attenuation was passaged 74 times on the CAM of chick embryos and 28 passages in Ferret [10]. In this study, Lederle strain formed a fork on CAM, but CD1901 strain did not, indicating that CD1901 strain is believed to be pathogenic in dogs that don't have CDV antibodies.

The nucleoside analogue, ribavirin (1- $\beta$ -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is well known as antiviral activity against a broad range of DNA and RNA viruses and has been used for the therapy for viral disease in human [23]. The proliferation of CD1901 strain was inhibited in medium containing various concentration of ribavirin as evaluated by viral titration in Vero/dSLAM cells, indicating that ribavirin may be useful for CDV therapies. Inhibition of CDV growth by ribavirin is presumed to block the cleavage of viral glycoproteins for the virus-cell fusion process [24].

Phylogenetic analysis of CDV H gene has been performed to study evolutionary relationships and to find genetic variation among CDV strains including vaccine strains. Sequence analyses of the H gene of 31 CDV strains including the CD1901 strain revealed that the CD1901 strain was classified into Asia 1 group and was genetically distinct from Lederle strain showing relatively low homology of 90.8%. As other researchers have already reported, these results based on genetic characterization may be fully expected [5,25]. Large differences in genetic homology of H gene between the vaccine strain and field CDV may incompletely protect dogs from CDV infections [17]. In this respect, the isolation of CDV from a naturally infected dog may be the first step in identification of the virulence of CD1901 strain and in evaluating the efficacy of current CDV vaccines in experimental and target animals.

Alignment of the 240–609 amino acid sequence of H protein with the same regions of the Lederle strain indicated that there were 2 N-glycosylation differences between CD1901 strain and Lederle strain at position 309 and 584, respectively. Furthermore, there were 11 amino acid differences in the SLAM binding H protein between the 2 CDVs. Several studies have investigated the specific amino acid substitutions within SLAM binding sites. In particular, residues 530 and 549 are predominantly related to host range [26]. Deduced amino acid sequences of the SLAM binding region of CD1901 strain revealed a glycine (G) and a histidine (H) at position 530 and 549. The G530 and H549 were founded in CDV strains obtained from Korean raccoon dogs [5]. This suggests that the CD1901 strain was transmitted from wild raccoon dogs rather than domestic dogs.

In conclusion, we isolated a CDV from a naturally infected dog using Vero cells expressing dSLAM gene and confirmed it to be CDV closely related to Asia 1 group based on biologic and genetic feature. The Vero/dSLAM cells will be useful for the isolation of field CDV and the CD1901 strain will be the raw material for the development of new CDV vaccine. However, further studies on genetic variation caused by continuous passage in various cells, virulence in experimental and target animal, are required. Nectin4 is also known as another receptor for CDV on the surface of epithelial and neurotropic cell [14]. Therefore, Vero cells expressing nectin4 may be useful to isolate wild CDVs belonging to Asia 2 group.

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